Pharmacology Biochemistry & Behavior, Vol. 37, pp. 219–225. © Pergamon Press plc, 1990. Printed in the U.S.A. 0091-3057/90 $3.00 + .00

Peripheral Serotonergic Inhibition of Suckling

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Received 11 April 1990

BATEMAN, S. T., A. H. LICHTMAN AND C. P. CRAMER. *Peripheral serotonergic inhibition of suckling*. PHARMACOL BIOCHEM BEHAV **37**(2) 219–225, 1990.—Systemically administered serotonin, which does not pass the blood-brain barrier, inhibited nipple attachment behavior in 20- and 30-day-old rat pups. Xylamidine, a peripheral serotonin antagonist, attenuated the effects of serotonin, quipazine, and fenfluramine on nipple attachment behavior. Thus, serotonin receptors in the periphery may play an important role in the serotonergic inhibitory mechanism that has been hypothesized as the developing system leading to weaning. However, unlike more general 5-HT antagonists, xylamidine given alone failed to facilitate suckling, suggesting different sites of action for facilitation and inhibition of this infantile behavior.

Xylamidine Weanling rats Quipazine Fenfluramine Serotonin Nipple attachment

SUCKLING is neonatal rat pups' most important behavior during the first 2–3 weeks of life; their sole means of getting nutrition is attachment to the dam's nipples. This behavior, so essential to their early survival, begins to decline at Day 20 and disappears completely after about 35 days of age (2,10). Attempts to identify mechanisms underlying this transition have focused on both experiential (31) and pharmacological (29) factors.

Pharmacological work has concentrated primarily on the neurotransmitter serotonin. Serotonin receptor sites occur throughout the brain, including in the hypothalamic areas, which have been implicated in adult feeding behavior (1). Serotonin receptors also occur extensively in the gut, where they might be directly involved in food intake. Manipulations of serotonergic metabolism have been shown to have significant effects on adult food consumption (4).

Acute injections of serotonin agonists and antagonists also alter nipple attachment behavior (29,35). This relationship between suckling and serotonin appears to change with transitions in suckling behavior. Spear and Ristine (35) found that 3- or 4-day-old rats would inhibit nipple attachment if given serotonin antagonists. On the other hand, Nock *et al.* (29) found that serotonin antagonists administered to 20-day-old rats would facilitate nipple attachment behavior. These two studies demonstrated that a drug acting to block serotonin receptors had opposite effects at different stages of development. Williams, Rosenblatt and Hall (37) found that the inhibitory effect of serotonin agonists seems to appear around 15 days of age. They theorized that the pups eventually wean because a developing serotonin system increasingly inhibits suckling after 15 days of age. Leshem and Kreider (22) found similar ontogenetic trends following chronic depletion of serotonin with the neurotoxin 5,7-dihydroxytryptamine on Day 3. However, Lichtman, McLaughlin and Cramer (24) found that such central depletion on Day 20 had little effect on methysergide-induced nipple attachment on Day 25, suggesting that additional sites or neurochemical systems may be involved.

Although suckling and adult feeding are very different behaviors (18), they both are affected by serotonergic manipulations. Understanding the mechanisms underlying transitions in suckling behavior at weaning could clarify both adult feeding behavior and fundamental developmental processes. The goal of the present experiments was to increase understanding of the role of neurotransmitters on nipple attachment behavior in weanling rats by localizing serotonin effects in either the brain or the periphery. In 20-day-old rat pups, the serotonin agonist, quipazine, inhibits nipple attachment, and the serotonin antagonist, methysergide, facilitates nipple attachment (29). However, both methysergide and quipazine pass into the brain; serotonin inhibition of suckling could be either centrally or peripherally controlled. Because 98% of all serotonin receptors are located in the periphery (21), it should be elucidated whether this change in behavior results from stimulation in the area of most receptors, such as the gut region, or the 2% of the receptors that are in the brain. The use of serotonin and xylamidine, which do not readily pass the blood-brain barrier, allowed this distinction to be made.

EXPERIMENT ONE

Serotonin, given subcutaneously, does not pass through the blood-brain barrier (30). Pollock and Rowland (33) found that anorectic doses of serotonin produced a dose-related decrease in

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food intake in hungry adult rats and that these doses had no effect on locomotion, activity, sensorimotor performance, or taste aversion. Previous studies linking serotonin to suckling behavior have primarily used serotonin agonists and antagonists instead of serotonin itself (29,37). The use of serotonin, which acts only peripherally when injected subcutaneously, allows more specificity than these previous experiments.

METHOD

Subjects

Long-Evans rats and their pups were used in all experiments. All were housed in polypropylene tub cages (25 × 45 × 20 cm) with pine shavings as bedding and stainless steel wire lids. They were fed with Prolab 3000 Animal chow, and tap water was always available. They were kept on a 14:10 LD cycle with lights on at 0600 EST. Room temperature ranged from 21 to 26°C with humidity uncontrolled. Litters were culled to 10 pups 3 days after birth. Four pups from each of 9 litters were age 20 days (day of birth = Day 0) on the day of testing. The same procedures were used for 10 litters of 30-day-old pups. The tested litters were deprived of food and maternal care for 8 hr (20-day-old) or 24 hr (30-day-old) prior to testing. They were injected and tested between 1800–2000 hr. An additional group of dams, whose own pups were 16–23 days of age, were used as stimulus dams during testing.

Procedure

Eight hr prior to testing, 4 pups were removed from the dam's cage and placed together in a similar cage. After 13 days of age, only food-deprived pups reliably attach and suckle (16); therefore, the pups did not have further access to either a lactating dam or solid food until after the experiment.

Fifteen min prior to testing, the pups were weighed and marked with colored ink for easy identification. They were injected with isotonic saline or serotonin (1, 5, or 10 mg/kg, SC). The experimenter had no knowledge of the contents of any injection.

For the tests the stimulus dam was anesthetized (0.25 cc, Nembutal, IP) to remove active maternal contributions to suckling (16,17), placed supine to expose all nipples, and a Plexiglas divider fitted over her neck to protect her head from the pups. Because an anesthetized dam will not let down milk (25), milk extraction by the pups was not a factor when analyzing the results.

The latency to attach was computed from the time of placement in the test cage. In addition, at 2.5-min intervals for a total of 30 min, the rats were classified as attached or not attached to the dam's nipple. The pups were not handled during this time except that they occasionally were moved slightly to confirm attachment. Data were analyzed by ANOVA, with drug condition treated as a within-litter (repeated) factor.

RESULTS AND DISCUSSION

Figure 1a represents the total attachment time for the 20-day-old pups. An analysis of variance of the data revealed a significant effect of drug, $F(3,32) = 5.1$, $p<0.01$. This decrease in nipple attachment time was dose-dependent [linear-trend analysis, $F(1,32) = 15.24$, $p<0.01$]. Post hoc analysis revealed that both the 5 mg/kg and the 10 mg/kg doses were significantly different from saline controls (Newman-Keuls, $p<0.05$).

Figure 1b presents the latency to attach for the 20-day-old pups. Again, analysis of variance revealed a significant effect of drug, $F(3,32) = 3.64$, $p<0.05$. The increase in latency time was also dose-dependent [linear-trend analysis, $F(1,32) = 10.19$, $p<0.01$]. The post hoc analysis revealed that the 10 mg/kg dose

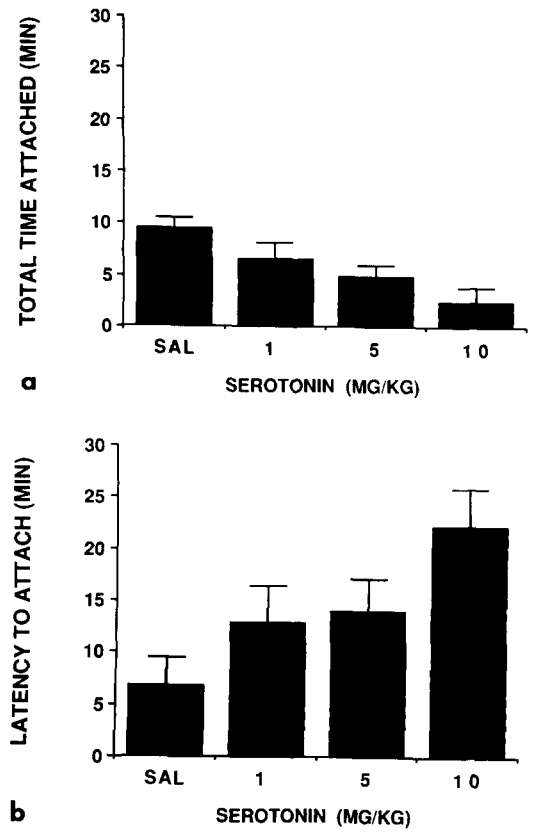

FIG. 1. Effects of different doses of serotonin on nipple attachment in 20-day-old rat pups. Graph a represents the mean (±SEM) total time attached during the 30-min test. Graph b presents the mean (±SEM) latency to attach.

was significantly different from the saline control (Newman-Keuls, $p<0.05$).

The data on total attachment time in the 30-day-old pups is presented in Fig. 2a. There was a significant effect of drug, $F(3,36) = 2.93$, $p<0.05$. The effect on decrease in total attachment time was dose-dependent [linear-trend analysis, $F(1,36) = 8.42$, $p<0.01$].

Figure 2b represents the data on the latency to attach in the 30-day-old pups. The effect of drug was not significant, and none of the groups differed significantly after post hoc analysis. There was, however, a dose-dependent increase in latency time [linear-trend analysis, $F(1,36) = 4.89$, $p<0.05$].

Inhibition of nipple attachment by serotonin is consistent with other agonist effects on suckling behavior. Because serotonin does not enter the brain, this inhibition appears to be influenced by peripheral receptors.

The serotonin-injected pups had high incidences of both scratching at the site of injection and nose- and foot-biting. Also apparent was a general inactivity and drooping eyelids. These characteristics have been called the "serotonin syndrome" (32), and might have influenced the results. The rat pup's system may still be more sensitive than that of adult rats. Pollock and Rowland (33) found no secondary, or "syndrome," behaviors from comparable anorectic doses of serotonin in adult rats.

EXPERIMENT TWO

Systemically administered xylamidine has been shown to

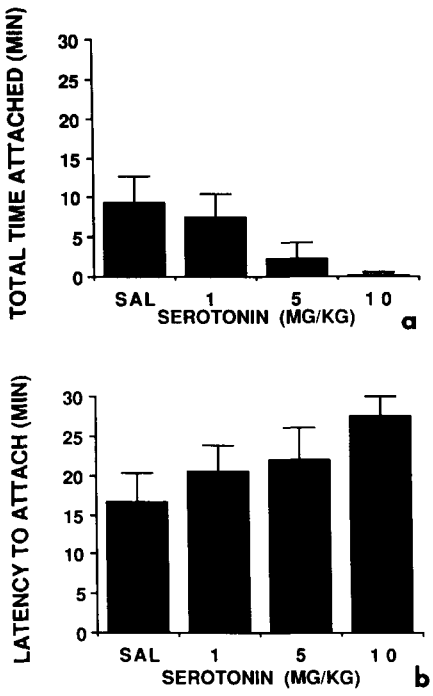

FIG. 2. Effects of different doses of serotonin on nipple attachment in 30-day-old rat pups. Graph a represents the mean (±SEM) total time attached during the 30-min test. Graph b presents the mean (±SEM) latency to attach.

selectively block only peripheral serotonin receptors (8,14). Fletcher and Burton (12,13) found a dose-dependent anorexia similar to that found by Pollock and Rowland (33) after injecting serotonin SC into adult rats. They determined that peripheral serotonin receptors can inhibit feeding behavior because xylamidine attenuated serotonin's anorectic effects.

The present experiment uses procedures similar to those of Fletcher and Burton (12), but with infant rats, using nipple attachment behavior as the dependent variable. Xylamidine is hypothesized to have similar effects on suckling behavior as it does in adult feeding behavior. If increased serotonin activation in the periphery is the cause of inhibition, then the effects of serotonin should be attenuated by xylamidine pretreatment.

METHOD

Subjects and procedures were similar to those described in Experiment 1; only 20-day-old rats were tested. The procedural changes occurred 5 hr after initial food deprivation, which was 3 hr prior to testing. At this time, the 4 pups were weighed and marked as in the first experiment. Half the pups then injected with xylamidine (3 mg/kg, IP), and the other half received an equivalent volume injection of isotonic saline. The pups were returned to their deprivation cages for another 3 hr.

Fifteen min prior to testing, the pups were treated exactly as they had been in the first experiment, but in this case half of the xylamidine-treated pups and half of the saline-treated pups received an injection of serotonin (7.5 mg/kg, SC), while the other half received saline. The 5-HT dosage chosen was well within the effective range in Experiment 1. The testing and analysis procedures were identical. Thirty-two pups from 8 litters provided N = 8/cell.

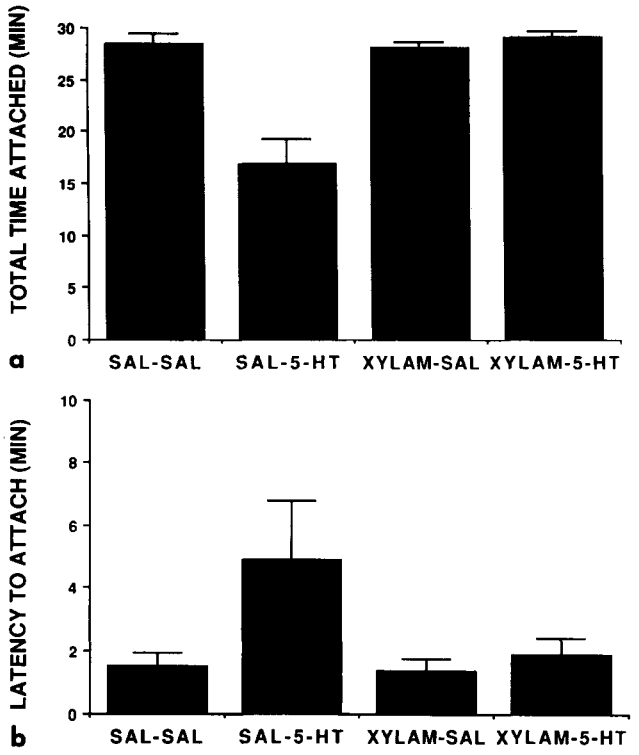

FIG. 3. Effects of serotonin and xylamidine on nipple attachment in 20-day-old rat pups. Graph a represents the mean (±SEM) total time attached during the 30-min test. Graph b represents the mean (±SEM) latency to attach.

RESULTS AND DISCUSSION

Figure 3a presents the mean total time attached for all 4 groups. Serotonin inhibited nipple attachment behavior in the food-deprived rat pups, $F(1,7) = 31.64$, $p<0.001$, replicating the results of Experiment 1. More important, xylamidine significantly interacted with serotonin, $F(1,7) = 23.40$, $p<0.002$.

Figure 3b presents the latency to attach for all four of the groups. Although none of the differences were significant, probably due to high levels of variability, the trends were consistent with those reported for total time attached.

Because xylamidine effectively blocked the inhibitory effect of serotonin on nipple attachment behavior, the mechanisms of action for this inhibitory response are apparently influenced peripherally. Nonspecific effects of 5-HT on the other systems in the periphery also do not appear to influence suckling. For example, if the irritating effect of serotonin in the area of subcutaneous injections triggered an endogenous endorphin release, and this was the cause of the inhibition, then pretreatment with xylamidine would not show decreased inhibition.

The xylamidine-pretreated pups that received serotonin exhibited the "serotonin syndrome" characteristics, yet their nipple attachment rate was near baseline. Because xylamidine counteracted suckling inhibition but failed to attenuate the "syndrome" behaviors, these "syndrome" behaviors are apparently independent of the nipple attachment response. Serotonin may thus have other effects that do not influence suckling inhibition besides the irritation at injection sites.

EXPERIMENT THREE

Experiment 2 demonstrated that xylamidine reversed serotonin-

induced inhibition. To compare peripheral and central control of suckling inhibition, centrally active serotonin agonists, quipazine and fenfluramine, were also studied.

Quipazine passes into the brain when injected IP and has been shown to be a selective and effective central serotonin agonist (36). Williams *et al.* (37) found a dose-response curve for the effect of quipazine on inhibition of nipple attachment behavior analogous to the dose-response curve found with serotonin in Experiment 1. If the mechanisms for this inhibition are only peripherally controlled, then xylamidine should counteract quipazine's effects even though quipazine also stimulated central serotonin receptors.

Fenfluramine is known to have both peripheral and central serotonergic effects (28). It has been widely used in studies of adult feeding because of its properties as a serotonin agonist producing dose-dependent anorexia in adult rats (4,6). Davies *et al.* (11) postulated that the effects of fenfluramine are specific to the mechanisms that control meal size, but it does not affect the initiation of feeding. Meal size control is linked to direct peripheral action, which reduces the rate of gastric emptying (3). Fletcher and Burton (13) and Carruba and Mantegazza (7), however, found that fenfluramine induced anorexia, which was not attenuated by xylamidine pretreatment. This suggests that the anorectic effect of fenfluramine is not mediated by peripheral serotonin receptors. Methysergide did attenuate fenfluramine-induced anorexia (5). Fenfluramine apparently influences feeding both centrally, where the main anorexia might originate, and peripherally, where the effect appears to be on satiation related to gastric emptying. Fenfluramine also affects glucose metabolism (4).

The effect of fenfluramine on nipple attachment has been studied by Williams *et al.* (37). They found that, like quipazine, it inhibited nipple attachment. Fenfluramine also passes into the brain and has mild depressant properties. It should therefore have similar effects on suckling inhibition. In the paradigm used to study suckling, no meal is received because the anesthetized dam gives no milk (25), therefore, meal size cannot be a factor in recording nipple attachment.

METHOD

The same procedures were used as in Experiment 2 except that quipazine (20 mg/kg, IP) or fenfluramine (7 mg/kg, IP) replaced serotonin. To be consistent with other studies utilizing quipazine or fenfluramine, the subjects were injected 30 min before testing instead of 15 min as with the serotonin. All testing, recording, and analysis were as described in Experiment 2. Forty pups from 10 litters provided N = 10/cell for the quipazine study, and 32 pups from 8 litters provided N = 8/cell for the fenfluramine study.

RESULTS AND DISCUSSION

Figure 4a presents total time attached for the quipazine study. The quipazine-injected animal exhibited reduced attachment time, $F(1,9) = 28.40$, $p < 0.01$. This was consistent with other findings (37) and was very similar to the inhibition induced by serotonin. As with serotonin-treated animals, the inhibitory effect of quipazine was effectively attenuated by pretreatment with xylamidine, as evidenced by a significant interaction, $F(1,9) = 11.81$, $p < 0.01$.

Figure 4b illustrates the latency to attach for all four groups. As in Experiment 2, this dependent variable failed to reach significance, although the quipazine-treated animals appeared to have longer latencies.

Figure 5a presents total time attached for the fenfluramine study. Fenfluramine produced a significantly decreased amount of

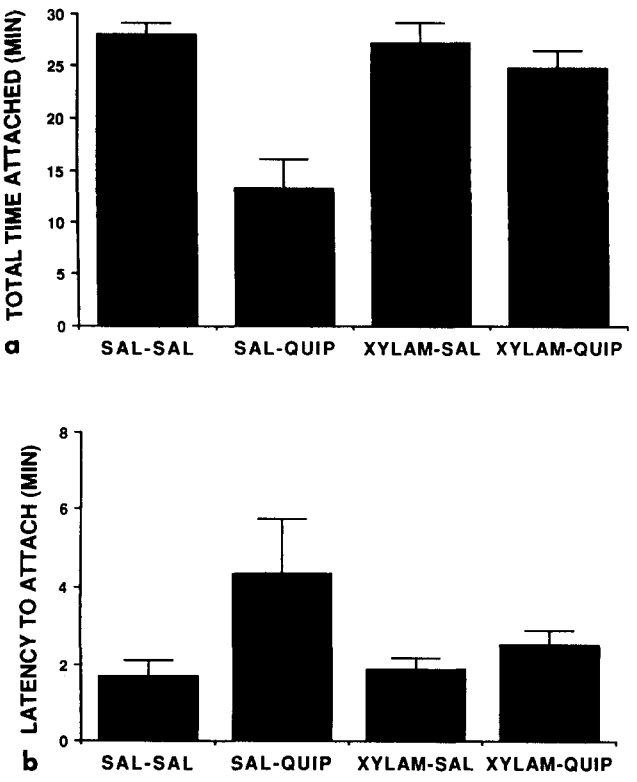

FIG. 4. Effects of quipazine and xylamidine on nipple attachment in 20-day-old rat pups. Graph a represents mean (±SEM) total time attached during the 30-min test. Graph b represents the mean (±SEM) latency to attach.

time attached, $F(1,7) = 157.07$, $p < 0.01$. Consistent with the quipazine and serotonin data, xylamidine pretreatment effectively counteracted fenfluramine's inhibitory influence, as evidenced by a significant interaction, $F(1,7) = 43.97$, $p < 0.01$.

Figure 5b represents the latency to attach. Fenfluramine demonstrated a marginal effect on latency to attach, $F(1,7) = 5.43$, $p = 0.053$.

Experiment 3 demonstrated that peripheral receptors stimulated by either of these agonists play an important role in the inhibition of nipple attachment. Xylamidine attenuated the agonist effects in the periphery; any central serotonin receptor stimulation was insufficient to counter the peripheral antagonism.

Although the results of Experiments 2 and 3 were identical, the pups treated with quipazine behaved differently from the serotonin-treated pups. As stated earlier, the serotonin pups exhibited signs of what has been called the "serotonin syndrome," which included drooping eyelids, excessive scratching at injection site, foot biting, and inactivity on the whole. In contrast, the quipazine-treated animals were very active and displayed none of the above behaviors. The fenfluramine-treated pups also did not display any other behaviors associated with the "serotonin syndrome"; these pups were indistinguishable from saline controls.

EXPERIMENT 4

Experiments 2 and 3 have clearly shown that xylamidine affects serotonin-inhibited nipple attachment behavior. Other serotonin antagonists have also demonstrated influence on this

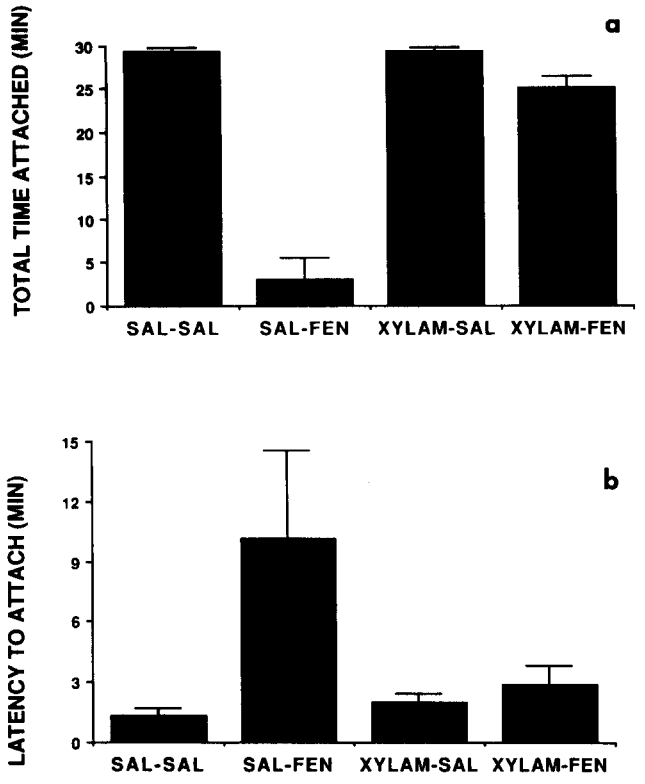

FIG. 5. Effects of fenfluramine and xylamidine on nipple attachment in 20-day-old rat pups. Graph a represents the mean (±SEM) total attachment time during the 30-min test. Graph b represents the mean (±SEM) latency to attach.

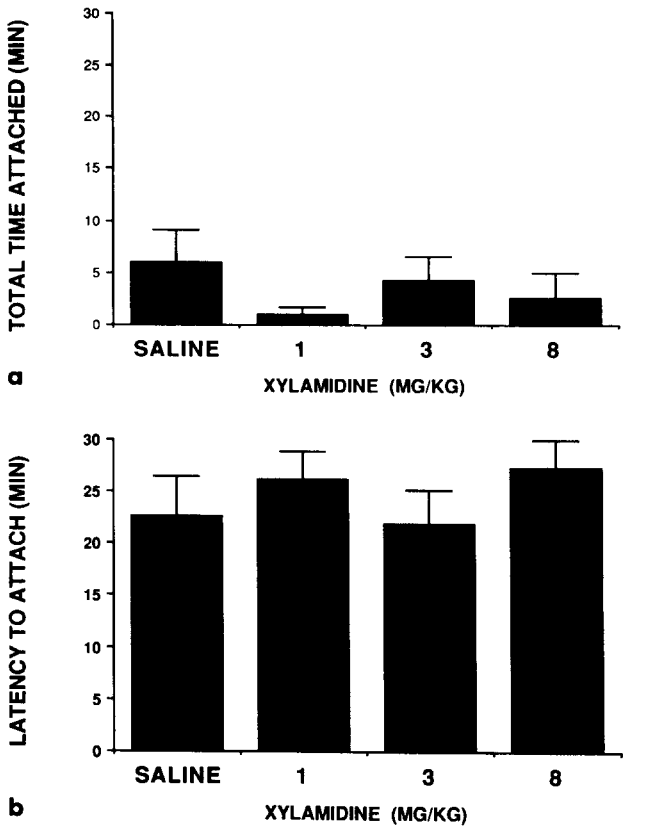

FIG. 6. Effects of different doses of xylamidine on nipple attachment in 20-day-old rat pups. Graph a represents the mean (±SEM) total time attached during the 30-min test. Graph b represents the mean (±SEM) latency to attach.

behavior, most notably methysergide. Williams *et al.* (37) showed that methysergide attenuates quipazine's inhibition of nipple attachment. Furthermore, they were able to show that methysergide alone promoted suckling in pups in a dose-response manner. Methysergide is unlike xylamidine in that it does pass into the brain. It is unclear whether its facilitatory effect is peripheral, as the inhibitory effects of serotonin, quipazine, and fenfluramine appear to be. The previous experiments failed to assess the possibility of *increasing* nipple attachment in 20-day-old rats by xylamidine. The pups were food-deprived 8 hr; unfortunately, such deprivation may have created a ceiling effect that masked facilitation. If xylamidine alone stimulated suckling behavior in rat pups that had not been so deprived, it could be further concluded that the peripheral serotonin receptors are directly involved in promoting as well as inhibiting suckling.

METHOD

Subjects

To avoid ceiling effects, 25-day-old pups were used, as they do not attach as readily as do 20-day-old pups. At this age, Williams *et al.* (37) demonstrated that methysergide is extremely effective in promoting nipple attachment. The change in age was also due to the fact that xylamidine needs to be administered 3 hr prior to testing in order to be effective. During this 3-hr period, it was important that the pups had no opportunity to feed or suckle. They could not therefore be returned to their dam's cage. But if 20-day-old pups are deprived for 3 hr, then the same ceiling effect of attachment might conceal any effect of xylamidine. Therefore, 25-day-old pups, who are less likely to attach after a 3-hr deprivation, were used for the tests.

Procedures

Three hr prior to testing, 10 sets of 4 littermate pups were given one of 4 treatments: isotonic saline or xylamidine (1, 3, or 8 mg/kg, IP). The dosages chosen include the effective attenuation dose from the other xylamidine experiments (3 mg/kg), and a dose below and above that level.

During testing, a dam of 15- to 20-day-old pups was anesthetized for attachment tests because a dam of 25-day-old pups is not lactating as readily, which could affect the attractiveness of her nipples. As before, the test lasted 30 min. Both the latency to attach and total time attached were recorded.

RESULTS AND DISCUSSION

Xylamidine failed to produce an increase in nipple attachment for any of the dosages used (Fig. 6). None of the conditions were significantly different from saline controls for either dependent measure. The xylamidine doses did not produce any dose-related facilitation.

The xylamidine failed to produce any significant differences in nipple attachment, but this in itself is significant. The lack of facilitation suggests that peripheral serotonin receptors are not responsible for initiating the suckling response. Cinanserin, another serotonin antagonist, has demonstrated a similar pattern.

Williams *et al.* (37) found that it too disinhibited quipazine-treated animals, yet did not facilitate nipple attachment alone. They did not address the apparent dichotomy of facilitation and inhibition. The results here suggest different sites of action for facilitation and inhibition.

The link may be recent findings that specific receptor subtypes in the brain (5-HT_2) are the same receptors that mediate smooth muscle contraction in the gut. Methysergide is known to affect both of them (19), and xylamidine is relatively selective for 5-HT_2 receptors (14). There are, however, many different subtypes of serotonin receptors and the exact specificity of each is unknown. Nevertheless, initiation of suckling is a more complex behavior, requiring interpretation of different signals from the body, and thus might be controlled in the brain. Inhibition of suckling, on the other hand, may not be as complex, requiring only peripheral information from the gut. Studies utilizing intraventricular administration of serotonin agonists and antagonists should help resolve this issue.

GENERAL DISCUSSION

The results of the first three studies clearly demonstrate that peripheral serotonin receptors play a role in the inhibition of suckling. Serotonin, quipazine, and fenfluramine all agonize serotonergic systems, but they exert their effects by an assortment of neurochemical mechanisms. Serotonin only acts in the periphery; quipazine act both peripherally and centrally; and fenfluramine not only acts peripherally and centrally, but also affects other mechanisms such as glucose metabolism. Because xylamidine, the peripheral serotonin antagonist, effectively attenuated the effects of all three of these agonists, peripheral serotonin receptors must be influencing this behavior.

While xylamidine attenuated inhibition of nipple attachment, it did not facilitate suckling on its own. The dosage chosen to study facilitation was sufficient to block the induced inhibition, but perhaps these dosages were too low to increase nipple attachment. However, similar doses of methysergide, which counteracted inhibition of suckling, have been shown to facilitate attachment (37).

The validity of these results relies on the assumption that xylamidine and serotonin do not pass the blood-brain barrier in pups. Some features of the blood-brain barrier are immature at birth and may not be completely developed at 20–25 days (20). However, its permeability to monoamines appears to have achieved adult-like status by 2, or at most 3 weeks of age (15,26), that is, before the ages studied here. Nonetheless, xylamidine penetration into CNS has not been explicitly studied in infant rats, and thus further studies, possibly with radiolabelled xylamidine, might be necessary to determine the permeability of the blood-brain barrier to this compound in weanlings.

Although serotonin alone may be sufficient to inhibit suckling, it may not be entirely necessary. Increasing serotonin levels may not be the unitary cause of the inhibition of nipple attachment; therefore, basing conclusions solely on serotonergic manipulation may disregard other important variables that contribute to this behavior. Environmental factors and serotonergic systems may interact to modulate weaning through a common mechanism. Lichtman and Cramer (23) found that the effects of methysergide can be modified by suckling experience. Early forced weaning attenuates the effects of methysergide, while continued opportunity to suckle produces long-lasting sensitivity to pharmacological manipulation. These results clearly indicate a combination of both experience and neurotransmitters in the process of weaning.

The effect of quipazine on inhibition of suckling might be due to increased activity from its stimulant similarities (36). Stimulants are known to reduce appetite, and amphetamine does reduce nipple attachment in rats of this age (34). Moreover, as with the problem of the "serotonin syndrome" in the second experiment, because xylamidine attenuated the inhibitory effect, the stimulant properties appear to be independent of nipple attachment.

The effects of fenfluramine on feeding behavior in adults are complex, with apparently different sites of action for anorexia and for satiation. Fenfluramine also has no effect on initiation of adult feeding (11). Suckling behavior, or nipple attachment, could be seen as initiating the milk extraction response from the dam's nipple. Fenfluramine inhibited this weanling feeding initiation response. The anorexia associated with fenfluramine may be independent of its effects on suckling inhibition. Xylamidine effectively returned the attachment time to baseline of pups who apparently had fenfluramine-induced anorexia, or reduced appetites.

Suckling inhibition may relate more to satiation than hunger. Because fenfluramine reduces gastric emptying rate in adults (3), it can be theorized that suckling inhibition is modulated in the periphery because the signal to stop suckling is linked to receptors in the gut. For instance, serotonin stretch receptors in the gut may be activated by increased concentration of serotonin or agonist, and this may induce the cessation of nipple attachment. Xylamidine pretreatment would block these receptors in the periphery and block the increased satiation the influx of serotonin or agonist would produce.

Although nipple-shifting behavior was not explicitly recorded, the fenfluramine pups pretreated with xylamidine appeared to have more instances of this behavior. Because this response is associated with milk extraction (9), it may be related to fenfluramine's effect on meal size in adults.

The results of this study offer a new direction for future attempts to localize the effects of serotonin on suckling. Xylamidine's attenuation of serotonin, quipazine, and fenfluramine effects on nipple attachment indicates that peripheral serotonin receptors at least influence the inhibition mechanisms. Yet, xylamidine's failure to facilitate nipple attachment indicates that these same receptor sites are not involved in promoting suckling. Clearly, a distinction exists between the mechanism of inhibition and facilitation. If increasing amounts of serotonin in the developing rat pup contribute to weaning at 25 days, then a further clarification of the sites of action on suckling behavior is necessary to determine the extent of the influence of this neurotransmitter.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Richter Foundation to S.T.B. and a Faculty Research Grant from Dartmouth College to C.P.C. We thank The Wellcome Research Laboratories for generously supplying the xylamidine.

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