Brief Communication

Pheromone-induced olfactory memory in newborn rabbits: Involvement of consolidation and reconsolidation processes

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Mammary pheromone (MP)-induced odor memory is a new model of appetitive memory functioning early in a mammal, the newborn rabbit. Some properties of this associative memory are analyzed by the use of anisomycin as an amnesic agent. Long-term memory (LTM) was impaired by anisomycin delivered immediately, but not 4 h after either acquisition or reactivation. Thus, the results suggest that this form of neonatal memory requires both consolidation and reconsolidation. By extending these notions to appetitive memory, the results reveal that consolidation and reconsolidation processes are characteristics of associative memories of positive events not only in the adult, but also in the newborn.

A new memory is initially labile and stabilizes over time through the process of consolidation (McGaugh 1966, 2000). However, consolidated memories become labile again when reactivated through recall and need to be reconsolidated to persist (Nader et al. 2000; Sara 2000). This has been evidenced by various treatments impairing the neural activity. Among them, protein synthesis inhibitors such as anisomycin induce severe memory impairment when delivered immediately after acquisition or reactivation.

These evidences mainly derive from studies in adult mammals. However, consolidation and reconsolidation processes have been shown recently in rat pups exposed to conditioned taste aversion (Gruest et al. 2004; Languille et al. 2008). The involvement of such processes nevertheless remained to be assessed in positively reinforced neonatal learning.

In the newborn rabbit, an original appetitive learning exists: Pups acquire a conditioned response to a new odorant (conditioned stimulus, CS) 24 h after its synchronous presentation with the mammary pheromone (MP) used as unconditioned stimulus (US) (Coureaud et al. 2006, 2008). This appetitive learning functions efficiently in a single and brief pairing between the CS and the US. The goal of the present experiments was to assess whether this original form of learning, which seems highly prepared (Seligman 1970), requires a memory processing similar to other types of memories, i.e., whether early appetitive memories involve, as early aversive memories, processes of consolidation and reconsolidation.

We evaluated the sensitivity of this early memory (n = 167 newborn rabbits, from 37 females) to the action of anisomycin (AN, 42 mg/kg, i.p., Aldrich) after both acquisition and reactivation. A single conditioning session (5 min) with the odorant E (ethyl acetoacetate; Aldrich) + MP (2-methylbut-2-enal; Aldrich) was carried out in 1- or 2-d-old pups (previous description in Coureaud et al. 2006, 2008). For the reactivation, odorant E was presented alone for 2 min min only. The conditioning and reactivation occurred around 10:30 am, 1 h before the nursing, which happened only daily in this species (Zarrow et al. 1965). The behavioral assay consisted of an oral activation test (Schaal et al. 2003; Coureaud et al. 2006, 2008), during which the pup was immobilized in one gloved hand of the experimenter, its head being left free, and the test odor was presented for 10 sec with a glass stick 0.5 cm in front of the nares. A test was considered positive when the stimulus elicited head-searching movements (vigorous, low-amplitude horizontal and vertical scanning movements of the head, displayed after stretching of the neck toward the stick) followed by oral grasping movements (labial seizing of the stick extremity). On the contrary, nonresponding pups displayed no response but sniffing to the stimulus. Thus, the dependent variable was the proportion of pups responding at least once by the typical orocephalic behavior. During the retention test, the responsiveness to the MP was also verified in each pup; in all groups, this responsiveness was always >83.3% (and therefore not detailed below). To avoid any interference with their prandial state (Montigny et al. 2006), the pups were tested before sucking. To minimize the litter effect, only two pups from a same litter were included in a given group. The percentages of pups responding to the stimuli were compared by the χ2 test of McNemar or the χ2 test of Pearson (comparisons for dependent or independent groups, respectively), with Yates correction when necessary. Data were regarded as significant when the tests (two-tailed) ended with P < 0.05.

To determine whether MP-induced odor learning requires a consolidation process, four groups of 2-d-old pups (n = 10/group) were conditioned by exposure to the odorant E+MP mixture, and injected with AN or saline immediately or 4 h after the conditioning. The day after (Fig. 1A), saline-injected groups and the AN-4 h-injected group responded strongly to the odorant E, whereas the AN-4 h-injected group responded strongly to the odorant E, whereas the pups immediately injected with AN did not (χ2 > 10.2, P < 0.001). Thus, injection of AN blocked the MP-induced odor memory when AN was administrated just after the conditioning, but not at 4-h delay.

Moreover, we tested whether a short-term memory (STM), usually considered immune from protein synthesis inhibitor (Davis and Squire 1984), follows the MP-induced odor conditioning: Two groups of pups were exposed to E+MP, immediately injected with AN or saline (n = 10 and 8, respectively), and tested 4 h (still unsuckled) and 24 h after the conditioning. The AN-treated pups
treated with AN did not (AN-0 vs. other groups: ***
strongly to the odorant E, whereas reactivated pups immediately
reactivated group, and the AN-4 h-injected group responded
their response to the odorant E. Saline-injected groups, the not-
before the injection of AN on day 2. On day 3, pups were tested for
larly responded to odorant E at 4 h and 24 h (Fig. 2B). The AN-
activation phases (Fig. 1A). The AN-injected pups exhibited a poorer retention than the saline-injected pups (\( \chi^2 = 9.8, P = 0.002 \); Fig. 1C). The amnesic effect of AN was
confirmed in these pups when tested at 24 h (\( \chi^2 = 20, P < 0.001 \)). To sum up, the impact of AN on STM was undetectable at 4 h delay, but when the conditioned response began to be measurable, after
8 h, the AN treatment clearly impaired the memory.
To determine whether the MP-induced odor memory becomes labile when reactivated, four groups of pups were conditioned to the odorant E+MP mixture on day 1, briefly exposed to the odorant E on day 2, and injected with AN or saline immediately or 4 h after reactivation (n = 10/group). Pups of another group (n = 9) were similarly conditioned, but not reactivated before the injection of AN on day 2. On day 3, pups were tested for their response to the odorant E. Saline-injected groups, the not-reactivated group, and the AN-4 h-injected group responded strongly to the odorant E, whereas reactivated pups immediately treated with AN did not (AN-0 vs. other groups: \( \chi^2 > 9.2, P < 0.01 \) in all the comparisons; Fig. 2A). Thus, one day after acquisition, AN abolished the long-term memory only when it was injected just after a reactivation.
Finally, to evaluate the possibility of a STM after reactivation, two groups of conditioned pups were reactivated, immediately injected with AN or saline (n = 10/group), and tested 4 h after the reactivation. The saline-injected pups highly and similarly responded to odorant E at 4 h and 24 h (Fig. 2B). The AN-

**Figure 1.** Consolidation. (A) Impact of anisomycin (AN) on long-term memory. Rabbit pups were conditioned to odorant E by pairing with the mammary pheromone (MP) on day 2 and injected with saline or AN immediately (saline-0, AN-0) or 4 h after conditioning (saline-4 h, AN-4 h). The behavioral response to E was tested 24 h later. Long-term memory was impaired by immediate injection of AN, but not at 4-h delay. (B) Impact of AN on short-term memory. Pups were conditioned, immediately injected with saline or AN, and tested 4 and 24 h after the conditioning. Memory is not expressed 4 h post-
conditioning. (C) Impact of AN on the first expressed memory. Pups were conditioned, immediately injected with saline or AN, and tested 8 and 24 h later. Memory expressed at 8-h delay is already sensitive to AN-induced impairment. (***) \( P < 0.001 \); (**) \( P < 0.01 \).

**Figure 2.** Reconsolidation. (A) Post-reactivation impact of anisomycin (AN) on long-term memory. Pups were conditioned on day 1, reactivated by exposure to odorant E on day 2, and injected with saline or AN immediately (saline-0, AN-0) or 4 h after reactivation (saline-4 h, AN-4 h). Another group was similarly conditioned but not reactivated before AN injection (No R). Retention test occurred 24 h after the reactivation. AN abolishes long-term memory only if memory is previously reactivated. (B) Post-reactivation impact of AN on short-term memory. Conditioned pups were reactivated and immediately injected with saline or AN. They were tested 4 and 24 h after the reactivation. AN leaves intact short-term memory displayed post-reactivation, but impairs long-term memory. (***) \( P < 0.001 \); (**) \( P < 0.01 \).
conditioned aversion induced by LiCl, but rather abolishes it (Gruest et al. 2004; Languille et al. 2008); (2) if AN acted as a negative reinforcer, it should be associated with the MP, and then should reduce the behavioral response to the MP; this is not the case; (3) 4 h after the contingent presentation of the odorant and the AN injection in the reactivation episode, an aversion to the odorant would be detected. On the other hand, AN injected 4 h after acquisition or reactivation has no effect on retention, ruling out a nonspecific anterograde effect of this drug on later memory performance. Thus, our results show that the stabilization in the long term of the MP-induced odor memory requires a time window that begins with conditioning and reactivation, and ends in less than 4 h. A similar time window for amnesia induced by hypothermia has been noted after odor acquisition in another form of conditioning in newborn rabbits (Kindermann et al. 1991). These considerations lead to admit that AN, whatever its mechanism of action (Routtenberg and Rekart 2005; Alberini 2008; Gold 2008), impairs the memorization processes engaged after both learning and retrieval in the newborn rabbit, thus ending in the retention impairment of the pheromone-induced associative memory.

Such retrograde amnesia is in line with an enormous corpus of data on adult rodents, showing that a memory remains labile after its formation and needs time to be consolidated. Moreover, recent experiments have shown that retrieval of consolidated memory requires reconsolidation to be maintained (Nader 2003; Dudai 2004; Alberini 2008; Lee et al. 2005; Bracewell and Bruner 2005; Milekic et al. 2006). If this memory requires new protein synthesis (Davis and Squire 1984; McGaugh 2000), then, after acquisition, no STM is expressed at 4 h measurement time, but at the same delay, the LTM appears already stabilized, since AN is not any longer effective. After reactivation, a memory is already expressed at the 4-h delay, while AN is no more effective on the LTM; the memory trace is therefore restabilized. To sum up, after both acquisition and reactivation, the temporal manifestation of the STM is dissociated from the LTM formation. Thus, STM and LTM would be parallel rather than sequential processes. Such parallelism, previously proposed for consolidation (McGaugh 1966, 2000; Izquierdo et al. 2002), might thus be extended to the reconsolidation process.

The delay needed for the memory to gain control over behavior after learning is not an exclusive property of the pheromone-induced neonatal memory investigated here. An improvement of memory expression during the hours following the acquisition has been described in adult mammals (Spear 1978), namely, in the cases of “reminiscence” and “incubation.” In the present case, however, the memory improvement of newborn rabbits is a consequent neither of multiple training nor of fear conditioning, and thus appears as an original example of spontaneous evolution of memory occurring after encoding. One may recall that rabbit pups usually interact with the mother only once per day and very briefly (Zarrow et al. 1965), and that missing two consecutive opportunities of sucking jeopardizes survival (Coureaud et al. 2000). Thus, although the pheromone-induced acquisition of maternal odors needs to be extremely rapid, the elaboration of the resulting memory traces benefits of several hours to mature, as it will be of adaptive use only 24 h later. In conclusion, the present study reveals that the mammary pheromone-induced appetitive odor memory, in spite of particularities presumably commensurate with the extreme social constraints met by newborn rabbits, shares some commonalities with processes functional in adults from other species. It suggests that consolidation and reconsolidation processes are constitutive of the associative memory, positive as negative, from very early in life.

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| Table 1. Summary of anisomycin (AN) effects on the pheromone-induced appetitive memory |
|------------------------|------------------------|------------------------|------------------------|
|                        | Test at 4 h            | Test at 8 h            | Test at 24 h            |
| Post-acquisition       | No memory              | Impaired by AN         | Impaired by AN immediate, but not by AN at 4 h |
| Post-reactivation      | Not impaired by AN     | —                      | Impaired by AN immediate, but not by AN at 4 h |
Pheromone-induced odor memory

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