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Altered Development of the Rat Brain Serotonergic System after Disruptive Neonatal Experience

(brain 5-hydroxytryptamine/gonadal axis/adrenal axis)

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ABSTRACT **A single disruptive experience, a cold treatment, on postnatal day 1 elevated open-field activity and reduced reaction to handling in rats tested more than 240 days later. Neurochemical mechanisms underlying these behavioral phenomena were examined by monitoring the development of the rat brain serotonergic system. After cold treatment, elevations in 5-hydroxytryptamine levels of a preparation of forebrain plus midbrain could be first detected on postnatal day 16 in both sexes. A more detailed regional dissection of the brain showed that such increases occurred in the cerebrum, midbrain, septum-thalamus, and hypothalamus-preoptic area. Rats treated on postnatal days 1 or 6 showed increased 5-hydroxytryptamine levels, whereas animals treated on postnatal day 10 did not, a finding that points to a possible "critical period" of sensitivity. Results of adrenalectomy and corticosterone injections suggest that the influence of the adrenal gland cannot account for the elevation in brain serotonin. Furthermore, the gonadal steroids, estradiol and testosterone, alter brain 5-hydroxytryptamine independently of early experience, as distinguished by the time of appearance, periods of sensitivity, and the direction of monoamine changes. These findings are discussed in relation to the maturation of the neuroendocrine systems governing adrenal and gonadal function.**

Environmental events during sensitive periods of early animal development are known to have profound and often persistent influences on subsequent behavior and physiology (1–4). A number of studies have shown that adult rats that have been given various forms of stimulation during particular periods in neonatal life show marked behavioral differences when compared with unstimulated controls (5–7). These "disruptive early experiences," such as exposure to temperature extremes or electric shocks, have been reported to alter adult behaviors, including open-field activity, active avoidance, startle responses, and "emotionality" (6–8). Disruptive early experience also affects several neuroendocrine parameters that involve the hypothalamic–pituitary–adrenal response to stress (5, 9, 10).

We sought to examine neurochemical phenomena that might help to elucidate the environmental influence upon developing brain mechanisms. Since the putative neurotransmitter 5-hydroxytryptamine (5HT, serotonin) has been linked to functions of the hypothalamic–pituitary–adrenal axis and to "emotionality" (11), we chose to monitor the brain level of this amine in developing rats after a single disruptive experience.

METHODS AND MATERIALS

Animals. Pregnant rats, timed for a specific parturition date by the supplier (Holtzman, Madison, Wisc.), were received on the 16th–18th day of gestation and housed individually in a temperature-controlled room (21°) with natural lighting. Litter sizes were not allowed to exceed 10.

Disruptive Early Experience. A disruptive cold treatment was given on the first postnatal day. Pups were removed from the nesting box, placed on crushed ice for 15 min, and then warmed under a lamp for 15 min. After this 30-min disruptive event, animals were returned to the nesting box and were not disturbed until they were killed. Controls received no cold treatment. Animals raised to adulthood were separated according to sex before day 30 and housed individually before day 80.

Hormone Treatments and Adrenalectomy. We tested the possibility that the effects of cold treatment may be mediated through the steroid hormones—testosterone, estradiol, or corticosterone—which play important roles in early development of the rat (6). Neonates were removed from the nesting box during the first postnatal day of life and injected subcutaneously. Gonadal hormone-treated groups received 100 μg (suspended in 0.05 ml of sesame oil) of the androgen, testosterone propionate (Calbiochem), or of the estrogen, estradiol benzoate (Steraloids, Pawling, N.Y.). Corticosterone groups were injected with 250 μg of corticosterone (Steraloids), suspended in 0.05 ml of a 1.5% (v/v) benzobenzoate–sesame oil solution. We selected these pharmacological doses of the steroids (known to have long-term neuroendocrine influence) in order to parallel other developmental studies (12–14). Control animals were injected with the oil vehicle.

Bilateral adrenalectomy was performed by micropipette aspiration on the first or the sixth day of postnatal life. Cold-treated controls were sham-operated. We did not attempt to separate the effects of surgical trauma from those of cold treatment. All the animals in this particular experiment, including animals that did not receive cold treatment, were injected on day 1 with 250 μg of deoxycorticosterone pivalate (Percorten®, from Ciba, suspended in 0.1 ml of water) to replace lost mineralocorticoids.

Behavioral Testing. At about 240 days of age, animals were tested for reaction to handling and open-field activity. The former was rated by the reactivity score described by Ader and Friedman (15); low-rated animals (the less-reactive ones) are generally considered to be less emotional. Open-field activity was chosen as one way to measure an animal's adaptability to a novel situation (16). Movements in an open field, measuring 110 × 110 cm, were monitored by an electronic detection system (17) for 10-min intervals on three consecutive days. Rats were rated on a 0–6 scale as they

Abbreviation: 5HT, 5-hydroxytryptamine (or serotonin).

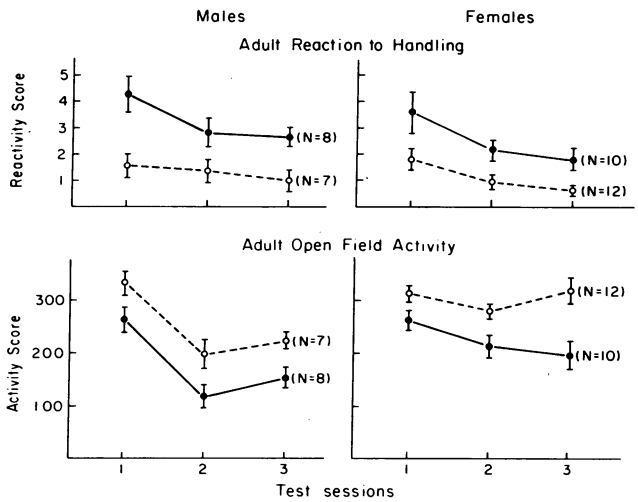

FIG. 1. Comparison of mean behavioral scores (± standard error of mean) between groups of rats given cold treatment on postnatal day 1 (O) and untreated controls (●) when tested for three consecutive days as adults at about 240 days of age. They were rated on a scale of 0–6 for reaction to handling and monitored electronically in the open field to determine activity levels. Cold-treated groups, when compared with untreated controls, show significant decreases in reactivity scores (males: $P < 0.01$; females: $P < 0.02$), and increases in open-field activity (males; $P < 0.01$; females: $P < 0.01$), as determined by analysis of variance.

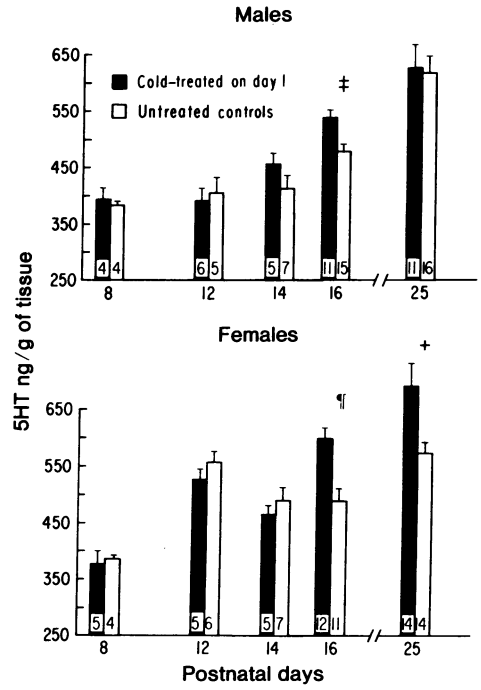

FIG. 2. Mean 5HT concentrations (ng of 5HT/g of frozen tissue ± SEM) in the forebrain plus midbrain, at various ages after birth, of rats given neonatal cold treatment and of untreated controls. In Figs. 2–6, sample sizes of each group are displayed within the bars. Two brains were pooled per sample. Analyses for this and subsequent experiments were determined by Student's t-test. $+P < 0.02$; $\ddagger P < 0.01$; $\P P < 0.001$.

reacted to handling when transferred to and from the open-field apparatus. Behavioral tests were carried out between midnight and 5 a.m.

Tissue Preparation. Animals of a desired age were removed from the nesting box and decapitated, at about the same time of day for a given experiment. A combined fore- and midbrain section was prepared by careful separation of the cerebellum and brainstem just rostral to the pons and by removal of the olfactory bulbs. This tissue preparation included all major forebrain structures that contain 5HT as well as the midbrain raphe. In one experiment, the fore- and midbrain was further dissected into four tissue blocks: "cerebrum" (including neocortex, basal ganglia, hippocampus, and amygdala), "midbrain," "septum plus thalamus," and a "preoptic plus hypothalamus" section. Excised tissue was quickly wrapped in aluminum foil and frozen on dry ice until analyzed. Two weighed fore- and midbrain sections, or three of the smaller tissue blocks, were pooled for each sample; the sections were taken from animals selected from different litters and balanced according to body weight. Runts were not used.

Assays. Brain levels of 5HT were measured by the *o*-phthaldehyde method of Maickel, with modifications as described earlier (12, 18). Duplicate determinations of pooled brain tissue were carried out with reagent blanks and standards of 5-HT-creatine sulfate (Regis). Standards were also added routinely to tissue samples for recovery corrections. A linear fluorescence yield was obtained for standards ranging from 15 to 250 ng. No differences in emission peaks could be detected between samples and standards.

Plasma corticosterone was determined by a competitive protein-binding method (19) with reagent blanks and standard solutions of steroid-containing ethanol. Pooled blood samples (from two to three animals) were collected from decapitated animals into heparinized tubes and frozen until analyzed.

RESULTS

Adult Behavioral Changes. We sought to establish whether our cold treatment, a 30-min event on the first day of postnatal life, resulted in long-lasting behavioral changes such as are produced by other disruptive manipulations. Fig. 1 shows that cold-treated animals were less reactive to handling than controls (females: $F = 8.03$, $df = 1/20$, $P < 0.02$; males: $F = 15.29$, $df = 1/13$, $P < 0.01$) when tested 240 days later. These results are in good agreement with changes brought on by neonatal handling or electric shock (5, 15). Open-field activity, on the other hand, was elevated in both cold-treated males ($F = 10.08$, $df = 1/13$, $P < 0.01$) and females ($F = 10.06$, $df = 1/20$, $P < 0.01$), as has been also reported by other investigators (5, 6).

Effect of Disruptive Experience on Brain Serotonin. Having demonstrated the occurrence of long-term behavioral changes, we next sought to examine neurochemical events in the developing brain produced by the same early experience. As noted earlier, we chose to follow postnatal 5HT levels in a sample of fore- and midbrain tissue.

Fig. 2 shows that cold treatment on day 1 did not produce detectable changes in the 5HT levels of the fore- and midbrain until day 16. The increase occurred in both sexes on day 16, and was still present in females on day 25. No differences in body weight, brain weight, or time of eye-opening could be

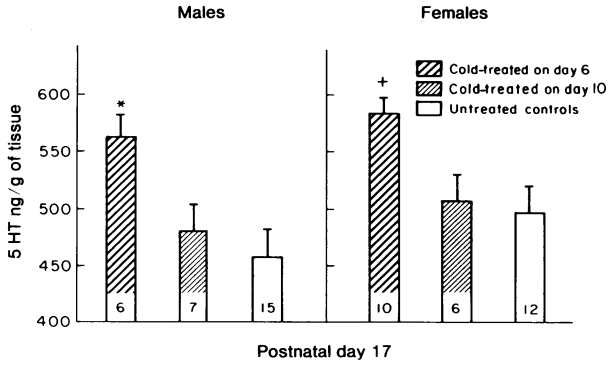

FIG. 3. Mean 5HT concentrations (ng of 5HT/g of frozen tissue ± SEM) in the forebrain plus midbrain of rats on postnatal day 17. Some rats were given cold treatment on day 6, others on day 10; controls received no cold treatment. Two brains were pooled per sample, $*P < 0.05$; $\dagger P < 0.02$ (as compared with controls).

detected between cold-treated and untreated groups throughout development. On day 12, female 5HT levels were elevated above male levels, as we reported earlier (12).

Critical Period. One important aspect of disruptive experience is that it must occur early in life in order to have profound consequences (6). We tested this notion of a "critical period" by administering cold treatment on either postnatal day 6 or day 10. Treatment on day 6 brought about elevations in 5HT levels in the fore- and midbrain in both sexes on day 17, as shown in Fig. 3. Treatment on day 10, however, had no apparent effect. Results point to a period of greater sensitivity of the brain to cold treatment between day 1 and day 6 in both sexes (see ref. 6).

Visual Deprivation. Eye-opening takes place in a neonatal rat on about day 15 and, as a new source of sensory stimulation, may be related to the induced rise in 5HT that appears by postnatal day 16. To test the effect of blocking visual input, rats that received neonatal cold treatment were reared in total darkness until they were killed. The pooled male brains ($n = 6$) of this group showed an elevation in 5HT levels over controls ($n = 7$) that were reared in the dark but did not receive cold treatment (537 ± 10 ng/g compared with 437 ± 20; $P < 0.01$). The pooled female brains ($n = 9$) of the cold-treated group also showed higher 5HT levels than their controls ($n = 9$) (571 ± 29 ng/g compared with 428 ± 40; $P < 0.02$).

Gonadal Steroids. When given to newborn rats, the gonadal steroids (androgens and estrogens) produce persistent changes in adult sex behavior and physiology (20). Disruptive early experience, on the other hand, appears to bring about adult behavioral and physiological changes regardless of sex (6). Since transient sex differences in cerebral 5HT levels of neonatal rats (12, 13) can be modified by androgens and estrogens (12), we compared the altered patterns in development of brain serotonin after treatments with gonadal steroids with those after disruptive early experience. On day 1, female rats were injected with 100 μg of either testosterone propionate or estradiol benzoate. A third group received cold treatment. Brain weights were not significantly altered by the steroid treatments. Fig. 4 compares 5HT levels in hormone- and

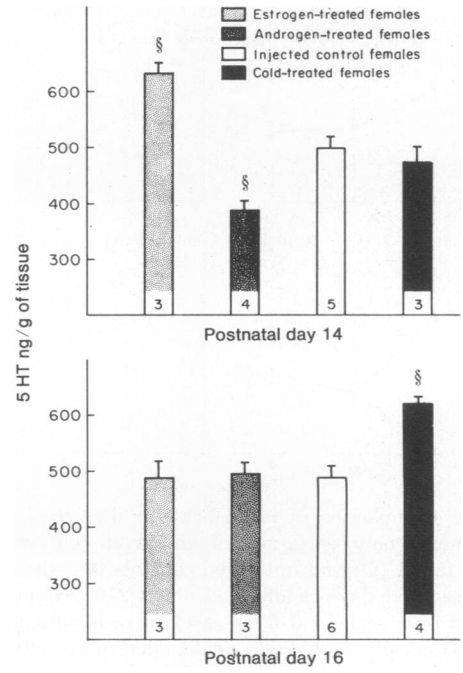

FIG. 4. Mean concentrations of 5HT (ng of 5HT/g of frozen tissue ± SEM) in the forebrain plus midbrain, on days 14 and 16, of female rats that had received on postnatal day 1 either cold treatment or 100 μg of either estrogen or androgen. The estrogen was estradiol benzoate; the androgen was testosterone propionate. Controls received only the oil vehicle. Two brains were pooled per sample. $\S P < 0.005$.

cold-treated groups on day 14 and on day 16. On day 14, estrogen increased the monoamine level and androgen decreased it, as previously reported (12). Elevations of 5HT caused by cold treatment appeared only on day 16, by which time sex hormones had little effect. These results agree with the general notion that disruptive experience phenomena are not sex-dependent (6).

Influence of the Adrenal Gland. Another possible neuroendocrine event related to the disruptive experience phenomenon is the stress-induced release of corticosterone in neonates, which may be responsible for permanent changes in the brain after disruptive treatments (6). In order to distinguish between immediate and prolonged adrenal influence, animals given cold treatment on day 1 were adrenalectomized on either day 1 or day 6. Cold-treated controls were sham-operated on the same days.

By presenting the levels of plasma corticosterone on day 16, the lower panel of Fig. 5 confirms the success of bilateral adrenalectomy. The upper panel shows that treatment-induced increases in 5HT occur with or without the presence of the adrenal glands.

In agreement with the foregoing, it was also found that the mean serotonin levels in corticosterone-treated male and female rats did not differ significantly from their controls, which received only the oil vehicle. In pooled brain samples of corticosterone-treated ($n = 5$) and control ($n = 5$) males, 5HT levels were 470 ± 38 ng/g and 436 ± 47 ng/g, respectively; for pooled brain samples of corticosterone-treated ($n = 7$) and control ($n = 6$) females, 5HT levels were 477 ± 19 ng/g and 450 ± 12, respectively.

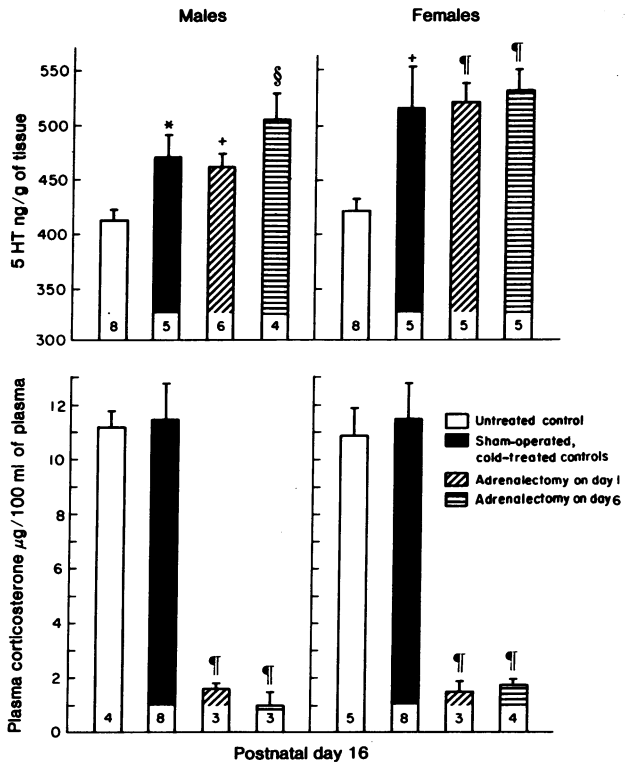

FIG. 5. (Upper panel) Mean 5HT concentrations (ng of 5HT/g of frozen tissue ± SEM) in the forebrain plus midbrain of animals on postnatal day 16. All groups, including controls, received 250 μg of deoxycorticosterone pivalate on day 1. Sham-operated controls and adrenalectomized groups had received cold treatment on day 1 of postnatal life. The surgical treatments were performed on either postnatal day 1 or 6 under cold anesthesia. Controls were not given cold treatment. Two brains were pooled per sample. (Lower panel) Mean plasma corticosterone concentrations (μg of corticosterone/100 ml of plasma ± SEM) at the time of killing for the groups described above. Two to three blood samples were pooled per determination. $*P < 0.05$; $\dagger P < 0.02$; $\S P < 0.005$; $\P P < 0.001$ (as compared with controls).

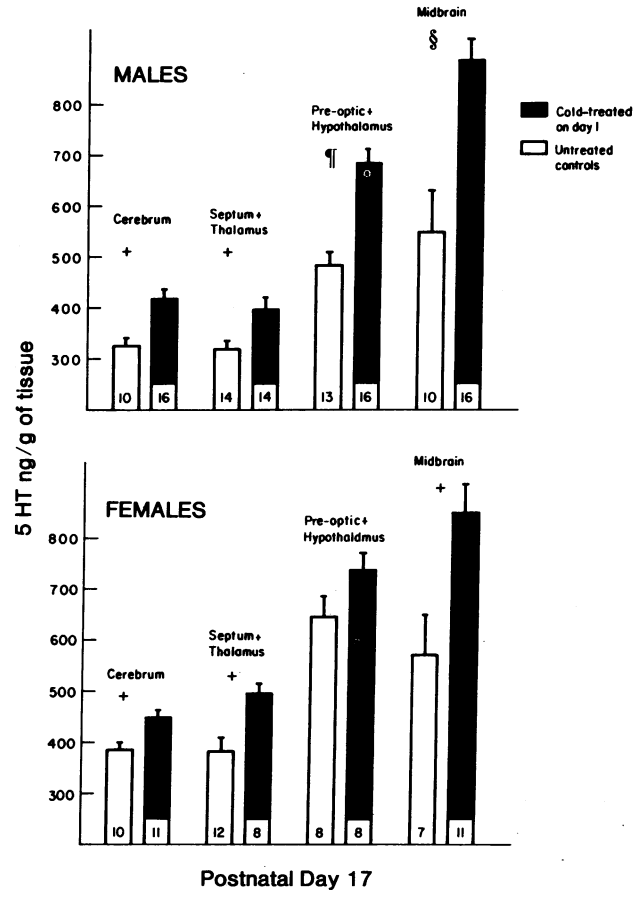

FIG. 6. Mean 5HT concentrations (mg of 5HT/g of tissue ± SEM) in the cerebrum, septum plus thalamus, preoptic region plus hypothalamus, and midbrain of rats on postnatal day 17. The experimental animals had received cold treatment on postnatal day 1; the controls did not receive cold treatment. Three tissue blocks were pooled per sample. $\dagger P < 0.02$; $\S P < 0.005$; $\P P < 0.001$ (as compared with controls).

Regional Serotonin Elevations. To gain insight concerning possible neurochemical mechanisms and behavioral correlates, we dissected the fore- and midbrain section into four smaller tissue blocks: cerebrum, septum plus thalamus, preoptic plus hypothalamus, and midbrain. Fig. 6 illustrates that cold treatment on day 1 elevated 5HT levels in all four regions by day 17. All increases were statistically significant, with the exception of the sample of female preoptic plus hypothalamus.

DISCUSSION

As reported here, a single disruptive experience during neonatal life produced behavioral changes in adult rats tested more than 240 days after the treatment. These changes, elevated open-field activity and reduced reaction to handling, occurred in both sexes. Other investigators have found similar effects using multiple neonatal treatments (5–7).

The serotonergic system was selected as a possible neurochemical index of the developing disruptive experience phenomenon. We found that cold treatment caused a delayed elevation in 5HT levels in our fore- and midbrain preparation. Analysis of separated regions within the fore- and midbrain showed that the increase occurred in all of them. The induced rise in serotonin level first appeared on day 16, the time in postnatal life of the rat associated with maturation of several parameters of the hypothalamic–pituitary–adrenal axis, including diurnal variation of corticotrophin releasing factor and stress-induced release of ACTH and corticosterone (21–24). A serotonin-related locomotor behavior is also reported to develop around postnatal day 15 (25).

Despite studies that have examined sensory components of various neonatal simulation procedures, the factors critical for producing effects in adulthood remain unresolved (6, 26). In our experiments, visual deprivation did not block the rise of 5HT on postnatal day 16. Moreover, since brains were pooled across litters, it seems unlikely that our results can be explained in terms of litter differences. Nor are differences in the maturational rate apparent between groups, for times of eye-opening, body weights, and brain weights were the same in treated and control animals throughout development. We do note, however, that the neurochemical events appear to depend on the kind and amount of neonatal stimulation given, for rats that received a vehicle injection showed less change in 5HT levels than those animals that received cold treatment. Similar observations have been made in regard to adult physiological and behavioral parameters (5, 27). Although the

manipulations used to stimulate neonates generally are rather severe, our results provide a means by which to consider more biologically meaningful early experiences and their effects upon specific regions of the brain.

The role of the adrenal glands with regard to disruptive treatment was examined by adrenalectomizing rats or by injecting them with corticosterone. Results showed that neither the presence of the adrenal glands nor additional corticosterone appears to mediate the phenomenon. It is interesting that, in contrast to early disruptive events, injections of corticosterone are reported not to alter adrenal responsivity to stress, enhance active avoidance, reduce emotionality, or increase open-field activity (14, 28). It would seem, therefore, that although pituitary-adrenal function and related behaviors are altered by an early disruptive experience, adrenocortical secretion is not the causal agent. Since all groups were injected with deoxycorticosterone pivalate, we have not ruled out permissive effects of the mineralocorticoids.

As described in an earlier report (12) and again in this paper, gonadal steroids alter the developing serotonergic system. However, we found here that neither androgen nor estrogen caused a pattern of elevation in brain 5HT like that produced by cold treatment. For example, a transient elevation in cerebral 5HT on postnatal days 10 through 14 occurs naturally only in female brains. Moreover, this rise appears to parallel a transient release of ovarian estrogen (29), and is consistent with our results on the effects of ovariectomy and estrogen injections (ref. 12; and this paper). Androgens reduce 5HT levels and seem to block the effects of estrogen (12), perhaps by inhibiting ovarian secretion (29). In contrast, the cold treatment, as reported here, produces elevations in 5HT levels in both sexes, though not before day 16—the time at which maturation of pituitary-adrenal function occurs (21–23). Cold treatment may also bring on persistent changes in other components of the serotonergic system. For example, the activity levels of monoamine oxidase (EC 1.4.3.2.) in several brain regions of both sexes are markedly different when cold-treated animals are compared with controls on about day 25 (Luine and Giulian, unpublished data). With regard to critical periods, female brains showed sensitivity to estrogen injections, though not to cold treatment, on postnatal day 10 (12). Male brains were not sensitive to either treatment at this age (12). It becomes possible, therefore, to distinguish the effects of gonadal hormones on the neonatal brain from those of disruptive experience by their time of appearance, direction of the effects, and different periods of sensitivity.

We note that such early experience phenomena, when examined at the cellular level, will help to elucidate mechanisms of long-term information storage within the central nervous system.

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