Brief Communication

Independence of first- and second-order memories in newborn rabbits

Grard Coureaud,^{1,3} Solne Languille,² Virginie Joly,¹ Benost Schaal,¹ and Bernard Hars²¹Equipe Ethologie Dveloppementale et Psychologie Cognitive, Centre des Sciences du Got et de l'Alimentation, UMR 6265CNRS/INRA/Universit de Bourgogne/Agrosup Dijon, 21000 Dijon, France; ²Laboratoire de Neurobiologie de l'Apprentissage, de la Mmoire et de la Communication, UMR 8620 CNRS, Universit Paris XI, 91405 Orsay, France

The mammary pheromone promotes the acquisition of novel odorants (CS1) in newborn rabbits. Here, experiments pinpoint that CS1 becomes able to support neonatal learning of other odorants (CS2). We therefore evaluated whether these first- and second-order memories remained dependent after reactivation. Amnesia induced after CS2 recall selectively blocked this memory, when recall and amnesia of CS1 left the souvenir of CS2 safe; this finding partially differed from results obtained in adult mammals. Thus, in this model of neonatal appetitive odor learning, second-order memory seems to depend on first-order memory for its formation but not for its maintenance.

The mammary pheromone (MP) emitted by lactating rabbit females helps newborn rabbits to orient to the mother, then rapidly to localize and grasp the nipples (Coureaud 2001; Schaal et al. 2003). It is a determinant for survival and growth in the context of mother–young interactions daily limited (<5 min) (Zarrow et al. 1965). The MP also acts as a cognitive organizer promoting the rapid (single trial) appetitive learning of new odor stimuli, initially neutral. After pairing with the MP, the learned stimulus gains significance (i.e., releases the sucking behavior of neonates) in less than 24 h, following rules of first-order conditioning where the MP constitutes the unconditioned stimulus (US) and the novel odorant the conditioned one (CS1) (Coureaud et al. 2006, 2008, 2009).

Consolidation and reconsolidation processes, usually required in long-term memories (McGaugh 1966, 2000; Nader et al. 2000b; Sara 2000), are also involved in MP-induced odor memory; retention is impaired by injection of a protein synthesis inhibitor (anisomycin), and anisomycin induces amnesia also after reactivation of the previously consolidated memory (Coureaud et al. 2009). Nevertheless, properties of learning and memory promoted by the MP remain poorly known. The present experiments aimed to go further, to determine whether second-order odor learning exists in this model (CS2–CS1 pairing) and to examine whether first- and second-order memories remain dependent in the neonatal brain. To date, Debiec et al. (2006) showed in adult rats that following first-, then second-order conditioning, CS2 memory remains dependent of CS1 memory for its expression in the long-term. Since mechanisms which support learning present specificities in newborns (Wilson and Sullivan 1994; Languille 2010), we suspected that early memories could be less dependent on each other after formation based on chain-like associations.

We investigated these early memory processes ($n = 115$ neonates, from 32 females) and their sensitivity to the action of anisomycin (AN; 42 mg/kg i.p.; Aldrich) after reactivation. Like other authors, using other species—e.g., adults (for review, see Davis and Squire 1984; Desgranges et al. 2008; Merhav and Rosenblum 2008) and newborns (Gruest et al. 2004; Languille

et al. 2008, 2009), we defend that the blocking effect of AN on odor memory in newborn rabbits cannot be explained by an aversive effect but by amnesia (Coureaud et al. 2009). A first-order conditioning (5 min) with the odorant E (ethyl acetoacetate; Aldrich) + MP (2-methylbut-2-enal; Aldrich) was carried out in 1-d-old pups (described previously in Coureaud et al. 2006, 2009). A second-order conditioning was similarly conducted the day after by the pairing of CS2 (odorant EM or F, ethyl maltol or furaneol, respectively; Aldrich) + CS1 (odorant E). For reactivation, odorants E or F were presented alone for two minutes on day 3. Conditioning and reactivation occurred at 10:30 a.m., one hour before nursing, which happened only daily in this species (Zarrow et al. 1965). The behavioral assay consisted of, on day 4, a validated activation test (e.g., Schaal et al. 2003; Coureaud et al. 2006, 2008, 2009) during which the pup was immobilized in one 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. The response was an on/off response; a test was considered positive when the stimulus elicited specific scanning movements of the head (vigorous, of low amplitude) displayed after stretching toward the stick and followed by labial seizing; nonresponding pups displayed no response except sniffing. Thus, the dependent variable was the proportion of pups displaying at least once the typical orocephalic behavior. Each pup participated in only one experiment but was successively tested to three stimuli (inter-trial interval of 120 sec; soft drying of the nose after positive response). The order of presentation of the stimuli was counterbalanced, except for the MP being always the last-presented (control). To avoid interference with their prandial state (Montigny et al. 2006), the pups were tested before sucking. To minimize litter effect, only two pups per litter were included in a given group. The percentages of pups responding to the stimuli were compared by the Cochran Q test or χ^2 test of McNemar for dependent samples, and by the χ^2 test of Pearson for independent groups, with Yates' correction when necessary. Data were regarded as significant when the tests (two-tailed) ended with $P < 0.05$.

To determine whether a MP-learned odorant (CS1) promotes second-order odor learning, two groups of 1-d-old pups ($n = 12$ /group) were first conditioned to the odorant E by exposure to the E + MP blend, exposed on day 2 to CS2 + CS1 mixtures, i.e., to odorants EM + E (Group 1) or F + E (Group 2), and behaviorally

³Corresponding author.E-mail gerard.coureaud@u-bourgogne.fr; fax 33 380681601.Article is online at <http://www.learnmem.org/cgi/doi/10.1101/lm.2145111>.

tested on day 4. Nine other pups exposed to E + MP on day 1 highly responded to E and MP on day 2 (>88.9%). This level of conditioning, equivalent to that observed in previous studies (Coureaud et al. 2006, 2009), allowed us to consider that pups from Groups 1 and 2 have efficiently learned the odorant E before being conditioned to CS2 by pairing with odorant E. The Group 1 (Fig. 1A) highly responded to odorant EM, as Group 2 did after presentation of odorant F (Fig. 1B; no difference between the stimuli: $\chi^2 = 0.54$, $P > 0.05$). Thus, after pairing with odorant E, the initially neutral EM and F odorants triggered responses in pups. Second-order conditioning was thus functional in newborn rabbits, independently of the chemical nature of CS2. The proportion of pups responding to CS2 on day 4 was similar to that of pups responding to MP ($\chi^2 < 0.5$, $P > 0.05$), and pups from both groups responded as well to CS2 as to CS1 ($\chi^2 < 0.05$, $P > 0.05$). Thus, pups were able to build CS2–CS1 association after the initial association between CS1 and US (MP). Since similar results were obtained with our two pairs of CSs, we pursued our investigations only with odorants E–F as CS1–CS2.

To assess whether the second-order conditioning was really the consequence of an associative CS1-induced learning, 41 pups were distributed in three groups. Group 1 formed a positive control with pups conditioned to E + MP on day 1 and to F + E on day 2. In Groups 2 and 3, the respective exposure to E and MP or to F and E was successive (2×2.5 min/stimulus, 1 min of delay between the stimulations). Control pups highly and similarly responded to odorants F, E, and MP ($\chi^2 < 0.05$, $P > 0.05$) (Fig. 2A). Pups from Group 2 responded only to the MP (comparisons of F or E vs. MP: $\chi^2 = 9.1$, $P < 0.01$) (Fig. 2B). Thus, the absence of simultaneity in the exposure to E and MP prevented the learning of E and consequent learning of F; this result underlined the associative nature of the first-order conditioning and suggested that F acquired the value of signal only when E previously became significant for neonates. Pups from Group 3 responded to E and to MP but not to F (E or MP vs. F: $\chi^2 = 10.1$, $P < 0.01$) (Fig. 2C); this finding highlighted the associative nature of the second-order conditioning.

Finally, to evaluate whether CS2 and CS1 odor memories remained linked after CS2–CS1 association, 41 pups were MP-conditioned to odorant E on day 1, E-conditioned to odorant F on day 2, and then separated into three different groups. On day 3, pups from Group 1 (control) were submitted to a brief recall

of odorant F before contiguous injection of saline. Pups from Group 2 were also cued with F but immediately injected with AN. Pups from Group 3 were re-exposed to odorant E before being injected with AN. The step of reconsolidation aimed to make the consolidated memory of CS2 or CS1 labile and therefore sensitive to amnesia induced by a pharmacological agent, such as AN (Nader et al. 2000a; Gruet et al. 2004; Languille et al. 2008; Coureaud et al. 2009). Consequently, it should allow determination of whether the disturbance of CS2 memory also impacted the memory of CS1 (and conversely, after amnesia of CS1). Tested on day 4, pups from the three groups strongly responded to MP (Fig. 3A–C), a result showing that neither the injection by itself nor the injected product disturbed the general physiology and behavior of the newborns. Moreover, pups from Group 1 strongly responded to odorants F and E (comparison F vs. E vs. MP: $Q = 2$, $df = 2$, $P = 0.34$) (Fig. 3A). Thus, the saline injection after CS2 recall had no consequence on the CS2 and CS1 memories. On the contrary, AN injection after brief recall of CS2 was followed by complete amnesia of CS2 in pups from Group 2 (responsiveness to odorant F in Groups 2 vs. 1: $\chi^2 = 25$, $P < 0.001$) (Fig. 3B). Concurrently, these pups still responded to odorant E, as control pups did (Group 2 vs. Group 1: $\chi^2 = 0.06$, $P > 0.05$). After recall of CS1 followed by AN injection, pups from Group 3 did not respond to CS1 but still responded to CS2 (responsiveness to odorants E vs. F: $\chi^2 = 8.1$, $P < 0.01$) (Fig. 3C). Thus, amnesia concerned exclusively the CS1 in this group.

The present study highlights for the first time that second-order conditioning is functional in rabbit pups. A new stimulus, initially neutral, acquires an attractive value when it is paired with another odorant previously associated with the MP. It may be surprising that an incompletely mature organism establishes such a high form of learning, including a CS2 which has never been directly associated with the primary reinforcer (MP, here) but which acquires its predictive value. However, complete sensory, cerebral, and cognitive maturity appears unnecessary for second-order conditioning. Cheate and Rudy (1979) suggested second-order odor-aversive conditioning in 6-d-old rats, Miller et al. (1990) demonstrated odor-induced secondary aversion of texture in 4-d-old rats, and Cheslock et al. (2003) even showed that rats exhibit second-order olfactory conditioning three hours after birth. The present study confirms the functionality of this mnemonic process in newborns from another species and provides original results concerning odor memories built in a chain and their link in the neonatal brain.

The associative aspect of CS1-induced odor learning is confirmed. Unpaired presentations of CS2 and CS1 or CS1 and MP (i.e., sensitization or generalization) failed to induce any conditioning. Thus, CS2 acquires a signal value only after simultaneous pairing with CS1, as CS1 after perceptual contingency with MP (Coureaud et al. 2009). Coherently, the formation of second-order memory strictly depends on pre-existing first-order conditioning (Rescorla 1980) following a chain of memories illustrated by MP → CS1 → CS2. According to this chain, when CS2 acquires a positive value, this value does not alter the CS1 value, a suggestion coherent with the strong responsiveness displayed by pups to CS1 after CS1-induced learning of CS2. Moreover, second-order learning is generally supposed to be weaker than

Figure 1. Second-order conditioning. Rabbit pups were first conditioned to odorant E by pairing with the mammary pheromone (MP) on day 1 (d1). Then, they were conditioned to odorant EM (A) or odorant F (B) by pairing with odorant E on d2. The behavioral responses to E, EM, and MP or to E, F, and MP were tested on d4. Pups highly respond to the first and the second conditioned stimuli, and similarly respond to the unconditioned one (MP).

Figure 2. Associative nature of the second-order learning. (A) Pups were conditioned to odorant E by pairing with MP on day 1 (d1) and to odorant F by pairing with odorant E on d2 (Group 1). (B) Pups were successively exposed to odorant E and MP on d1 and simultaneously exposed to odors F+E on d2 (Group 2). (C) Pups were MP-conditioned to odorant E on d1 and then successively exposed to odors F and E on d2 (Group 3). All the pups were tested for their responsiveness to odors E, F, and MP on d4. Pups strongly respond to odorant F only after association with odorant E, previously MP-learned. $**P < 0.01$.

first-order (Rescorla 1980). Here, the responsiveness of pups is strong and similar both to CS2 and CS1. The strength of the second-order conditioning may be due to our paradigm, which involves a US of high biological value, close temporal proximity between CSs, and a single sensory modality (Gewirtz and Davis 2000). Taken together, these findings highlight that conditioned memories coexist, allowing both CS1 and CS2 to become predictive signals of a critical social event (nursing) and releasers of a decisive individual behavior (sucking).

Regarding the dependence of CS1 and CS2 memories, (1) the injection of AN after recall of CS2 is followed by amnesia of CS2. It demonstrates the effective reactivation of CS2 memory and, subsequently, the requirement of a reconsolidation process (new protein synthesis) for stabilization in the longer term (Sara 2000; Nader 2003; Dudai and Eisenberg 2004; Sara and Hars 2006). However, the recall of CS2 does not trigger (or only weakly triggers) the reactivation of CS1 memory, since this latter stays immune from the amnesic effect induced by AN. (2) When CS1 is recalled, the corresponding memory is reactivated and impaired by AN. This result confirms that MP-induced odor memory, like other kind of memories, needs reconsolidation (Coureaud et al. 2009). In addition, there is no bottom-up reactivation from the first- to the second-order memory; impairment of CS1 memory occurring after consolidation of CS2 memory is selective and leaves the second memory immune from the abolishment of the first one. Thus, first- and second-order memories appear independent on postnatal day 4, even if the formation of CS2 memory directly requires the previous formation of CS1 memory.

These results partially differ from those obtained in adult rats by Debiec

et al. (2006) in a paradigm of fear conditioning. In their study, as in the present one, blockage of CS2 reconsolidation does not alter the first-order memory. However, recall, then amnesia of CS1, induced after shaping of associative CS2–CS1 memories, impairs the second-order memory. The authors consider that the CS2 memory, dependent on the CS1 memory for its formation, remains dependent on the first-order memory for its expression in the long-term, according to the associative chain CS2 → CS1 → US. Here, in newborn rabbits, this chain necessary for CS2 formation appears broken at the time of reactivation; the CS2 memory becomes rapidly independent of the first-order one, as if they do not form a chain anymore but stay separately linked with the US as follows: CS2 → MP, CS1 → MP. This discrepancy may be due to several differences with the study by Debiec et al. (2006), i.e., the animal species, the appetitive vs. aversive conditioning, the olfacto-olfactory vs. auditory-somesthetic CS-US conditioning, and the use of a US engaged in survival in rabbit pups. One may also suggest that in fear conditioning, due to the strong emotional value of the US, it is easier to reactivate the CS2 memory when reactivating the CS1 memory, making the second memory also sensitive to amnesia. Finally, the difference of age and neurophysiological maturity of the animals may also be underscored. As amply highlighted by Sullivan and colleagues, the circuits involved in acquisition of odor memory change during postnatal development (for extinction, see also Kim and Richardson 2010): Structures like the amygdala and piriform cortex may be involved differently depending on their level of maturation (e.g., Moriceau and Sullivan 2005; Raineke et al. 2009). On one hand, similar learning situations can thus generate

Figure 3. Reactivation of odor memories created by first- and second-order conditionings. Pups were conditioned to odorant E by pairing with MP on day 1 (d1), and to odorant F by pairing with odorant E on d2. On d3, pups were reactivated by exposure to odorant F and, respectively, injected with saline (Group 1) (A) or anisomycin (AN, Group 2) (B), or were reactivated by exposure to odorant E and immediately injected with AN (Group 3) (C). All the pups were tested for their responsiveness to odors E, F, and MP on d4. Post-reactivation administration of AN impairs only the conditioned response to the odorant used as the retrieval cue. $**P < 0.01$.

different conditioned responses, and, on the other hand, similar responses may depend on different neural structures during ontogeny (Languille et al. 2009). Such variations could create differences in bonding of first- and second-order memories as a function of age. For instance, adults could establish a stronger link of causality between CS1 and CS2 as compared to newborns, making, therefore, CS2 more dependent on CS1.

Anyway, the long-term dependence of CS2–CS1 memories created by first-, then second-order conditionings does not appear as an absolute rule in mammals. Our result on CS1 retrieval is more in accordance with those observed in the odor aversion paradigm in the slug (*Limax flavus*), showing that cooling treatment administered after reactivation of first-order memory impairs the souvenir of CS1 but not of CS2 (Sekiguchi et al. 1997). However, these authors also observe that amnesic treatment administered after CS2 retrieval affects first- and second-order memories (Sekiguchi et al. 1994, 1997), suggesting that reactivation of CS2 may propagate to CS1 in this invertebrate (a result that differs from our results and those of Debiec et al. 2006).

In conclusion, the fact that CS2 and CS1 memories become rapidly independent in a context of chain odor learning could be particular to neonates. This independence could allow for high-order memories recently created to survive to erasure or modification of first-order ones, and thus for distinct acquired cues and significant rewards to remain connected. In newborns looking for maternal nipples and milk, who have to behave in an olfactory environment subjected to permanent variations, such rapid learning and independent retention of novel information may enlarge their knowledge about, and therefore improve their responsiveness and adaptation to, the environment.

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References

- Cheatle MD, Rudy JW. 1979. Ontogeny of second-order odor-aversion conditioning in neonatal rats. *J Exp Psychol Anim Behav Process* **5**: 142–151.
- Cheslock SJ, Varlinskaya EI, High JM, Spear NE. 2003. Higher order conditioning in the newborn rat: Effects of temporal disparity imply infantile encoding of simultaneous events. *Infancy* **4**: 157–176.
- Coureaud G. 2001. "Olfactory regulation of sucking in newborn rabbit: Ethological and chemical characterization of a pheromonal signal." PhD thesis, University Paris 13.
- Coureaud G, Moncomble AS, Montigny D, Dewas M, Perrier G, Schaal B. 2006. A pheromone that rapidly promotes learning in the newborn. *Curr Biol* **16**: 1956–1961.
- Coureaud G, Thomas-Danguin T, Le Berre E, Schaal B. 2008. Perception of odor blending mixture in the newborn rabbit. *Physiol Behav* **95**: 194–199.
- Coureaud G, Languille S, Schaal B, Hars B. 2009. Pheromone-induced olfactory memory in newborn rabbits: Involvement of consolidation and reconsolidation processes. *Learn Mem* **16**: 470–473.
- Davis HP, Squire LR. 1984. Protein synthesis and memory: A review. *Psychol Bull* **96**: 518–559.
- Debiec J, Doyere V, Nader K, Ledoux JE. 2006. Directly reactivated, but not indirectly reactivated, memories undergo reconsolidation in the amygdala. *Proc Natl Acad Sci* **103**: 3428–3433.
- Desgranges B, Lévy F, Ferreira G. 2008. Anisomycin infusion in amygdala impairs consolidation of odor aversion memory. *Brain Res* **1236**: 166–175.
- Dudai Y, Eisenberg M. 2004. Rites of passage of the engram: Reconsolidation and the lingering consolidation hypothesis. *Neuron* **44**: 93–100.
- Gewirtz JC, Davis M. 2000. Using Pavlovian higher-order conditioning paradigms to investigate the neural substrates of emotional learning and memory. *Learn Mem* **7**: 257–266.
- Gruest N, Richer P, Hars B. 2004. Memory consolidation and reconsolidation in the rat pup require protein synthesis. *J Neurosci* **24**: 10488–10492.
- Kim JH, Richardson R. 2010. New findings on extinction of conditioned fear early in development: Theoretical and clinical implications. *Biol Psychiatry* **67**: 297–303.
- Languille S. 2010. "Dynamics of memory during postnatal development, study in rat pup." PhD thesis, Editions Universitaires Européennes, Sarrebruck.
- Languille S, Gruest N, Richer P, Hars B. 2008. The temporal dynamics of consolidation and reconsolidation decrease during post-natal development. *Learn Mem* **15**: 434–442.
- Languille S, Richer P, Hars B. 2009. Approach memory turns to avoidance memory with age. *Behav Brain Res* **202**: 278–284.
- McGaugh JL. 1966. Time dependent processes in memory storage. *Science* **153**: 1351–1358.
- McGaugh JL. 2000. Memory—A century of consolidation. *Science* **287**: 248–251.
- Merhav M, Rosenblum K. 2008. Facilitation of taste memory acquisition by experiencing previous novel taste is protein-synthesis dependent. *Learn Mem* **15**: 501–507.
- Miller JS, Molina JC, Spear NE. 1990. Ontogenetic differences in the expression of odor-aversion learning in 4- and 8-day-old rats. *Dev Psychobiol* **23**: 319–330.
- Montigny D, Coureaud G, Schaal B. 2006. Rabbit pup response to the mammary pheromone: From automatism to prandial control. *Physiol Behav* **89**: 742–749.
- Morel S, Sullivan RM. 2005. Neurobiology of infant attachment. *Dev Psychobiol* **47**: 230–242.
- Nader K. 2003. Memory traces unbound. *Trends Neurosci* **26**: 65–72.
- Nader K, Schafe GE, LeDoux JE. 2000a. The labile nature of consolidation theory. *Nat Rev Neurosci* **1**: 216–219.
- Nader K, Schafe GE, LeDoux JE. 2000b. Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature* **406**: 722–726.
- Raineki C, Shionoya K, Sander K, Sullivan RM. 2009. Ontogeny of odor-LiCl vs. odor-shock learning: Similar behaviors but divergent ages of functional amygdala emergence. *Learn Mem* **16**: 114–121.
- Rescorla RA. 1980. *Pavlovian second order conditioning: Studies in associative learning*. Erlbaum, Hillsdale, NJ.
- Sara SJ. 2000. Retrieval and reconsolidation: Toward a neurobiology of remembering. *Learn Mem* **7**: 73–84.
- Sara SJ, Hars B. 2006. In memory of consolidation. *Learn Mem* **5**: 515–521.
- Schaal B, Coureaud G, Langlois D, Ginies C, Semon E, Perrier G. 2003. Chemical and behavioral characterization of the rabbit mammary pheromone. *Nature* **424**: 68–72.
- Sekiguchi T, Suzuki H, Yamada A, Mizukami AM. 1994. Cooling-induced retrograde amnesia reflexes Pavlovian conditioning associations in *Limax flavus*. *Neurosci Res* **18**: 267–275.
- Sekiguchi T, Yamada A, Suzuki H. 1997. Reactivation-dependent changes in memory states in the terrestrial slug *Limax flavus*. *Learn Mem* **4**: 356–364.
- Wilson DA, Sullivan RM. 1994. Neurobiology of associative learning in the neonate: Early olfactory learning. *Behav Neural Biol* **61**: 1–18.
- Zarrow MX, Denenberg VH, Anderson CO. 1965. Rabbit: Frequency of suckling in the pup. *Science* **150**: 1835–1836.

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