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2-NAP, a peripheral CCK-A receptor antagonist, modulates the development of a preference for the mother by the newborn lamb

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Abstract

The aim of this study was to investigate the effect of devazepide (DEV), a cholecystokinin (CCK)-A receptor antagonist, which crosses the blood–brain barrier (BBB) and prevents the development of a preferential relationship with the dam, and compare it to that of 2-NAP, which acts peripherally. At birth, lambs received either an intraperitoneal injection of saline (CTL), DEV (0.1 mg/kg), or 2-NAP (0.01, 0.1, or 1 mg/kg). No major side effects were observed after the injection, however, there was a trend for lambs receiving CCK-A antagonists to be more vocal in the first 2 h and to loose more weight between birth and 3 h. When tested in a two-choice test situations at 24 h of age, the latency to reach a ewe and the total time spent next to them were not affected by the treatments. On the other hand, unlike CTLs, lambs receiving DEV or 0.1 mg of 2-NAP did not spend significantly more time near their dam than near the alien ewe. 2-NAP had no effect at 0.01 mg/kg, but at 1 mg/kg, it improved the preference score. There was no relationship between preference scores and the variations in weight recorded during the neonatal period. The effects of DEV, but not those of 2-NAP, persisted at 48 h of age. Thus, these results support the hypothesis that peripheral CCK receptors are involved in the development of a preference for the mother, but the effects differ according to the amount of 2-NAP that the lambs receive. © 2000 Elsevier Science Inc. All rights reserved.

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1. Introduction

The role of cholecystokinin (CCK) as a hormonal and a neural regulator of the gastrointestinal tract is well recognized [23]. This peptide is released from the proximal intestine in response to food entering the duodenum, and has been found both in the brain and in the periphery [24]. The effects of CCK are mediated by two types of receptors named CCK-A and CCK-B [6]. CCK-A receptors are mainly localized in the gastrointestinal tract (pancreatic acini, gall bladder, pyloric sphincter, smooth muscle cells of the ileum), but are also present in regions of the peripheral (vagus nerve) and central nervous systems [46]. CCK-B receptors are dominantly in the central nervous system, but exist in the gastrointestinal tract as well (pancreatic acini, stomach, vagus nerve) [46]. Both are members of the G protein-coupled superfamily of receptors [47], and exist in multiple affinity states and modes of G-coupling [16].

The post-prandial release of CCK from the small intestine has been proposed as a peripheral signal involved both in food-rewarded learning and in satiety. It has been demonstrated that intraperitoneal injection of CCK facilitates learning [12,40,49] and reduces food intake [7,13]. In both biological processes, the effect of exogenous CCK is mediated via Type-A receptors since the effects are blocked by devazepide (DEV), a selective CCK-A receptor antagonist [7,11,41,48]. Furthermore, recent findings have shown that rats of the OLETF strain, which lack CCK-A receptors, do not respond to the satiating effect of CCK [28] and show a deficit in learning and memory functions [31]. Lastly, the most convincing argument for a peripheral site of action of CCK is the fact that the effect of intraperitoneal injections of CCK on satiety and memory retention is abolished by vagotomy [12,18,27]. In conclusion, it appears that peripheral Type-A receptors and vagal afferent fibers are necessary both for the mediation of the satiating effect of CCK and for food-rewarded learning.

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In sheep, the facilitatory effect of suckling on the development of a preference by the neonate for its dam is mediated by CCK-A receptors. Twenty-four hours after birth, most lambs choose their mother rather than an alien ewe in a two-choice test [38]. Unrestricted access to the udder is a necessary step for the development of this preferential relationship: lambs prevented from suckling during the first 6 h after birth do not show any discrimination at 24 h despite the fact that they are left with their mother all the time, and have access to the udder from 6 h onwards [35]. On the other hand, when lambs deprived of neonatal suckling are tube-fed with colostrum, they develop a preference for their mother in an identical way to lambs that have been suckled by their dam from birth [14]. Furthermore, Nowak et al. [36] have shown that CCK is released in the plasma after suckling, and only lambs whose CCK levels increase in the first 6 h after birth display a preference for their mother at 24 h [35]. Pharmacological studies with selective CCK-receptor antagonists produced evidence of the involvement of Type-A but not Type-B receptors in the development of this preferential relationship [34]. Thus, the results obtained in newborn lambs suggest strongly that CCK is a physiological factor stimulating learning of maternal cues by acting on CCK-A receptors. The initial site of action of CCK is not known but, as in rodents, could well be peripheral. The use of DEV by Nowak et al. [34] did not resolve the issue since this antagonist crosses the blood–brain barrier (BBB) [15,40], and could have prevented the development of a preference for the mother by acting at brain level.

The use of vagotomy or capsaicin as a means of destroying vagal afferent fibers is not possible in our experimental situation unless performed in utero, an extremely difficult procedure. Therefore, we chose to use pharmacological tools instead. The aim of the present study was to test the involvement of peripheral CCK-A receptors in the development of a preference for the mother by comparing the effect of DEV to that of 2-NAP, which does not cross the BBB [2,17].

2. Methods

2.1. Animals

Forty-six multiparous Préalpes-du-Sud ewes were synchronized at estrus and treated with 30 μg (300 IU) of Pregnant Mare Serum Gonadotropin to induce multiple ovulation. One week before lambing, ewes were put in individual pens (1 × 2 m) and accustomed to human presence and handling. Parturition was induced by an intramuscular injection of 16 mg of dexamethasone (Intervet, Angers, France) on Day 143 of gestation. Only ewes displaying complete maternal behavior and lambs born without difficulties were included in the experiment. By these means, we obtained 71 lambs.

2.2. Treatments

All the procedures used in this experiment were in compliance with the European Community Council Directive of 24 November 1986 (86/609/EEC). At birth, lambs were ear-tagged, weighed, and allocated at random to one of five experimental groups. A single intraperitoneal injection (1 ml/kg) of a test or control substance was administered within minutes following birth. In twin pairs, each animal received a different treatment.

2.2.1. Controls

Lambs were given an injection of saline (CTL, $n=14$).

2.2.2. Devazepide

This benzodiazepine analogue (gift from Merck Sharp & Dohme Research Laboratories, West Point, PA) is a CCK-A receptor antagonist, which crosses the BBB efficiently. DEV was dissolved in CTL with one or two drops of Tween 80 at a concentration of 0.4 mg/ml, and suspended by ultrasonic dispersion for 10 min. Lambs received an injection of 0.1 mg/kg of $\mathrm{DEV}_{0.1}$ ($n=12$).

2.2.3. 2-NAP

The 2-naphtalenesulfonyl-L-aspartyl-2-(phenethyl) amide (gift from Dr. R.A.D. Hull, James Black Foundation, London, UK) is a selective CCK-A receptor antagonist, which does not cross the BBB because of its hydrophilic properties. 2-NAP was dissolved in distilled water. Lambs received an injection of either 0.01 ($\text{2-NAP}_{0.01}$, $n=14$) or 0.1 ($\text{2-NAP}_{0.1}$, $n=16$) or 1 mg/kg (2-NAP_{1}, $n=15$) of 2-NAP.

2.3. Preliminary observation

Each lamb was observed for 5 min every 20 min from the time it was born until 4 h later. The following data were recorded: time spent lying, time spent exploring the mother's body (exploration was defined as the lamb's nuzzle in contact with the body of the ewe), time spent suckling, number of bleats emitted, latency to stand up (time between birth and the first time the lamb was seen standing for at least 10 s), and latency to suck (time between birth and the first time the lamb was seen sucking for at least 10 s). Lambs were weighed at birth and at the age of 3, 6, 12, 24, and 48 h. These variables were recorded in order to check that the pharmacological treatments did not induce any major side effects on postnatal activity, in particular, the suckling behavior, which could have affected the subsequent growth of the lambs. Because of birth synchronization, it was not possible to record systematically all the variables for all the lambs. Consequently, the number of animals observed differs from the number of animals used in the testing procedures, and is specified in the results.

2.4. Testing pen and procedure

These were the same as in Nowak et al. [34,35], and will only be described briefly here. Lambs were tested at 24 h of age for their capacities to choose between their mother and an alien ewe in a triangular pen (5 × 4 × 4 m) made of 1-m high metal hurdles (Fig. 1). The base of the triangle consisted of three pens side by side, two of which contained the post-parturient ewes. The two ewes were separated by an empty pen (1 × 1 m). The lamb was placed at the opposite corner at the beginning of the 5-min test. The testing pen was divided into six areas, and only the 50-cm wide areas in front of the ewes were considered to be zones of contact between the tested lamb and the two maternal ewes (Zones 3 and 5 on Fig. 1), the others were designated neutral zones. During the test, lambs could make contact with the ewes, as the bars of the hurdles left sufficient space to allow reciprocal nosing and sniffing, but they were unable to reach the udder. We recorded the following data: (1) time to enter each contact zone, (2) time spent in the contact zone near the mother, or (3) near the alien ewe, (4) time spent in the neutral zone, and (5) number of zones crossed by the test lamb. A second test identical to the first one was performed 48 h after birth to determine whether the lambs had recovered from the treatments.

Mother preference was assessed in two ways. First, the times spent in the contact zone near the mother and the alien ewes were compared within each group for two periods of the test: (a) during the first minute spent in the contact zones and (b) during the whole duration of the 5-min test [34]. Lambs that did not reach the ewes by the end of the test were excluded from the analysis. Secondly, for each subject, an index of preference (IP) was defined as follows: IP=(time spent near the mother – time spent near the alien ewe)/(total time spent near either ewe). This IP value expressed the ability of the lamb to discriminate between the two ewes, its maximum value is 1 when the lamb spent all of the time near its mother, and the minimum value is -1 if it spent all of the time near the alien ewe [14]. Three lambs spent only a few seconds near one of the two ewes, but were scored with minimum or maximum IP values. These extreme values were considered as meaningless in terms of a preference for an individual and therefore were excluded from the analysis. Only the lambs, which spent more than 1 min in the contact zones, were taken into consideration in the analysis of IP values. A lamb was considered to display a preference for its mother if $IP \geq 0.33$, a preference for the alien ewe if $IP \leq -0.33$, and no preference if $-0.33 < IP < 0.33$. The threshold (± 0.33) delimiting the three classes of lambs was defined according to the results obtained by Nowak et al. [37]. In this previous study, a lamb was considered to choose its mother correctly if it had spent more than 3 min (60% of the duration of the test) with the ewes and at least two-thirds of this time near the mother.

2.5. Statistical analysis

All the variables were analyzed by non-parametric tests. Behavioral data collected at 20-min intervals within the 4 h after birth were pooled in 1-h blocks as follows: 0–1 h, 1–2 h, 2–3 h, and 3–4 h. The blocks were compared for differences between and within groups. For independent samples, the Kruskal–Wallis test was employed followed by the Mann–Whitney *U* test for any pairwise comparisons to detect differences between groups. The Friedman test was chosen to assess the effect of time within each group. For dependent samples, the Wilcoxon test for pairwise comparisons was used. *P* values less than .05 were considered as statistically significant, and *P* values between .05 and .10 were considered as showing a statistical tendency.

3. Results

3.1. Neonatal activity (from birth to 4 h)

Complete sets of observations were made from birth to 4 h of age for 49 out of the 71 lambs (CTL: $n=6$; $DEV_{0.1}$: $n=9$; 2-$NAP_{0.01}$: $n=10$; 2-$NAP_{0.1}$: $n=12$; 2-NAP_1: $n=12$). The analysis did not reveal any significant effect of the pharmacological treatments on the latencies to stand up and to suckle, nor on the time spent lying or exploring the mother's body. Lambs from all the groups displayed the same basic behavioral pattern. There was a peak of activity between 1 and 2 h due to the search for the udder until the teat was found [mean latency to find the teat (min) ± S.E.M.: CTL: 53.0 ± 8.0; $DEV_{0.1}$: 63.0 ± 4.5; 2-$NAP_{0.01}$: 82.5 ± 13.5; 2-$NAP_{0.1}$: 73.5 ± 12.9; 2-NAP_1: 61.4 ± 6.4]; once the lambs had successfully suckled, they explored their mother's body very little. Although there was a tendency for 2-$NAP_{0.1}$ lambs to be seen suckling less often than CTLs (P=.053), the time spent suckling was not significantly affected by the treatment [mean (min) ± S.E.M.: CTL: 333.3 ± 78.8; $DEV_{0.1}$: 389.9 ± 103.8; 2-$NAP_{0.01}$: 190.9 ± 41.5; 2-$NAP_{0.1}$:

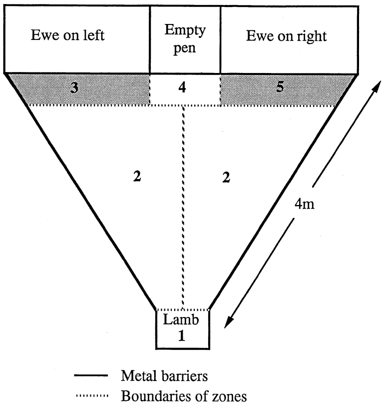

Fig. 1. Plan of the pen used to test mother preference. Zones 3 and 5 are the zones of contact with the ewes. Zones 1, 2, and 4 are the neutral zones.

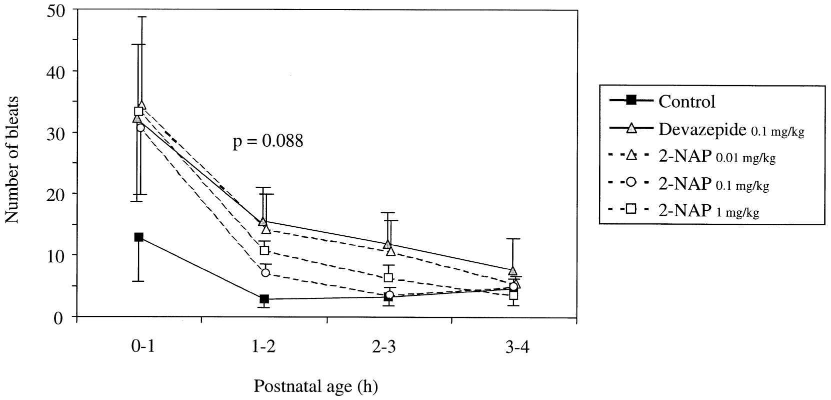

Fig. 2. Vocal activity of lambs during the first 4 h after birth. At birth, lambs received a single injection of CTL ($n=6$) or one of the two CCK-A antagonists: $DEV_{0.1}$ ($n=9$) or 2-NAP ($2\text{-}NAP_{0.01}$, $n=10$; $2\text{-}NAP_{0.1}$, $n=12$; $2\text{-}NAP_1$, $n=12$). Data (mean ± S.E.M.) are expressed in number of bleats per 1-h block.

139.8 ± 29.2; $2\text{-}NAP_1$: 209.6 ± 25.9]. The number of vocalizations decreased significantly over time for $2\text{-}NAP_{0.01}$ ($P=.002$), $2\text{-}NAP_{0.1}$ ($P=.001$), and $2\text{-}NAP_1$ ($P=.002$) but not for the other groups. Fig. 2 shows that overall, CTL lambs vocalized less that those receiving the CCK-A antagonist and the Kruskal–Wallis ANOVA revealed a tendency for a difference between groups during the 1–2-h period ($P=.08$). Pairwise comparisons revealed that CTL lambs were less vocal than $DEV_{0.1}$ ($P=.058$), $2\text{-}NAP_{0.1}$ ($P=.029$), and $2\text{-}NAP_1$ lambs ($P=.018$) at 1–2 h.

3.2. Weight and rectal temperature of the lambs (from birth to 48 h)

Lambs' body weight evolved similarly in each group: they lost between 50 and 250 g between birth and 3 h ($P<.05$, in all cases) after which they gained weight regularly. However, except for $2\text{-}NAP_{0.1}$ lambs, those receiving an injection of CCK-A antagonist lost more weight within 3 h of birth than did CTL lambs ($P<.05$, Fig. 3). Weight gain from 3 h of age onwards was nonetheless similar and significant ($P<.05$) in all groups. By 2 days of age, most lambs had recovered their birth weight. Rectal temperatures were stable; values ranged between 39°C and 40°C for all groups.

3.3. Preference for the mother at 24 h

When a lamb was released in the testing pen, it bleated immediately, and both ewes responded by emitting high-pitched bleats. Seven out of seventy-one lambs did not reach either contact zone by the end of the 5-min test, and were excluded from all the statistical analyses (one CTL, one $DEV_{0.1}$, one $2\text{-}NAP_{0.01}$, three $2\text{-}NAP_{0.1}$, and one $2\text{-}NAP_1$). Once near the ewes, lambs bleated less and occasionally tried to reach a ewe through the bars of the hurdles. The ewes investigated the lamb when it was close to them, mothers emitted low-pitched bleats (maternal bleats) as well as high-pitched bleats, whereas alien ewes emitted high-pitched bleats only. There was no difference between groups in the time taken to reach the ewes, total time spent in the contact zones, time spent in the neutral zones, nor in the number of zones crossed by lambs.

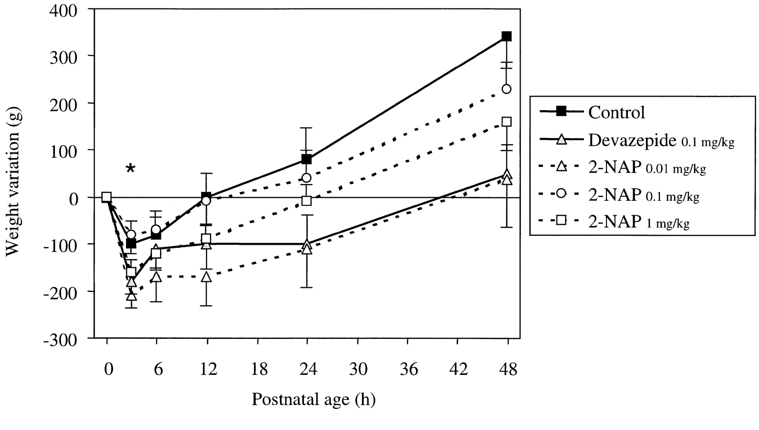

Fig. 3. Mean weight evolution of the lambs during the first 2 days following birth according to the pharmacological treatments. At birth, lambs received a single injection of CTL ($n=14$) or one of the two CCK-A antagonists: $DEV_{0.1}$ ($n=12$) or 2-NAP ($2\text{-}NAP_{0.01}$, $n=14$; $2\text{-}NAP_{0.1}$, $n=16$; $2\text{-}NAP_1$, $n=15$). Data are expressed as variation of the birth weight (* $P<.05$).

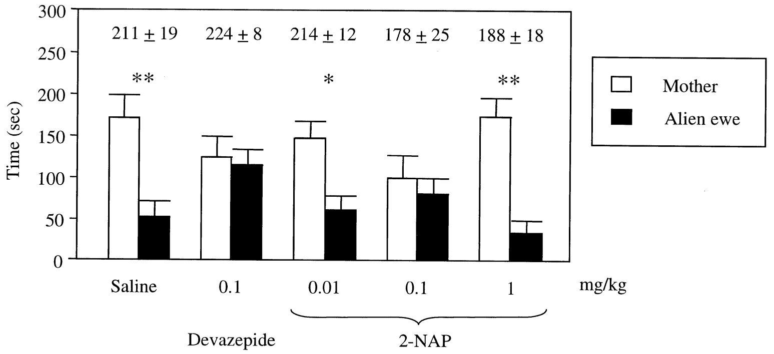

Fig. 4. Time(s) spent in the contact zone either near the mother (open bars) or near the alien ewe (black bars) in a two-choice test by 24-h-old lambs (mean ± S.E.M.), according to pharmacological treatments. At birth, lambs received a single injection of CTL ($n = 13$) or one of the two CCK-A antagonists: DEV$_{0.1}$ ($n = 11$) or 2-NAP (2-NAP$_{0.01}$, $n = 13$; 2-NAP$_{0.1}$, $n = 13$; 2-NAP$_1$, $n = 14$). Values above the bars represent the total time [mean(s) ± S.E.M.] spent in the contact zone during the entire duration of the test ($**P < .01$; $*P < .05$).

Fig. 4 shows that CTL, 2-NAP$_{0.01}$, and 2-NAP$_1$ lambs spent significantly more time near the mother than near the alien ewe ($P < .05$ in all cases). Furthermore, in the first minute of contact, there was a strong tendency for 2-NAP$_1$ lambs to spend more time near the mother than near the alien ewe (40.9 ± 6.8 vs. 17.6 ± 6.4, $P = .062$). This was not the case for CTL and 2-NAP$_{0.01}$ lambs. By contrast, DEV$_{0.1}$ and 2-NAP$_{0.1}$ lambs did not show any preference for the dam. Three lambs spent less than 1 min near the two ewes, and were therefore excluded from the analysis of the IP (one CTL, one 2-NAP$_{0.01}$, and one 2-NAP$_1$). The treatment effect on the time spent near the mother or the alien ewe within each group was confirmed by the analysis of the IP. Fig. 5 shows that 10/12 CTL, 7/12 2-NAP$_{0.01}$, and 10/13 2-NAP$_1$ lambs displayed a clear preference for their mother. Consequently, the mean IP score of these three groups was well above the set value of 0.33 (mean ± S.E.M.: IP = 0.55 ± 0.16, IP = 0.43 ± 0.14, and IP = 0.64 ± 0.16, respectively). Further comparison of individual cases showed that 8/13 2-NAP$_1$ lambs reached a maximum IP value of 1, whereas only 1/12 CTL lambs did so. This difference was significant ($P = .011$, Fisher test). By contrast, DEV$_{0.1}$ and 2-NAP$_{0.1}$ lambs were spread rather evenly between the two extreme IP values, and the means of these two groups were close to 0 (IP = − 0.03 ± 0.18 and IP = − 0.02 ± 0.21, respectively). IP values differed significantly between groups ($P = .013$): CTL and 2-NAP$_1$ lambs had higher scores than DEV$_{0.1}$ ($P < .01$ in both cases) and 2-NAP$_{0.1}$ lambs ($P = .068$ and $P = .018$, respectively), but did not differ between themselves nor from 2-NAP$_{0.01}$ lambs. No relationships were recorded between the preference scores and the variables measured during the neonatal period, the weight variations, or the rectal temperatures.

3.4. Preference for the mother at 48 h

When tested for the second time, CTL and 2-NAP$_{0.1}$ lambs spent significantly more time near the mother than near the alien ewe during the entire 5-min test [mean(s) ± S.E.M.: CTL: 156 ± 22 vs. 51 ± 12 and 2-NAP$_{0.1}$: 139 ± 19 vs. 46 ± 11, $P < .05$ in both cases]. 2-NAP$_{0.01}$ and 2-NAP$_1$ lambs spent more time near their mother than

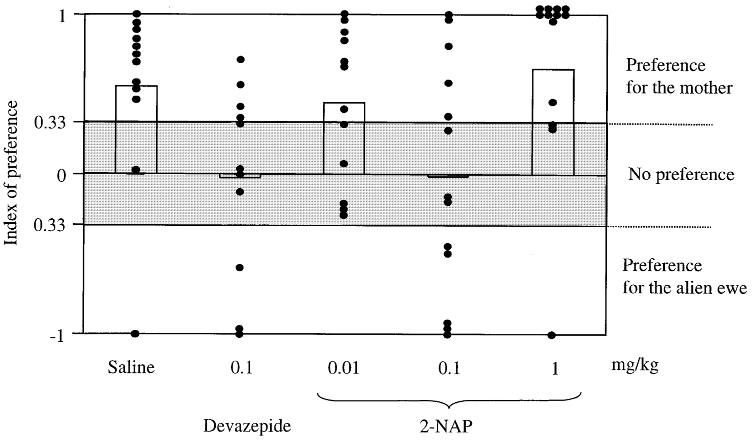

Fig. 5. IP at 24 h of age, according to pharmacological treatments. At birth, lambs received a single injection of CTL ($n = 12$) or one of the two CCK-A antagonists: DEV$_{0.1}$ ($n = 11$) or 2-NAP (2-NAP$_{0.01}$, $n = 12$; 2-NAP$_{0.1}$, $n = 13$; 2-NAP$_1$, $n = 13$). Individual values are represented by dots and the mean IP for each group by vertical bars.

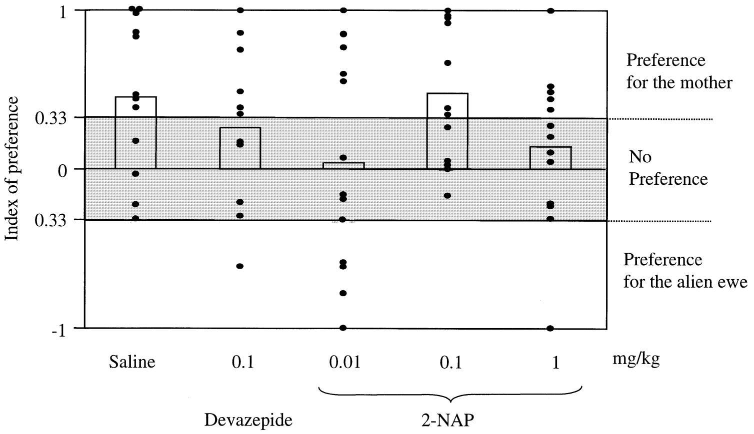

Fig. 6. IP at 48 h of age, according to pharmacological treatments. At birth, lambs received a single injection of CTL ($n=12$) or one of the two CCK-A antagonists: $DEV_{0.1}$ ($n=11$) or 2-NAP ($2\text{-}NAP_{0.01}$, $n=13$; $2\text{-}NAP_{0.1}$, $n=11$; $2\text{-}NAP_{1}$, $n=13$). Individual values are represented by dots and the mean IP for each group by vertical bars.

near the alien ewe only in the first minute of contact [mean(s) ± S.E.M.: $NAP_{0.01}$: 43.3 ± 6.5 vs. 15.6 ± 3.5 and $2\text{-}NAP_{1}$: 42.6 ± 5.9 vs. 16.6 ± 3.9; $P<.05$ in both cases]. Lambs treated with DEV did not display a preference for their mother at any moment of the test. For the analysis of the IP, four lambs spent less than 1 min near the two ewes and were excluded (one CTL, two $2\text{-}NAP_{0.1}$, and one $2\text{-}NAP_{1}$). Only CTL and $2\text{-}NAP_{0.1}$ lambs had a mean IP value above the threshold set for a preference for the mother (CTL: IP = 0.47 ± 0.14 and $2\text{-}NAP_{0.1}$: IP = 0.50 ± 0.13; Fig. 6). However, IP values did not differ significantly between groups, nor did the time to reach a ewe, the time spent in the contact or the neutral zones, or the number of zones crossed.

4. Discussion

Analysis of the behavior of the lambs during the two-choice tests, both at 24 and 48 h, did not suggest that their motivation to make contact with any ewe and stay in the contact zones were influenced by the pharmacological treatments. Of all the variables taken into account, only those relative to the expression of a preference for the mother were affected (time spent near the mother vs. time spent near the other ewe, and IP). DEV prevented the development of a relationship with the mother in a similar manner to that described in our previous study [34]. Concerning 2-NAP, its inhibitory effect appears across a narrow dose range, yielding a U-shaped curve. A clear inhibition due to 2-NAP was obtained with the intermediate dose of 0.1 mg/kg, and is similar to that of DEV. This result indicates that blocking peripheral CCK-A receptors is sufficient to prevent the development of a preference for the mother. Neonatal ingestive behavior [14] and the post-prandial release of CCK [35] seemingly facilitate neonatal learning of maternal cues by acting on CCK-A receptors, and the development of the relationship with the mother is more rapidly established. Like in rodents, the vagus nerve is likely to be the relay involved in the CCK-A receptor-mediated response [11,12,19]. The absence of an inhibitory effect with 0.01 mg/kg of 2-NAP suggests that this concentration was not sufficient to block all of the CCK-A receptors, and therefore did not impair neonatal learning of maternal cues. In our previous work [34], the use of 0.01 mg/kg of DEV prevented the development of a preference for the mother. Although we cannot compare directly these two results, the fact that 2-NAP displays less affinity for CCK-A receptors than DEV (K_i: 250 vs. 0.1 nM) [30] could explain its lack of effect at the lowest dose. More surprising is the fact that lambs treated with the highest dose of 2-NAP (1 mg/kg) had improved preference scores. This is supported by the fact that $2\text{-}NAP_{1}$ lambs displayed discriminative abilities as early as the first minute of contact with the ewes, which was not the case for CTLs. Moreover, during the 5-min test, 8/13 $2\text{-}NAP_{1}$ lambs spent all of the time near their mother, whereas the ratio was 1/12 for CTL lambs. In a two-choice situation, 24-h-old lambs usually approach a ewe at random and choose their dam once at close quarters (less than 50 cm) [35,37,38]. It is only at 3 days of age that they walk or run straight to their mother and do not make contact with the alien ewe [33]. In the present experiment, $2\text{-}NAP_{1}$ lambs had clearly better preference scores compared to CTLs suggesting that the treatment had facilitated the development of a preference for the mother instead of inhibiting it. Two hypotheses could explain this finding. The first concerns a possible loss of specificity of 2-NAP for CCK-A receptors, a case rather commonly observed in pharmacological studies. Lemaire et al. [20] described the same phenomenon for a CCK-B agonist used in a test of olfactory recognition in rats. Although highly selective, 2-NAP has lower selectivity for CCK-A receptors than DEV (ratio of CCK-A/CCK-B is 300:3750) [30]. Therefore, at high doses 2-NAP could bind to peripheral CCK-B receptors and have opposite effects. CCK-B receptors are indeed widespread in the gastrointestinal tract as they correspond to gastrin receptors [30]. In particular, autoradiographic studies in rats [5] and rabbits [25] have described the presence of CCK-A and CCK-B receptors on the vagus nerve. No data are available in lambs, but studies on adult sheep have shown a predominance of Type-B receptors [10]. From a behavioral point of view, intraperitoneal injections of CCK-B antagonists have facilitating effects on learning and memory. Lemaire et al. [20,21] have demonstrated that CI-988, L-365260, and LY-262691 enhance memory retention in a model of social olfactory recognition in rats. Similarly, we found that the performance of lambs treated with PD135158 was better than that of CTL lambs suggesting facilitatory properties of this CCK-B antagonist on the development of a preference for the mother [34]. The fact that 2-NAP$_1$ lambs behave in a similar way to PD135158-treated lambs suggests an action on CCK-B receptors. The second hypothesis is that 2-NAP might interact with different affinity states of CCK-A receptors. CCK-A receptors exist in multiple affinity states for CCK-8 [16,22,51]. The COOH-terminal heptapeptide analog CCK-JMV-180 is believed to interact with the different classes of CCK-A receptors, acting as an agonist at the high-affinity and low-capacity sites but as an antagonist at the low-affinity and high-capacity sites [22,44]. In lambs, 2-NAP could interact in a similar way with the different affinity states of CCK-A receptors, and cause opposite effects according to the dose used. Thus, at 0.1 mg/kg, it could act as an antagonist and inhibit the stimulating effect of endogenous CCK. By contrast, at 1 mg/kg, it could act as an agonist, facilitate neonatal learning, and consequently improve the development of a preferential relationship with the mother.

The effect of DEV persisted at 48 h of age confirming previous data [34] while 2-NAP had a transient effect. There is no obvious explanation for this differential effect, but it suggests that these two pharmacological agents do not have the same duration of action. Unlike CTLs, 2-NAP$_{0.01}$, and 2-NAP$_1$ lambs did no longer express a preference for their mother during the 5-min test at 48 h. This change in the lambs' performance is probably due to repetitive testing, a phenomenon already mentioned in previous studies [14,34] and not to a sudden impairment of their discriminative abilities. The second testing session is believed to be less stressful as the whole procedure is no longer novel to them, and therefore, lambs do not necessarily maintain proximity with their dam. This is supported by the fact that 2-NAP$_{0.01}$ and 2-NAP$_1$ lambs displayed a preference for their mother in the first minute of contact after which they left her and explored the testing pen. It was noticed that lambs were frolicking, sniffing the straw, and observing the alien ewe more frequently during the second than during the first testing session.

The behavioral differences observed during the choice test suggest that 24 h after birth, CTL, 2-NAP$_{0.01}$, and 2-NAP$_1$ lambs had memorized some maternal features and recognized their dam during the test, while DEV$_{0.1}$ and 2-NAP$_{0.1}$ lambs had not. On the other hand, the lamb's choice could also be influenced by the behavior of the ewes. Although we did not record it, the mother and the alien ewe indeed behaved differently during the test. Because of their selective maternal behavior, the mothers behaved in a way familiar to the lambs (emission of low-pitched bleats, licking, or nosing), whereas the alien ewes displayed unfamiliar behavior (emission of high-pitched bleats, threats, few attempts at licking, or nosing). Poindron et al. [39] have shown that acceptance behaviors are an important element in orienting the choice of the lambs: 24-h-old lambs express a strong preference for their mother when tested with two selective ewes, but not in the presence of two anosmic ewes, which are not selective. It appears that low-pitched bleats are strongly attractive to the lamb. The variability of the lambs' responses in the present work could well be explained in terms of differences in maternal behavior, and we cannot conclude whether lambs chose their mother on the basis of individual characteristics or general maternal features (acceptance behavior). Nevertheless, whatever information the lambs rely on to make a choice, our previous work using food reward [14] or suppressing it [35], or using pharmacological agents known for their amnesic properties [34] give strong indications that the preference score reflects a true recognition process.

Although CCK-A antagonists are known to influence food intake, several facts suggest that the lack of preference for the mother at 24 h is not due to a direct action on satiety. Firstly, though as a general trend, lambs treated with CCK-A antagonists lost more weight between birth and 3 h than did CTLs (see Fig. 3), this did not influence their performance during the choice test at 24 h. Thus, although 2-NAP$_{0.01}$ lambs lost more weight than CTLs, they displayed similar performances in the test. In addition, weight variations of CTL and 2-NAP$_{0.1}$ lambs follow a similar pattern, but only the former developed a preference for their mother. The same is observed for 2-NAP$_{0.01}$ and DEV$_{0.1}$ lambs: they both had a considerable neonatal weight loss and an identical subsequent low growth rate, yet the inhibitory effect was only obtained with DEV. The second objection to an effect of the pharmacological treatments on satiety in lambs is that in other species, whenever an effect was recorded, CCK-A antagonists always induced an increase in food intake since they block the satiating effect of CCK [1,9,17,29,43]. In all likelihood, DEV should have induced an increase in colostrum intake compared to control lambs in a similar way to what has been described for independent feeding in rat pups [50]. Instead, the reverse was observed. Lastly, the factors controlling food intake in suckled young are still poorly understood. Work concerning the ontogeny of the satiating effect of CCK in rodents has shown that it depends on the age of the pup and the mode of ingestion. According to Blass et al. [3], CCK does not reduce milk ingestion of 5-

to 10-day-old rats, but it does reduce the intake of 15-, and especially, of 25-day-old rats. Lorenz [26] has revealed that while CCK has no effect on milk intake in suckling pups, it does suppress the intake in pups of the same age ingesting either bovine light cream or chow pellets in the adult mode. The basis for the failure of CCK to influence suckling in young pups is unclear. But this points out that the suckling act is distinguished from the adult form of feed: internal stimuli do not play a significant role until the young reaches a developmental stage where eating and drinking free food begin. The greater neonatal weight loss in lambs treated with CCK-A antagonists, as noted in our previous study [34], cannot yet be explained but is unlikely to reflect lower feeding activity. 2-NAP also abolishes the inhibitory effect of CCK on food intake in adult rats [2], but unlike DEV, does not have an influence on food ingestion per se [8].

Another effect of the CCK-A antagonists during the neonatal period concerns the vocal activity. The decrease in the number of vocalization by newborn lambs has been described previously [32]. Lambs are usually most vocal in the hour following birth, before they explore their mother's body and locate the udder. After suckling, they bleat much less frequently. Blass and Shide [4] have shown that the calming effect of milk in 10-day-old rats is CCK-mediated: DEV-treated pups emitted more ultrasonic vocalizations that those receiving CTL. The lamb's neonatal vocal activity also seems to be under the influence of endogenous CCK as animals receiving a CCK-A antagonist were more vocal than CTLs in the first 2 h after the injection, although no statistical differences appeared from the analysis. The wide inter-individual variation associated with the unfortunate small sample size of the CTL group is likely to be responsible for this. A tendency for a difference was nonetheless obtained between 1 and 2 h after birth, in other words, after the lambs had found the teat and ingested some colostrum. This suggests that the potential calming effect of the post-prandial release of CCK has been lessened.

In conclusion, the finding that 2-NAP affects the establishment of mother preference supports the hypothesis that this behavior is mediated by peripheral CCK receptors. Although we have no experimental proof that 2-NAP unlike DEV does not act on the brain, the BBB of lambs is relatively mature at birth [42,45], and there is good anatomical and physiological evidence to expect that 2-NAP does not reach central receptors. Thus, the vagus nerve, which contains CCK receptors is a good candidate for relaying the information from the periphery to the brain.

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