ONTOGENY OF CHOLECYSTOKININ-INDUCED SATIETY IN RATS

by

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April 18, 1979
Date
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ABSTRACT

A putative satiety hormone cholecystokinin (CCK) was used for investigating the development of ingestive regulation in rat pups. In Experiment 1, 192 rat pups were selected from 24 litters to be tested on 12 age levels: 1, 3, 5, 7, 8, 9, 10, 11, 12, 13, 14, and 15 days after birth. CCK octapeptide (2 µg/kg body weight) or control saline were administrated intraperitoneally to the pups after 5 hr deprivation. The subsequent 1 hr milk intake measured by body weight increase was obtained to test the effect of CCK injection. CCK induced satiety was first seen in 9 day old rat pups. The satiety effect of CCK disappeared on days 10, 11, and 12, and then permanently reappeared on day 13. This developmental process of CCK-induced satiety appeared to be related to natural growth rate such as body weight increase. The rat pups showed a gradually developed compensatory ingestive ability between days 9 and 15 when intake was measured 5 hr after CCK injection. In Experiment 2, 80 rat pups were selected from 10 litters to be tested on the 5 critical age levels (9, 10, 11, 12, and 13) to reexamine the non-monotonic developmental process of CCK-induced satiety. The results confirmed those obtained from the Experiment 1. A morphological development of the rat pups was observed to parallel to the behavioral response to CCK injection.
INTRODUCTION

The intestinal hormone cholecystokinin (CCK) has recently been suggested to function as a short-term satiety factor (Mueller and Hsiao, 1978; Smith and Gibbs, 1975). CCK is dose-relatedly effective in decreasing food intake in subjects of various species (Gibbs, Young, and Smith, 1972, 1973; Gibbs, Falasco, and McHugh, 1976; Koopmans, Deutsch, and Branson, 1972; Sturdevant and Goetz, 1976). The CCK octapeptide not only decreases meal size but also prolongs the intermeal interval (Hsiao and Wang, in press; Kraly et al, 1978). Other evidences supporting the CCK being a satiety hormone include that CCK specifically suppresses liquid food intake but not water drinking (Mueller and Hsiao, 1977) and that CCK induced a complete behavior episode of satiety such as grooming and resting in rats (Antin et al. 1975). The satiety-inducing effects of CCK at the behavioral level have been well documented although several conflicting results have also been reported (Goetz and Sturdevant, 1975; Glick, Thomas, and Mayer, 1971; Mineka and Snowdon, 1978).

On the neurological level, the underlying mechanism by which CCK exerts its effects is still largely unknown. Vagus nerve was shown to be necessary for the CCK satiety mechanism (Lorenz and Goldman, 1978), whereas, Anika, Houpt, and Houpt (1977) reported that subdiaphragmatic
vagotomy failed to abolish the CCK satiety effect. Ventromedial hypothalamus has been suggested to be the site of CCK action (Stern, Gudillo, and Kruper, 1976). However, CCK is also effective in suppressing food intake in rats with electrolytic lesions of the ventromedial hypothalamus (Kulkosky et al. 1976). Exogenous CCK has been found to alter the EEG pattern evoked by auditory stimuli selectively in feeding related areas (Dafney, Jacob, and Jacobson, 1975). High levels of CCK-like peptides have been found in certain areas of the brain detected by radioimmunoassay (Dockray, 1976; Innis, Correa, and Snyder, 1978). The results of these investigations indicate that CCK may mediate its function through certain parts of the central nervous system; but no conclusion has yet been obtained in this area of study.

The investigation of CCK behavioral effects and their underlying neurological mechanisms can be pursued by way of ontogenic studies. The ontogenic approach has long been employed in the field of physiological psychology in studying the brain-behavior relationship. Several advantages accrue from studying the new-born subjects: a) the organisms' behavior undergoes a dramatic sequential change with rapid growth in early ages which allow reliable observation and description, b) the prematurity of central nervous system in the neonates provides a good model for studying the age-related physiological and anatomical changes, and c) by extraneously manipulating the
physiological and anatomical growth rate, the parallel behavioral changes provide inferences of the brain-behavior functional causality. Research taking this approach has provided comprehensive knowledge concerning the development of ingestive regulation (Epstein, 1976).

Suckling behavior of mammalian infants undergoes a sequential transition during the developmental processes. This transition prepares the neonates to develop from a unitary mode of suckling behavior to multimodal ingestive behaviors such as drinking and eating. Certain parameters of suckling episodes such as nipple attachment and nipple shifting were seen to change between eleven and fourteen days of age in rats (Hall, Cramer, and Blass, 1975, 1977). The control of initiation and termination of each suckling period were shown to be more effective and more directly oriented toward the nipples of the dam in pups of twenty days old than in younger animals (Hall and Rosenblatt, 1977). Epstein (1976) summarized several ontogenic studies and proposed a timetable to illustrate the sequential development of rat pups' ingestive regulation. Deprivation increases rat pups' milk intake during suckling (Friedman, 1975; Houpt and Epstein, 1973; Houpt and Houpt, 1975). The neonates are able to respond to deprivation as early as one day of age. Gastric loads of various substances decrease subsequent intake in rats of 3 to 7 days old (Houpt and Epstein, 1973; Houpt and Houpt, 1975). However, glucoprivation produced by 2-Deoxy-d-glucose (2-DG, a substance
similar to glucose in its chemical structure but is not metabo-
lizable and thereby, reduce the availability of cellular glucose)
did not increase milk intake until the week after natural weaning
(Houpt and Epstein, 1973).

Several approaches have been adopted in explaining the under-
lying mechanisms which regulate the postnatal ingestive behavior.
The first explanation involves a model of encephalization in regulat-
ing the ingestive behavior. Differentiation of infantile suckling
behavior into a normal adult's feeding and drinking pattern was shown
to parallel the distinct recovery stages of lateral hypothalamic
syndrome in adult rats (Teitelbaum, Cheng, and Rozin, 1969; de Castro
and Balagura, 1975). These studies suggest the involvement of a dien-
ccephalic mechanism that regulates the development of ingestive behavior.

A second approach considers milk intake as a behavioral re-
response to changes in the physiological mechanism of body fluid
regulation. Intravascular hypovolemia and extracellular dehydra-
tion has been shown to stimulate milk intake in preweaning rats
(Friedman, 1975; Friedman and Campbell, 1974; Wirth and Epstein,
1976). Milk intake is, hence suggested to be controlled by a
mechanism related to body fluid regulation.

The third approach emphasizes gastrointestinal load and the
stimulation of upper gastric stretch receptors. Intragastric intubation
of different substances decrease subsequent milk intake to various degrees not related to osmotic and caloric values (Friedman, 1975; Houpt and Epstein, 1973; Houpt and Houpt, 1974, 1975). Among these multidimensional regulators of milk intake, however, the hormonal satiety factors such as CCK have drawn little attention in the investigation of infantile ingestive regulation.

In the present study, the development of ingestive regulation in neonatal rats was investigated by using the putative satiety hormone-CCK to determine when the pups' satiety mechanism started to function in response to the exogenous CCK octapeptide. By observing the rat pups' response to CCK injection through successive age levels, the onset of CCK-induced satiety can be precisely located. Also, the development of the pups' ability to demonstrate compensation for a decreased intake induced by CCK administration is determined. The daily increase of body weight were measured and other signs of maturation were noted to correlate them with the onset of CCK-related intake regulation.
EXPERIMENT 1

Method

Subjects

The subjects were 24 litters of neonatal rats bred in our laboratory. One hundred and ninety two rat pups were chosen from those litters to be tested on 12 age levels. Multiparous pregnant females obtained from the Holtzman Company (Madison, Wisconsin) were housed individually in suspended wire mesh cages. At late pregnant each female was placed in a plastic nesting box (36 x 28 x 15 cm) with stainless steel mesh cover and wood shaving bedding. Females were checked for parturition late each afternoon. Pups discovered then were considered to have born that day and counted as 0 days old. Two days after birth, litter sizes were reduced to ten pups by random selection and then left undisturbed until the pups reached a designated test age. Litters with size smaller than 9 were not used. Only the eight heaviest pups were selected for test. All adult rats were maintained on Wayne laboratory pellets and tap water. Lights were on from 0800 to 2000 daily and the temperature kept at 22°C in the laboratory.

Testing Procedure

The 24 litters of rats were randomly assigned to be tested at one of the following 12 age levels with 2 litters in each: 1, 3, 5, 7,
8, 9, 10, 11, 12, 13, 14, and 15 days after birth. Within each litter the eight selected pups were divided into control and CCK-treated groups with four in each, matched with their body weight. Thus, the subjects used in each age level were 8 experimentals and 8 controls. Rats were tested only once. On each test day, only one litter was tested.

The pups were removed from their mother at 0900 on the scheduled test date for 4 hour deprivation. They were kept in an incubator maintained at 30°C with high humidity. About one hour before the end of deprivation, the pups were manually induced to defecate and urinate by gently stroking the anogenital area. The stroking was manipulated to imitate the licking of the dam seen in the normal nursing situation. This procedure was applied to eliminate the undesirable body weight fluctuation induced by mother-infant interaction during the nursing period. The pups were then weighed in an analytical balance to the nearest 0.01 gram.

Each pup in the experimental group received an intraperitoneal injection of CCK octapeptide (SQ 19844, Squibb and Sons, Inc.) at the dose of 2 µg/kg body weight which approximately equals to 54 Ivy dog unit/kg body weight. The control pups received the same amount of isotonic saline. As soon as the injection was completed, (usually around 1300) the eight selected rats were returned to their mother for nursing.
Measure of Milk Intake

Weight gain of the pups after a period of nursing was used for measuring the milk intake. The one hour intake was measured at 1400 to determine the effect of CCK injection. Rat pups were then returned to their mother immediately after being weighed. A second body weight measure was taken at 1800 (five hours after injection) to determine the possible compensatory intake of CCK-treated rats during the four hour nursing period.

As noticed in our pilot studies and reported by other researchers (Lincoln, Hill, and Wakerley, 1973; Rosenblatt, 1965), the intermittent milk let down of the dam and the complication of mother-infant interaction constitute a major difficulty in conducting experiments of this kind. Variation of intake among litters (even within the same age level) seems inevitable. The maternal activity contributes to a great extent of this variation. When a situation of underfeeding occurred (where the pups were not fed to satiation), a valid comparison of CCK-induced satiety with normal satiety cannot be achieved. Therefore, a criterion was established to determine the situation of being "underfed". When the mean one hour intake of the control group from one litter was less than 60% of the control mean from the other litter within the same age level, the entire litter was regarded as being underfed and the data of that litter were not used. New litters of the same age were replaced until the mean intake of the control group from the two litters met the criterion.
Results

Five litters out of 24 were determined underfed. Two of them were from the group tested at day 12 and one was from each of the groups tested at days 5, 13, and 15. Data of these age levels were completed by replacing new litters after the first experimental period. The underfed condition was usually caused by the mothers terminating the nursing too soon in the first hour testing period. Normally, the mothers would gather their pups and start nursing them as soon as the pups were returned from four hour deprivation. The first milk ejections (as determined by the pups' first stretch reflex) were always seen within 10 minutes and the mothers would stay with their pups for at least 40 minutes. However, in those underfed litters, the mothers would either fail to gather their pups to nurse or leave the pups too soon and did not go back to them within the test period. This caused their pups to ingest much less than those pups in other litters who stayed with their mother longer.

First Hour Milk Intake

The first hour milk intakes of the CCK and saline treated groups in each age level are listed in the Table 1. Comparisons between the two groups within each age level were analyzed by two factor analyses of variance. CCK injection significantly suppressed the first hour milk intake at days 9, 13, 14, and 15. All the pups seemed to attach to the nipples in the same rate within each age level and remained attached throughout the nursing period as long as
Table 1. First hour milk intake of the rat pups treated with CCK (2 μg/kg body weight) or control saline. Entries are mean (+ S.E.M.) milk intake of different age levels measured by body weight increase in grams.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age (Days)</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.41 0.63 0.72 0.84 1.02 1.10 1.02 1.16 1.64 1.20 1.24 1.38</td>
<td></td>
<td></td>
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<td></td>
<td>(0.05) (0.06) (0.07) (0.04) (0.11) (0.11) (0.07) (0.14) (0.10) (0.09) (0.10) (0.04)</td>
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</tr>
<tr>
<td>CCK</td>
<td>0.42 0.50 0.57 0.85 0.96 1.06 1.26 1.62 0.88 0.88 1.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>(0.03) (0.05) (0.08) (0.02) (0.07) (0.08) (0.05) (0.13) (0.12) (0.06) (0.08) (0.09)</td>
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</tr>
<tr>
<td>F</td>
<td>&lt;1 3.12 2.25 &lt;1 &lt;1 7.25** &lt;1 &lt;1 &lt;1 9.51*** 9.02** 5.00**</td>
<td></td>
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</table>

* p < 0.05  ** p < 0.025  *** p < 0.01
the mother stayed with them. Rarely were there any pup left behind or failed to attach to the nipple. Only occasionally did the mother fail to gather every pup and left one or two pups far away from her causing a failure of nipple attachment. In these cases the experimenter would carefully place the pups under the dam or near enough for her retrieval. The pups were always successful in achieving nipple attachment with these aids and caught the first milk ejection nicely.

Second Measure for Five Hour Intake

Mean 5 hr milk intake of CCK and control groups are shown in Table 2. Only at day 9 did these measure remain significantly different. At days 13, 14, and 15 (but not 9), the CCK pups ingested a compensatory amount of milk after the first hour suppression of intake by CCK injection. This compensation balanced out the suppression of first hour intake due to the CCK treatments.

Increase of Body Weight Along the Age Levels

Figure 1 shows the mean body weight increase along the age levels. There are two surges in body weight changes occurring at days 9 and 12. No significant difference of body weight was seen between the experimental and control groups within each age level.
Table 2. Five hour milk intake of the rat pups treated with CCK (2 µg/kg body weight) or control saline. Entries are mean (+ S.E.M.) milk intake of different age levels; measured by body weight increase in grams.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age (Days)</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.56</td>
<td>0.88</td>
<td>0.94</td>
<td>1.08</td>
<td>1.36</td>
<td>1.49</td>
<td>1.36</td>
<td>1.73</td>
<td>2.09</td>
<td>1.62</td>
<td>2.01</td>
<td>1.76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.06)</td>
<td>(0.08)</td>
<td>(0.06)</td>
<td>(0.10)</td>
<td>(0.10)</td>
<td>(0.12)</td>
<td>(0.09)</td>
<td>(0.09)</td>
<td>(0.13)</td>
<td>(0.16)</td>
<td>(0.18)</td>
<td>(0.10)</td>
<td></td>
</tr>
<tr>
<td>CCK</td>
<td>0.52</td>
<td>0.82</td>
<td>0.85</td>
<td>1.09</td>
<td>1.39</td>
<td>1.24</td>
<td>1.26</td>
<td>1.70</td>
<td>2.13</td>
<td>1.37</td>
<td>1.84</td>
<td>1.83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.05)</td>
<td>(0.07)</td>
<td>(0.09)</td>
<td>(0.09)</td>
<td>(0.16)</td>
<td>(0.09)</td>
<td>(0.05)</td>
<td>(0.12)</td>
<td>(0.21)</td>
<td>(0.07)</td>
<td>(0.15)</td>
<td>(0.11)</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>1.40</td>
<td>2.67</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>5.00*</td>
<td>1.33</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>2.66</td>
<td>1.14</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* p < 0.05
Figure 1. Body weight increase along the age levels in Experiment 1 and Experiment 2.
Discussion

Since the significant difference of intake between the CCK and control groups were first obtained at 9 day of age, the onset of CCK-induced satiety in the rat pups is apparently initiated at this particular age. The significant effect of CCK disappeared on days 10, 11, and 12 which then reappeared at day 13 and was consistently effective at days 14 and 15. The dramatic disappearance of CCK effect at age 10 through 12 may be attributed to a type II error of statistical testings that caused a failure to detect the CCK-induced satiety at these age levels. Likewise, the significant difference obtained at day 9 might have been a type I error that caused a false detection of the CCK effect at that particular age. However, this phenomenon may also due to a peculiar maturational effect. The sharp increase of body weight at days 9 and 12 happened to locate on (the 9 day) or 1 day preceding (12 day) the onset of CCK-induced satiety. This fact seems to suggest that there are some growth factors that, while promoting the rapid increase of body weight, also promote the manifestation of CCK effect. This speculation will be addressed in the following experiment.

The second measure of 5 hr intake indicates a development of compensatory ingestive ability. The rat pups developed a compensatory ingestion between day 9 and day 15. At day 9, the pups were not able to make up the suppressed first hour milk intake during the following 4 hr nursing period and left the difference remain significant as shown in Table 2. The compensatory ingestive ability was gradually developed
along the ages of 13 and 14 which finally reached a great amount of compensation at day 15 and cause the CCK group to ingest slightly more than the control group after 5 hr nursing. This development of compensatory intake ability between day 9 and 15 indicates a rapid growth of ingestive regulation during these age levels.

Considering the possible alternative statistical explanations as mentioned above and the need for a more close observation of the maturational processes, the following experiment was conducted to test the effect of CCK across the critical ages of days 9 through 13 to provide a more adequate result.
EXPERIMENT 2

This experiment was designed to reexamine the development of CCK-induced satiety during ages between day 9 and day 13. Phenomenon of the absence of CCK effect during days 10, 11, and 12 as seen in the Experiment 1 was closely measured. Other indicators of maturation such as body weight increase and morphological changes were also carefully observed.

Method

Subjects

The subjects were 10 litters of neonatal Holtzman rats. Breeding procedure and maintenance were the same as Experiment 1. The females were all primiparous. The 10 litters were randomly assigned to be tested at one of the following 5 ages with 2 litters in each: 9, 10, 11, 12, and 13 days after birth. All subjects were used only once.

Test Procedure

Test procedure was the same as that used in the Experiment 1.

Measure of Milk Intake

The same strategy was used to measure the milk intake so that the control groups from the two litters in the same age level would
reach the criterion of no variation greater than 40%. Only the first hour intake was measured.

**Results**

Two litters out of 10 were determined underfed in this experiment. They were those tested at ages of 9 and 11 days old with one in each. The data of these litters were replaced with those from litters tested at the same ages. The mean intake during the one hour nursing period is presented in the Table 3. The CCK injection again significantly suppressed the milk intake at days 9 and 13, but not at days 10, 11, and 12. Both differences between the CCK and control groups at day 9 and 13 were all significant at the level of 0.001 tested by two factor analyses of variance.

An interesting finding in this study is that the onset of CCK effect seems to match nicely with the morphological changes during the developmental process. At 12 days of age, the rat pups were observed to begin to open their ears. One of the two litters tested in this age had their ears opened and the CCK effect appeared to be much more potent in this litter as compared with the other litter tested at the same age with their ears closed. Therefore, even the overall result is still not significant at day 12, the effect of CCK seemed to begin to reappear at this age and reached a significant level at day 13 when all the rat pups had their ears opened.

The surge of body weight increase at day 9 as seen in Experiment 1 was also seen in the Experiment 2 except the second peak at
Table 3. Effect of CCK injection on the subsequent one hour milk intake measured after four hour deprivation. -- Entries are mean (± S.E.M.) in grams measured by body weight increase.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age (Days)</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.35</td>
<td>0.96</td>
<td>1.44</td>
<td>1.70</td>
<td>1.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.05)</td>
<td>(0.10)</td>
<td>(0.10)</td>
<td>(0.13)</td>
<td>(0.11)</td>
<td></td>
</tr>
<tr>
<td>CCK</td>
<td>1.10</td>
<td>0.95</td>
<td>1.26</td>
<td>1.56</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.05)</td>
<td>(0.05)</td>
<td>(0.09)</td>
<td>(0.11)</td>
<td>(0.12)</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>24.00 ***</td>
<td>&lt;1</td>
<td>2.00</td>
<td>1.83</td>
<td>32.41***</td>
<td></td>
</tr>
</tbody>
</table>

*** p < 0.001
12 days old which was not seen in this experiment (see Figure 1). The body weight of the rat pups used in this experiment were generally less, than those of pups used in the Experiment 1. This may be explained by the fact that all the breeding females used in this experiment were primiparous as opposed to the multiparous females used in the Experiment 1.

**Discussion**

CCK-induced satiety has been shown in weanling rats (Bernstein, Lotter, and Zimmerman, 1976). Forty Ivy dog units/kg of CCK octapeptide suppresses intake of liquid and solid diet in fasted 21-day-old rats. The authors suggested that CCK injection might induce satiety in even younger rats. The results of the present studies indicated that the onset of CCK-induced satiety was located at 9 days of age. The CCK effect disappeared on days 10, 11, and 12 and then reappeared on day 13. This phenomenon has been replicated in the Experiment 2. The data confirmed those obtained from the Experiment 1 and largely reduced the possibility that either type I or type II errors might have contributed to the results obtained from the Experiment 1. The absence of CCK effect in 10 day old rat pups has been obtained by Hall (1978). The study was investigated by using artificial feeding techniques devised to achieve better control and more precise observation of neonatal milk ingestion. Anesthetized mothers with no milk let down were used to eliminate the mothers' active contribution to the observational difficulties in the testing situation. Surgically implanted tongue cannulae
were used for manual milk delivery to the pups. The results indicated that 40 Ivy dog units/kg of CCK octapeptide suppressed rat pups' intake at 15 and 20 but not 10 days of age (Hall, 1978). However, the CCK influence on milk intake measured in that study was dependent upon the development of the pups' ability to refuse nipple reattachment. The pups had to develop an ability to refuse reattaching to the nipples in order to prevent the experimenter's milk injection. This ability of showing satiety by rejecting the nipple attachment, however, was not apparent at day 10 and earlier ages (Hall and Rosenblatt, 1977). Therefore, a valid observation of CCK-induced satiety might not have been achieved. To observe the onset of a satiety mechanism the voluntary control of intake seems crucial. This goal has been achieved in the present study by using a natural nursing situation. The continuous observation through each preweaning age levels have also provided a more complete picture of the sequential development of ingestive regulation.

The observation of the correspondence between the morphological transformation and the behavioral change in response to the CCK injection suggested that the disappearance of CCK effect during the days 10 through 12 was influenced by a natural growth factor. The mechanism of neurological maturation by which CCK exert its satiety function need to further investigated.

The high CCK radioimmunoreactivities found in certain areas of the brain (Dockray, 1976; Innis, Correa, and Snyder, 1978) suggested
that CCK receptors may exist in the brain by which the CCK exerts its function as a neuromodulator. The developmental processes of those CCK receptors may modified by or in some way interact with other neurotransmitters to show a non-monotonic development as shown in the present experiments. The major neurotransmitters in the rat brain such as the catecholamines and serotonin undergo a dramatic increase during the second and third postnatal weeks (Loizou and Salt, 1970; Loizou, 1972). It is possible that the sharp increase of these neurotransmitters can interfere with or override the CCK effect until CCK receptors in the brain regain their affinity to the exogenous CCK. Research in the nature of CCK receptors within the brain, their specificity, and the developmental processes of these receptors that relate to a behavioral manifestation need to be further investigated before the full understanding of the present results can be achieved.
REFERENCES


