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# Glucocorticoids, the Hippocampus, and Behavioral Inhibition in the Preweanling Rat

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**Endogenous corticosteroids influence brain development and behavioral expression. In rat pups, a corticosteroid-dependent developmental response is behavioral inhibition, which occurs in situations involving threat. Behavioral inhibition consists of freezing and a reduction in ongoing behavior. It is presently unknown which brain region(s) that bind corticosterone (CORT) is involved in the development of freezing. The hippocampus (HC), however, is the principal target site of CORT that regulates the postnatal development of HC dentate granule cells. Therefore, this study examined whether the HC, and in particular, the dentate granule cells, plays a major role in the early appearance of behavioral inhibition.**

**On postnatal day 9, rat pups received bilateral HC electrolytic lesions, or bilateral HC infusions of colchicine, a neurotoxin selective for dentate granule cells, or bilateral HC infusions of kainic acid, a neurotoxin selective for pyramidal cells in the CA3 field. Control rats received sham operations. After the operations, all rats were adrenalectomized (ADX) and injected daily with 3.0 mg/kg CORT, except on the day of the behavioral test. On day 14, all pups were tested for behavioral inhibition, which consisted of removing the pup from the nest box and placing it in a temperature-controlled enclosure subdivided into two compartments by a wire-mesh partition. The pup was placed in one compartment and an unfamiliar anesthetized adult male rat was placed in the adjacent compartment. Results indicated that preweanling rats with electrolytic lesions ranging from the dorsal to the ventral HC exhibited significant deficits in freezing. Importantly, similar deficits in freezing were present in pups treated with colchicine but not KA. Hence, administration of exogenous CORT is not effective in facilitating the occurrence of freezing in preweanling pups lacking dentate granule cells. To determine whether the dorsal HC dentate gyrus is an essential target site of CORT in facilitating freezing, 9-d-old rats were implanted bilaterally with 30 gauge cannula filled with either CORT or cholesterol. After the operation, all rats were ADX and tested for behavioral inhibition on day 14. During testing, ADX pups with CORT-filled cannulae showed significantly higher levels of freezing than ADX control pups. Taken together, results suggest that during the early postnatal period, the action of endogenous CORT in the HC influences the development of dentate granule cells that play an essential role in mediating the appearance of behavioral inhibition.**

***[Key words: adrenalectomy, behavioral inhibition, corticosterone, dentate gyrus, freezing, glucocorticoids, hippocampus, preweanling rat, ultrasonic vocalization]***

Behavioral inhibition is an adaptive response exhibited by vertebrates when threatened (Palmer, 1909; Ratner, 1967; Schaller, 1972, Curio, 1976). It consists of immediate cessation of ongoing behavior accompanied by a prominent immobile posture or freezing response. Understanding the neurobiology of behavioral inhibition may offer critical insights into the pathophysiology of anxiety disorders (Gray 1982; Kagen et al., 1988; Biederman et al., 1990). Therefore, identification and characterization of the neural systems mediating freezing are currently the focus of intense research (LeDoux, 1987; Blanchard and Blanchard, 1988; Davis, 1992).

This laboratory has adopted a developmental approach to the study of behavioral inhibition in an effort to obtain much-needed information on the basis underlying early individual differences in stress-induced responses. Preweanling rodents removed or isolated from the nest often emit ultrasonic vocalizations (Zippelius and Schleidt, 1956; Hart and King, 1966; Noirot 1966, 1968; De Ghett, 1974) that are capable of attracting the attention of the nursing dam (Allin and Banks, 1972; Noirot, 1972; Smotherman et al., 1974). In preweanling rats, this propensity to emit ultrasounds during social isolation, however, is reduced in the presence of an unfamiliar adult male rat (Takahashi, 1992a,b), a potentially infanticidal threat (Rosenberg et al., 1971; Takahashi and Lore, 1982). Concurrent with the reduction in ultrasound production is the display of freezing. This ability of rat pups to exhibit these reciprocal patterns of behavior appears near the end of the second postnatal week (Takahashi 1992a,b), which may reflect the emergence of recently matured physiological systems.

Although the neural system underlying the development of behavioral inhibition remains to be elucidated, it is especially notable that rats adrenalectomized (ADX) on postnatal day 10 exhibit pronounced deficits in behavioral inhibition when tested subsequently on day 14 (Takahashi and Rubin, 1993). This ADX-induced impairment in behavioral inhibition is reversed after administration of exogenous corticosterone (CORT), the major glucocorticoid of the rat, either systemically or directly into the brain (Takahashi and Rubin, 1993; Takahashi and Kim, 1994). Because glucocorticoids are implicated in brain development (Sze et al., 1976; Doupe and Patterson, 1982; Meyer,

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1985), it is possible that neural sites that bind endogenous CORT have a major involvement in the developmental expression of behavioral inhibition.

A likely neuroanatomical target of CORT whose development appears to coincide with the ability of rats to express behavioral inhibition is the hippocampus (HC). Anatomical studies indicate that the HC, in particular, dentate granule cells, shows considerable postnatal development during the early preweaning period (Altman and Das, 1965; Schlessinger et al., 1975; Bayer 1980a,b; Cowan et al., 1980). Furthermore, prior to the end of the second postnatal week, adrenal steroids play a prominent role in regulating granule cell genesis and cell death (Gould et al., 1991a,b). The mechanism(s) by which adrenal steroids produce their effects on HC cells is not clear but may be via hormone receptor activation because of the high density of adrenal steroid receptors located in the HC (Stumpf, 1971; Gerlach and McEwen, 1972; Rosenfeld et al., 1988a,b, 1990; Sarrieau et al., 1988; Lawson et al., 1991; Van Eekelen et al., 1991). Finally, behavioral studies suggest that the HC is involved in controlling some forms of response suppression or inhibition (Douglas 1967; Altman et al., 1973; De Kloet et al., 1988). Taken together, data suggest that CORT-induced developmental changes occurring in the HC may have a critical role in facilitating the ontogeny of behavioral inhibition. Therefore, the purpose of this study was to provide the first step to address the fundamental question of whether or not the developing rat HC plays a major role in the appearance of behavioral inhibition.

Some of these results appeared in abstract form (Takahashi, 1994a).

## Materials and Methods

*Animals.* Rat pups were offspring of Sprague–Dawley female rats (75–90 d old) derived from a stock obtained from Sasco, Madison, WI. Rats were maintained on a 12 hr/12 hr light/dark cycle, with lights on at 0600 hr. After mating, female rats were housed singly in stainless steel hanging cages until day 20 of pregnancy when they were transferred to plastic breeding cages (31 × 22 × 18 cm) with wire-mesh tops. Each cage was provisioned with food, water, and a layer of wood shavings. Breeding cages were checked daily for the presence of pups (day of birth = postnatal day 0). Litters were left undisturbed except for routine cage cleaning. Sexually experienced adult male Sprague–Dawley rats, housed in an adjacent room, were used as stimulus animals.

*Electrolytic lesions.* On postnatal day 9, two male pups were taken from each litter ($n$ = 11) and assigned to either the lesion or the sham-operation group. Pups were anesthetized with methoxyflurane (Pitman-Moore, Mundelein, IL) and placed in a stereotaxic apparatus adapted for neonatal rats (David Kopf Instruments, Tunjunga, CA). A stainless steel 0.2 mm diameter wire insulated with Epoxylite except for 0.5 mm at the tip was used for passing anodal current from a constant dc source (Model DCLM5A, Grass Instrument Company, Quincy, MA). Lesions were made by passing 1.5 mA current for 15 sec with the electrode positioned at three sites within each HC. The following flat-skull coordinates were used: A-P = −2.0 mm from bregma, M-L = ±1.0 mm, D-V = −3.0 mm from the skull surface; A-P = −3.8 mm from bregma, M-L = ±3.5 mm, D-V = −3.4 mm and −5.4 mm from the skull surface. Identical electrode placement procedures were used for sham-operated rats except no current was passed. After stereotaxic surgery, all pups were ADX and treated with CORT.

Two sham-operated controls died prior to testing and two HC-lesioned rats appeared sick and were not tested. Therefore, data were obtained from nine sham-operated (body weight = 25.8 ± 0.9 gm) and nine HC-lesioned (23.9 ± 0.3 gm) rat pups.

*Destruction of hippocampal dentate granule cells.* Two male pups were taken from each litter ($n$ = 11) and prepared for stereotaxic surgery on day 9. Colchicine infusions were made with a 28 gauge stainless steel cannula connected to a microliter syringe by polyethylene tubing. The microliter syringe was driven by an infusion pump. Colchicine (Sigma Chemical, St. Louis, MO) was dissolved in deionized water and 25 ng was infused in a volume of 0.25 μl over a 60 sec period. The infusion cannula remained in place for an additional 90 sec. Control pups were infused with vehicle. Each HC was infused with vehicle or colchicine at two locations using the following flat-skull coordinates: A-P = −2.0 mm from bregma, M-L = ±1.0 mm, D-V = −3.0 mm from the skull surface; A-P = −3.8 mm from bregma, M-L = ±3.6, D-V = −4.4 mm from the skull surface. All rat pups were ADX and administered CORT after the operation.

Two sham-operated controls and one colchicine-treated pup died prior to testing. Thus, histological and behavioral data were obtained from nine controls (body weight = 26.7 ± 0.7 gm) and 10 (25.9 ± 0.2 gm) colchicine-treated rats.

*Destruction of hippocampal CA3 cells.* Two 9-d-old male pups were obtained from each litter ($n$ = 9) and received either sham operations or infusions of KA in the HC. KA infusions were made with a 28 gauge stainless steel cannula. KA (Sigma Chemical, St. Louis, MO) was dissolved in sterile saline and 100 ng was infused in a volume of 0.5 μl over a 120 sec period. The infusion cannula remained in place for an additional 90 sec. Control pups were infused with vehicle. Each HC was infused with vehicle or KA at two locations using the following flat-skull coordinates: A-P = −2.0 mm from bregma, M-L = ±2.0 mm, D-V = −3.0 mm from the skull surface; A-P = −3.8 mm from bregma, M-L = ±3.6, D-V = −5.4 mm from the skull surface. After the operation, all pups were ADX and injected with exogenous CORT. In adult rats, systemic administration of KA elicits seizures (Cherubini et al., 1983; Albala et al., 1984; Ben-Ari et al., 1984; Tremblay et al., 1984; Sperber et al., 1991). Therefore, KA infused pups were observed periodically in the nest box during a 3 hr postoperative recovery period. Behavior indicative of seizure activity was not observed.

One sham-operated pup died prior to day 14. Therefore, a total of eight control pups (body weight = 24.3 ± 0.2 gm) and nine KA-treated rats (24.0 ±24.0 gm) were tested.

*Histological verification of lesions.* Upon completion of behavioral testing, pups were overdosed with sodium pentobarbital and perfused intracardially with 0.9% saline followed by 10% formalin. Brains were removed and kept in 10% formalin followed by 20% sucrose-formalin for cryoprotection. Frozen sections were cut at 60 μm and every third section was mounted throughout the extent of the lesion and stained with thionine. The magnitude and placement of lesions and cannulae were determined with the aid of a 10-d-old rat brain atlas (Sherwood and Timiras, 1970).

*Implantation of CORT in the dorsal HC.* Two 9-d-old male pups were obtained from each litter ($n$ = 11) and assigned randomly to either cholesterol or CORT implantation groups. Implantation procedures consisted of bilateral placements of 30 gauge stainless steel cannula containing cholesterol or CORT (Sigma Chemical) in the dorsal HC. Cannulae were filled with molten cholesterol or CORT. After solidifying, the outer tip of the cannula was cleaned with ethanol to ensure that hormone was available only at the surface of the lumen. The following flat-skull coordinates were used: A-P = −2.0 mm from bregma, M-L = ±2.0 mm, D-V = −3.0 mm from the skull surface. After the operation, ADX procedures were conducted.

Prior to returning the ADX pup to the nest box on day 10, an s.c. injection of 8 μg/100 g body weight of aldosterone (Sigma Chemical) was administered. A second aldosterone injection was administered on day 12. This dose of aldosterone facilitates survival in ADX pups and does not promote the development of behavioral inhibition (Takahashi and Rubin, 1993; Takahashi, 1994c).

After testing, pups were prepared for perfusion and brain histology as described previously, with the exception that a blood sample was taken from the heart prior to perfusion with a syringe. Blood samples were placed in ice-chilled microcentrifuged tubes containing EDTA. Tubes were centrifuged in an Eppendorf microcentrifuge (Brinkman Instrument, Westbury, NY) for a duration of 3 min. Plasma was aliquoted and stored at −70°C until the time of assay for CORT.

Cannulae were removed from the brain and the lumen examined using a microscope for the presence of hormone. Inspection of cannulae revealed that although hormone was recessed in the lumen, all cannulae contained hormone. Cannulae were then allowed to dry, weighed, refilled with CORT, and reweighed to determine the amount of CORT dissolved during the period of implantation.

Duplicate plasma samples were analyzed in one assay for CORT using a $^{125}$I CORT kit (Diagnostic Products, Los Angeles, CA). The antiserum exhibits a cross-reactivity to 11-deoxycorticosterone of < 2.9%. Cross-reactivity to other adrenal steroids was < 0.9%). The de-

tection limit of the assay was 1.5 ng/ml. The intraassay coefficient of variation was 4.0%.

One cholesterol-implanted pup died prior to day 14. In addition, two CORT-implanted pups had high plasma concentrations of CORT (mean = 49.5 ng/ml) and were excluded from the data analysis. Plasma concentrations of CORT in the remaining CORT-implanted pups as well as ADX pups implanted with cholesterol-filled cannulae were below the level of detection of the assay. Therefore, behavioral analyses were performed on data obtained from 10 cholesterol- (body weight = 24.9 ± 0.6 gm) and 9 CORT-implanted (24.4 ± 0.9 gm) rats.

*Adrenalectomy and CORT replacement.* The HC is a target for the negative feedback effects of glucocorticoids and its removal is associated with elevations in pituitary-adrenal hormones and their secretagogues (Knigge, 1961; Murphy et al., 1979; Feldman and Confronti, 1980; Wilson et al., 1980; Herman et al., 1989; Jacobson and Sapolsky, 1991). In preweanling rats, elevations in endogenous pituitary-adrenal hormones influence behavioral responding (Takahashi et al., 1991). Therefore, ADX and exogenous CORT administration procedures were conducted to eliminate the possibility that behavioral differences between sham-operated and lesioned groups, i.e., electrolytic, colchicine, and KA-lesioned rats, were due to effects produced by HC lesion-induced alterations in hypothalamic-pituitary-adrenal hormone secretion.

Immediately after stereotaxic surgery, a unilateral dorsal incision was made to extract one adrenal gland. The pup was then returned to the nest box containing the unoperated male and female littermates. During this postsurgical period, which generally lasted between 30 to 60 min, the dam was kept in another cage. Once operated pups began to move around the cage, they were reintroduced to their mother. After a 24 hr period, the operated pups were anesthetized with methoxyflurane and the remaining adrenal gland was extracted. Pilot work on rat pups indicated that removal of adrenal glands over a 2 d period improves survival after stereotaxic surgery. Prior to returning the pup to the nest box, an s.c. injection of 3.0 mg/kg of CORT was administered. CORT injections were repeated at 24 hr intervals on days 11, 12, and 13. This dose of CORT is effective in facilitating the development of behavioral inhibition in ADX pups (Takahashi and Rubin, 1993; Takahashi, 1994b).

*Apparatus.* Tests were conducted in a Plexiglas enclosure (26.5 × 26.5 × 20 cm) housed in a temperature-controlled incubator with a glass front. The Plexiglas enclosure was subdivided by a wire-mesh partition positioned with the two ends attached to the midportion of two adjacent walls, thereby forming a small triangular compartment. The top was open except for the area above the small triangular compartment, which housed the rat pup. The enclosure was placed on a cardboard floor that was changed after every test. The ultrasonic detector was positioned directly above. Ambient temperature in the incubator varied from 33 to 35°C. These temperatures are within the thermoneutral range for 14-d-old rats (Conklin and Heggeness, 1971).

*Behavioral tests.* Rat pups were tested on day 14. At this time, an adult male rat was anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed into the large compartment of the test apparatus. The rat pup was then placed in the adjacent triangular compartment.

*Behavioral measurements.* All behavioral tests were 10 min in duration and conducted in the first half of the light cycle. During testing, the duration (in seconds) of freezing was recorded using timers. Freezing was scored whenever the pup assumed an immobile posture with the head in a stationary and elevated position. Ultrasonic vocalizations were recorded with a counter. Ultrasounds were detected with headphones attached to the socket of a Mini-2 bat detector (Bat Conservation International, Austin, TX) tuned to 40 kHz. The bat detector transforms the ultrasound into the audible range of humans. It should be noted that in earlier pilot studies, 14-d-old rat pups consistently emitted ultrasounds that were within the 40 kHz range. Higher or lower frequency ultrasounds beyond the detectable limits of the 40 kHz setting were never heard.

Removal of adrenal hormones reduces the ability of organisms to thermoregulate (Deavers and Musacchia, 1979), which may affect behavioral expression (Allin and Banks, 1971; Blumberg and Alberts, 1990). Therefore, immediately after testing, the pup's rectal temperature was measured using a microprobe (IT-21, Physitemp, Clifton, NJ) attached to a BAT-12 digital thermometer, with a resolution of 0.1°C. The probe was inserted into the rectum to a depth of 10 mm and held in position until the temperature stabilized. Rectal temperature measurements were obtained within a 10 sec period.

*Statistics.* Statistical significance was determined using independent $t$ tests. Results are expressed as mean ± SEM.

## Results

### *Effects of hippocampal lesions on behavioral inhibition*

*Histological results.* The extent of electrolytic lesions produced in the HC formation is shown in Figure 1. Damage in the region of the dorsal HC was symmetrical and invariably included the dentate gyrus (see coronal sections A2.0 and A2.9 in Fig. 1). At these levels of the HC formation, the CA3 region underwent the least destruction. However, in posterior regions of the HC, the CA3 regions incurred extensive damage (coronal sections A0.4 and A1.2 in Fig. 1), whereas destruction to dorsal and ventral dentate gyrus was considerably less severe. Finally, the posterior boundary of the lesions extended into the dentate gyrus (coronal section A0.0 in Fig. 1). In addition to damage in the dorsal HC region, there was variable damage to the corpus callosum and overlying neocortex. In posterior HC regions, minor damage was occasionally found in the lateral and medial geniculate nucleus.

*Behavioral results.* Administration of exogenous CORT was ineffective in facilitating freezing in rat pups bearing HC lesions. As shown in Table 1, HC-lesioned pups spent significantly less time engaged in freezing than sham-operated animals, $t(16) = 6.90$, $p < 0.01$. No group differences were found in the number of ultrasonic vocalizations, $t(16) = 1.09$, $p > 0.05$, which were low. Body temperatures did not differ between sham-operated (37.0 ± 0.1) and HC-lesioned pups (37.7 ± 0.1).

### *Effects of hippocampal granule cell loss on behavioral inhibition*

*Histological results.* Marked destruction of dentate granule cells was observed 5 d after bilateral intrahippocampal infusion of colchicine (Fig. 2). In contrast, CA1 to CA3 regions incurred only minor damage. The extent of the damage decreased with distance from the site of injection. Examination of serial sections using low power magnification revealed that dentate granule cell loss occurred within an anterior–posterior distance of 360 to 540 μm from the site of greatest damage. In dorsal HC regions, the corpus callosum and neocortex overlying the site of injection was also damaged (see Fig. 2*B*). Colchicine infusions did not appear to produce major loss of dentate granule cells in the most posterior–ventral HC regions proximal to the entorhinal cortex.

*Behavioral results.* Analysis of behavioral data indicated that infusion of colchicine into the HC produced a significant reduction in freezing duration in ADX pups administered exogenous CORT, $t(17) = 8.45$, $p < 0.001$ (see Table 2). Ultrasonic vocalizations produced by both groups were low in occurrence and did not differ reliably, $t(17) = 0.10$, $p > 0.05$. Body temperatures were also similar between sham-operated and colchicine-treated groups (37.3 ± 0.1 vs 37.3 ± 0.1, respectively).

### *Effects of hippocampal CA3 cell loss on behavioral inhibition*

*Histological results.* Five days after bilateral intrahippocampal injections of KA, there was a pronounced loss of cells in the hilar region of the dentate gyrus (see Fig. 3*B,C*). At the site of injection, the majority of CA3 cells were destroyed. The destruction of hilar cells appeared to produce a reduction in the distance between the dentate granule cell layers that was most notable in the dorsal HC. Although CA3 cells immediately adjacent to those located in the hilar region were also destroyed, the majority of CA3 as well as CA1 and CA2 neurons appeared intact.

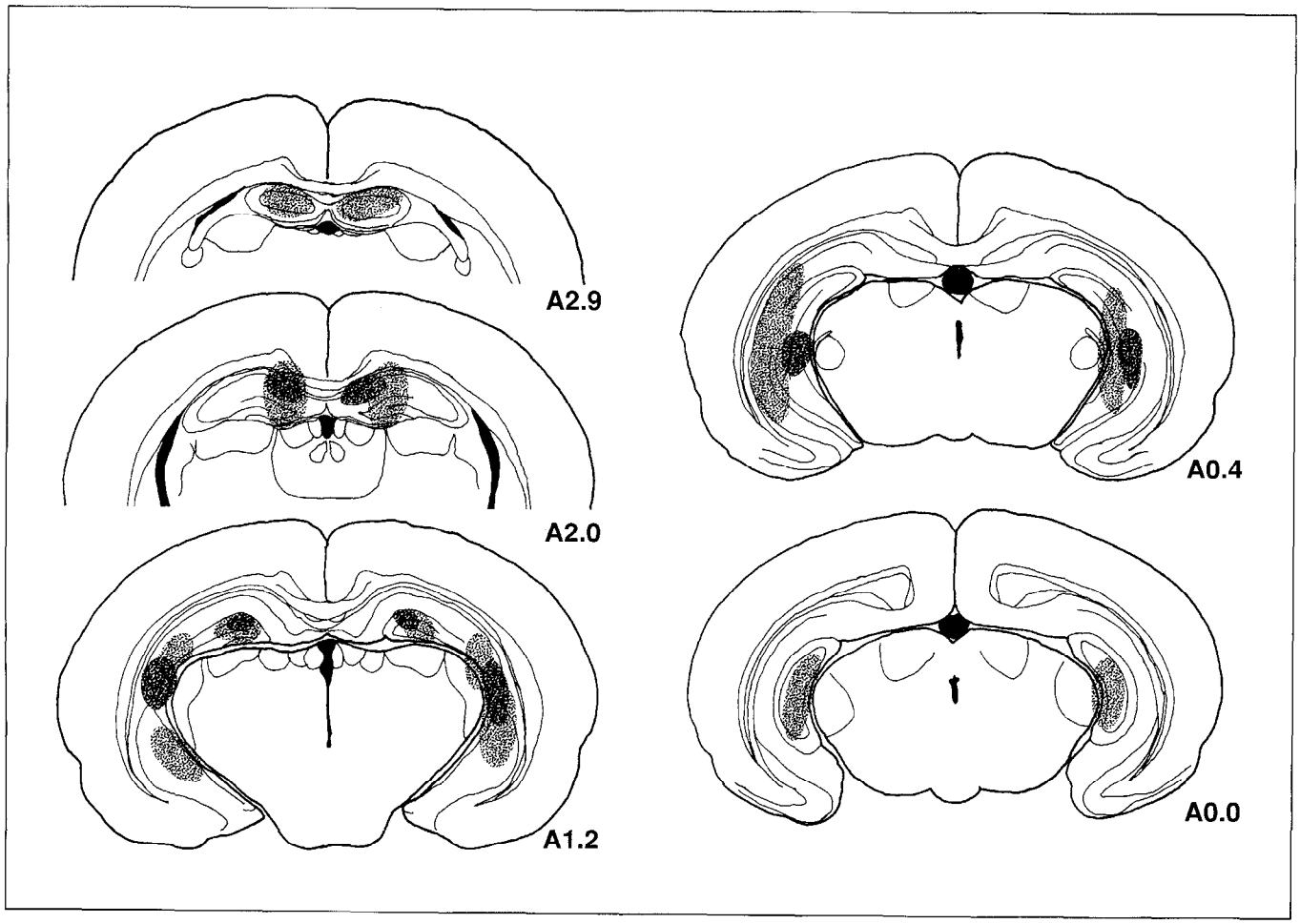


*Figure 1.* Reconstruction of the extent of hippocampal lesions of rat pups. (The *dark areas* represent the smallest lesions, whereas the *light stippled areas* indicate the largest lesions. The *number* designates the anterior position of the coronal section in mm relative to interaural zero. Plates were adapted from the 10-d-old rat brain atlas of Sherwood and Timiras (1970), with permission.)

Destruction of CA3 cells was present in an anterior–posterior distance of 360 to 450 μm from the hilar site of greatest damage.

*Behavioral results.* Intrahippocampal injections of KA produced no significant reduction in freezing, $t(15) = 0.01$ (see Table 3) in ADX rat pups treated with exogenous CORT. In addition, groups did not differ significantly in either ultrasound production, $t(15) = 0.25$ (Table 3), or body temperature, $t(15) = 0.03$ (data not shown).

### *Effects of CORT action in the dorsal HC on behavioral inhibition*

*Histological results.* Bilateral implantation sites of 30 gauge cannulae containing either cholesterol or CORT are shown in Figure 4. All cannula tips were located in the dorsal HC, with a majority in the region that contained the coronal section identified as A2.0. At this coronal level, a number of implantation sites were located in the molecular layer bordering the superior blade of the dentate granule cells. A few implants were also found in the hilus. Only three animals (one cholesterol- and two CORT-implanted pups) had implants located either anterior or posterior to coronal section A2.0. After a 5 d implantation period, the amount of CORT released from each cannula tip was 8.2 ± 1.6 μg.

**Table 1. Mean ± SEM of behavioral responses in 14-d-old rats after sham operations or HC lesions**

| | Sham-operated ($n = 9$) | HC lesions ($n = 9$) |
|---|---|---|
| Freezing (sec) | 268 ± 35 | 20 ± 8* |
| Ultrasonic vocalizations (no.) | 7 ± 3 | 3 ± 1 |

* Significantly different from sham-operated group, $p < 0.01$.

*Behavioral results.* CORT-implants were highly effective in facilitating freezing in ADX pups in comparison to cholesterol implants, $t(17) = 3.48$, $p < .0.01$ (Table 4). The emission of ultrasounds, however, did not differ significantly between cholesterol- and CORT-implanted pups $t(17) = 0.53$. Body temperature also did not differ reliably between groups, $t(17) = 1.02$ (data not shown).

## Discussion

### *Role of hippocampal dentate granule cells in behavioral inhibition*

Results suggest clearly that by the end of the second postnatal week the HC plays a prominent role in the expression of behavioral inhibition. More specifically, data suggest that HC dentate granule neurons play an essential role in mediating the ef-

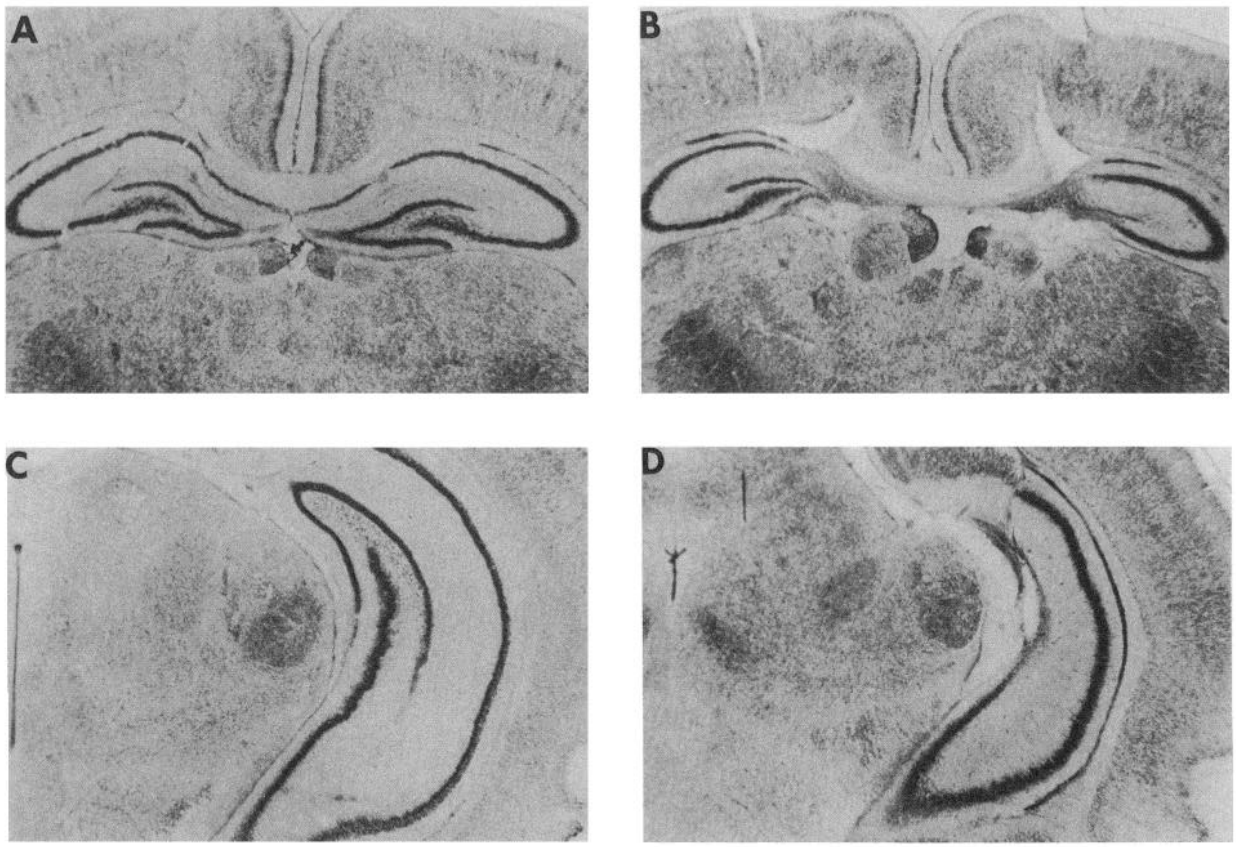


*Figure 2.* Photomicrographs of thionine-stained 60 μm sections of dorsal and posterior hippocampal regions in control (*A* and C) and colchicine-treated (25 ng/0.25 μl/site, *B* and *D*) rat pups. A typical dorsal hippocampal lesion induced by colchicine produced extensive damage, especially to the inferior blade of the dentate granule cell layer, as depicted in the right hippocampus of section *B*. In this rat pup, left dorsal hippocampal dentate granule cells were severely damaged approximately 120 μm anterior to plate *B*. Note in *D* the extensive loss of dentate granule cells in the region of the ventral hippocampus. At both dorsal and ventral hippocampal regions, hilar pyramidal cells also appeared to be damaged.

fects of CORT on freezing. This conclusion appears to be supported by the finding that KA-induced destruction of pyramidal CA3 cells in the hilar region was ineffective in attenuating freezing. Hence, the significant reduction in freezing produced by electrolytic lesions that nonselectively eliminated both dentate granule and pyramidal cells in the HC may be attributed to the specific destruction of dentate granule cells. By implicating the HC, these results now provide the basis to examine in detail the HC mechanisms responsible for mediating the emergence and display of behavioral inhibition.

The entorhinal cortex constitutes the major source of afferents to the dentate gyrus via the perforant pathway (Lorente de No, 1934; Blackstead, 1958; Raisman et al., 1965; Amaral and Witter, 1989). Within the dentate gyrus, granule cells and their mossy fiber axons are the main sources of output to cells in the hilus, CA3 and CA2 fields of the HC (Swanson et al. 1978; Gaarskjaer, 1986). Present results suggest that colchicine-induced destruction of dentate granule cells critically undermines the functional integrity of these intrinsic connections, thereby severely compromising the rat pup's ability to exhibit freezing. Furthermore, mossy fiber projections to pyramidal cells distal to those located in the hilus and CA3c regions appear to be sufficient to maintain freezing. This view is supported by the observation that pups appeared fully capable of freezing after KA-induced destruction of CA3 cells in the hilus. From a developmental and behavioral perspective, the importance of dentate granule cells and their mossy fiber projections is clear because it is not until the end of the second postnatal week that mossy terminals in field CA3 attain an adult-like appearance (Amaral and Dent, 1981) and the ability to freeze emerges in rats (Collier and Bolles, 1980; Takahashi, 1992b). In addition, the distribution of entorhinal afferents in the molecular layer of the dentate gyrus finally develops an appearance comparable to that of mature animals by postnatal day 12 (Cowan et al., 1980).

Although the posterior–ventral dentate granule cells adjacent to the entorhinal cortex appeared, for the most part, to be spared, it is not certain whether elimination of the dorsal HC dentate granule cells is sufficient to produce an attenuation in freezing. An early report demonstrated that after exposure to electrolytic lesions that produced extensive damage throughout the HC formation, adult rats exhibited a marked reduction in freezing in the presence of a cat (Blanchard and Blanchard, 1972). How-

**Table 2. Mean ± SEM of behavioral responses in 14-d-old rats after sham operations or HC colchicine lesions**

| | Sham-operated ($n = 9$) | Colchicine lesions ($n = 10$) |
|---|---|---|
| Freezing (sec) | 407 ± 27 | 66 ± 30* |
| Ultrasonic vocalizations (no.) | 3 ± 2 | 3 ± 2 |

* Significantly different from sham-operated group, $p < 0.001$.

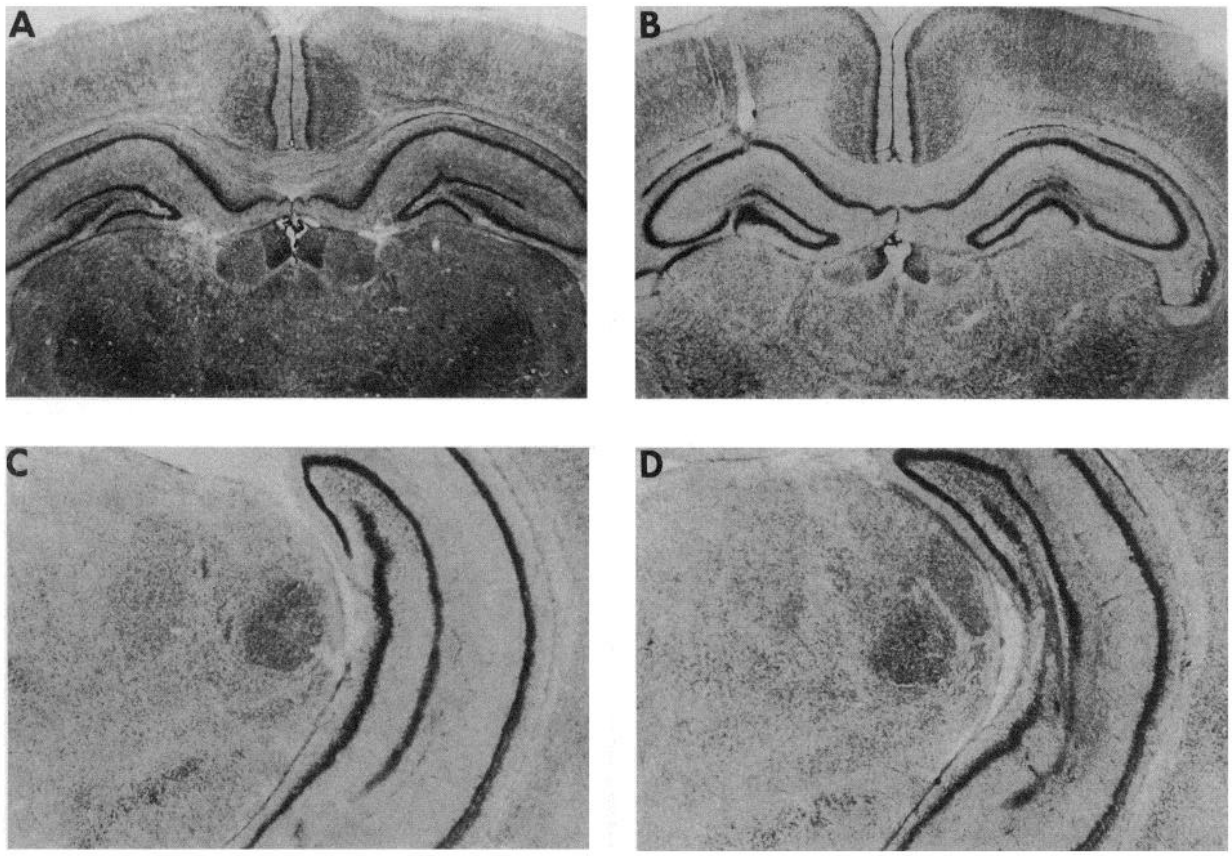


*Figure 3.* Photomicrographs of thionine-stained 60 μm sections of dorsal and posterior hippocampal regions in control (*A* and *C*) and KA-lesioned (100 ng/0.5 μl/site, *B* and *D*) rat pups. In both dorsal and posterior hippocampal regions, KA produced an extensive loss of cells in the hilar region. Variable damage was also evident in the immediately adjacent CA3 cells. Dentate granule cells appeared to incur only minor damage.

ever, in other studies using adult rats, electrolytic lesions confined to the dorsal HC that destroyed both the dentate gyrus and fields CA1 and CA3 were not sufficient to reduce freezing induced by stimuli associated with foot shock (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). Between-study differences in the stimulus used to elicit freezing may account for either the presence or reduction in freezing occurring after HC lesions. It is also possible that potential physiological differences between dorsal and ventral HC regions (e.g., Garcia Ruiz et al., 1993) differentially influence behavioral expression, depending on the nature of the test situation. For instance, some investigators reported that aspiration lesions of the ventral HC were effective in producing rats with deficits in a conditioned suppression water-lick test (Clark et al., 1992). In addition, cholinergic receptor antagonists infused into the ventral HC region, in the area of the entorhinal cortex, were more effective in producing behavioral alterations than infusions made into the dorsal HC (Blozovski, 1979; Blozovski and Hennocq, 1982). Other investigators reported that spatial learning deficits reflect the magnitude of dorsal HC damage, whereas ventral HC lesions are generally without effect (Moser et al., 1993).

**Table 3. Mean ± SEM of behavioral responses in 14-d-old rats after sham operations or HC kainic acid lesions**

| | Sham-operated ($n = 8$) | KA lesions ($n = 9$) |
|---|---|---|
| Freezing (sec) | 370 ± 53 | 369 ± 51 |
| Ultrasonic vocalizations (no.) | 4 ± 2 | 5 ± 4 |

The basis underlying the attenuation of freezing after loss of cells in the dentate gyrus remains to be precisely specified. Adult rats with large lesions of the HC showed an increase in locomotor activity in the presence of a cat but not prior to the cat's introduction (Blanchard and Blanchard, 1972). In other studies, dentate granule cell loss was associated with increased locomotor activity that may account for deficits reported to occur in passive avoidance tests (Haggbloom et al., 1974; Walsh et al, 1986). Although ambulatory movements were not measured in the present experiments, it is possible that colchicine-induced attenuation in rat pup freezing is due to a potentiated increase in behavioral movements. The inability to suppress certain behavioral responses coupled with an increase in movement-related behavior were among the cardinal manifestations reported in early studies of HC lesions (Issacson and Wickelgren, 1962; Douglas, 1967; Kimble, 1968). Subsequent research suggests that increased behavioral movements occurring after HC lesions may be due, in part, to alterations in dopamine activity in the nucleus accumbens (Reinstein et al., 1982), a target of HC projections (Raisman et al., 1966; Swanson and Cowan, 1977; Kelley and Domesick, 1982).

An increase in behavioral movements, however, is not the only interpretation that may account for a deficit in freezing. Alterations in neural function arising from the destruction of dentate granule cells may be occurring in a manner that biolog-

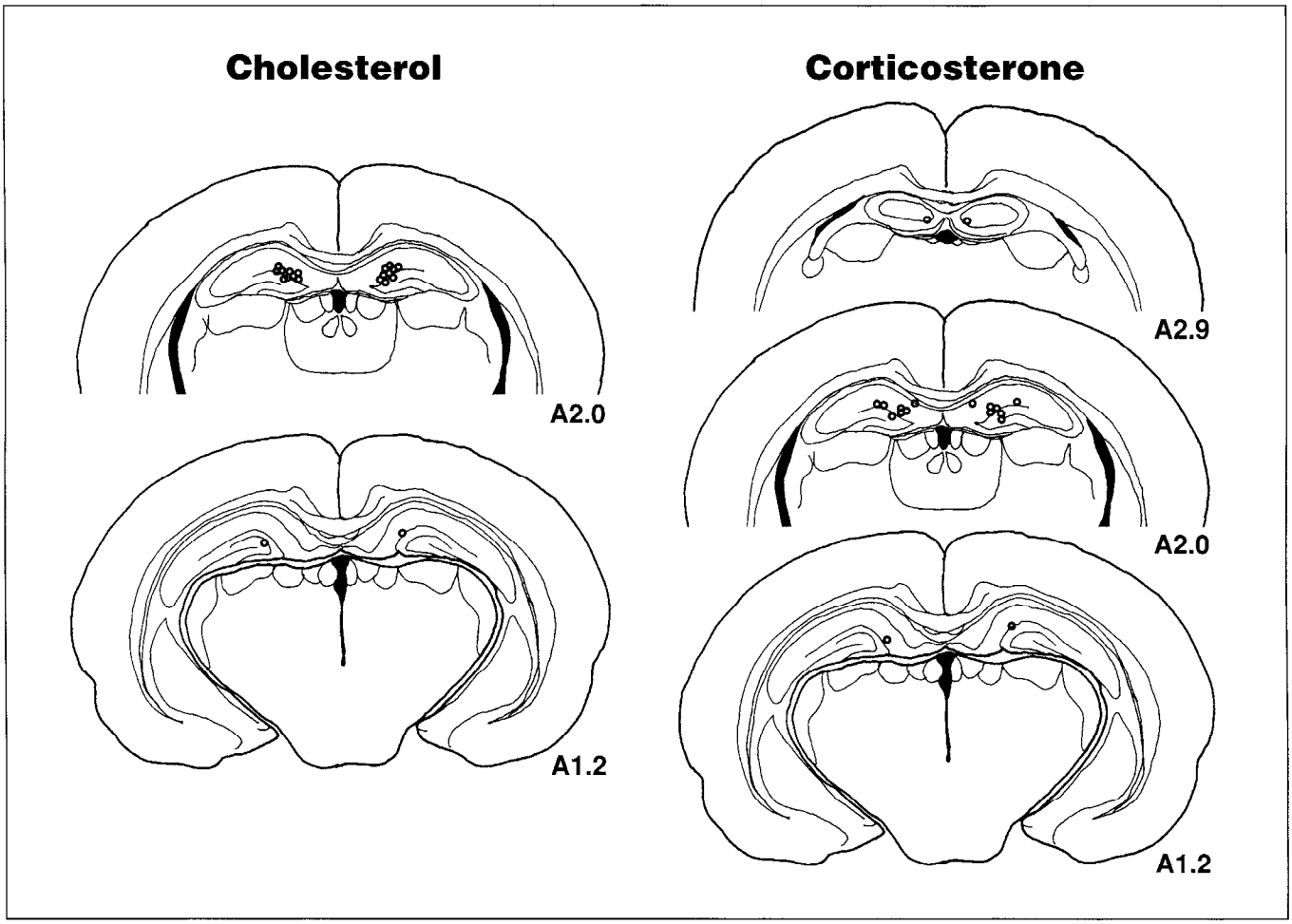


*Figure 4.* Location of bilateral 30 gauge cannula tips containing either cholesterol or corticosterone in the dentate gyrus of 14-d-old rat pups. (The *number* designates the anterior position of the coronal section in mm relative to interaural zero. Plates were adapted from the 10-d-old rat brain atlas of Sherwood and Timiras (1970), with permission.)

ically relevant stimuli are no longer integrated in an adaptive manner (Deadwyler et al., 1981). The HC system was initially proposed to play an important role in the processing of olfactory stimuli (Brodal, 1947), and recent electrophysiological studies demonstrate that cells of the dentate gyrus respond to olfactory stimuli (Vanderwolf, 1992). Of particular relevance are studies demonstrating that odors of predators potentiate fast wave bursts in the dentate gyrus of rats (Heale et al., 1994). In addition, odor-guided learning and memory appear to be dependent upon an intact HC system (Staubli et al., 1984; Eichenbaum et al., 1991). Accordingly, destruction of dentate granule cells may impair not only the rat pup's ability to respond appropriately to odors associated with threat but also the integration of unfamiliar with familiar odors that may further contribute to the observed disruption in freezing.

**Table 4. Mean ± SEM of behavioral responses in 14-d-old rats after implantation of 30 gauge cannulae containing cholesterol or CORT in the dorsal HC**

| | Cholesterol implants ($n$ = 10) | CORT implants ($n$ = 9) |
|---|---|---|
| Freezing (sec) | 142 ± 36 | 301 ± 9* |
| Ultrasonic vocalizations (no.) | 34 ± 20 | 21 ± 10 |

* Significantly different from cholesterol implants, $p < 0.01$.

It should be noted that although the pup could view the adult male through the wire-mesh partition, visual cues probably do not contribute significantly to the elicitation of freezing. In this strain of rats, approximately 40% of 14-d-old pups do not have fully opened eyes (Takahashi, 1994b). Importantly, they do not differ in freezing from pups with opened eyes. Additionally, previous studies (Takahashi, 1994b) demonstrated that rat pups readily exhibit behavioral inhibition in the presence of an unfamiliar adult male rat, whereas similar-sized but familiar adult male rats are ineffective in potentiating behavioral inhibition. Thus, HC dentate granule cells may be an important component of an odor-based memory system that guides or directs behavioral responses accordingly to the nature of the odor and its degree of familiarity.

In addition to a putative role underlying the integration of olfactory information, data suggest strongly an involvement of dentate granule cells in spatial and nonspatial memory. Colchicine-induced loss of dentate cells in adulthood was shown to produce deficits in both the Morris water maze (Sutherland et al., 1983) and radial-arm maze tasks (Walsh et al., 1986; Emerich and Walsh, 1990). Other experimental procedures that induce a loss of granule cells such as long-term ADX (Sloviter et al.,

1989; Sapolsky et al., 1991) were also effective in impairing spatial learning in the Morris water maze (Conrad and Roy, 1993). In studies using infant rats, x-irradiation–induced granule cell hypoplasia produced deficits in patterned alternation (Diaz-Granados et al., 1992), a form of nonspatial memory-based learning. Damage to the HC formation that includes the dentate gyrus also disrupts configural discrimination (Rudy and Sutherland, 1989; Sutherland et al., 1989), another form of nonspatial learning. Together, data suggest that the reduction in freezing is not a specific outcome of dentate granule cell loss. The potential relevance of these studies for understanding behavioral inhibition, however, is that it underscores the involvement of the HC in cognitive functions. The risk of predation exerts a profound influence on animal decision making (Lima and Dill, 1990), which, in turn, is responsible for the occurrence of overt patterns of behavior, e.g., freezing. Disruption of hippocampal function may influence cognitive integration in potentially harmful situations in number of different ways. As indicated earlier, important olfactory stimuli may not be readily perceived. Even if the stimulus is perceived, appropriate attention to the stimuli may not be forthcoming, resulting in the production of atypical responses. The HC formation may be a neural structure suitable for future developmental neurobiological studies of risk assessment.

*Role of hippocampal CA3 cells in behavioral inhibition*

Studies indicate that systemic administration of KA is less effective in producing widespread damage to CA3 cells in young rat pups (Albala et al., 1984; Nitecka et al., 1984; Sperber et al., 1991). Although the current study employed intrahippocampal infusion techniques, the amount of damage to the CA3 field of the HC still appears less severe than that observed in intrahippocampal KA-treated adult rats in which cells throughout the CA3 field were damaged (Fornnum and Walaas, 1978; Nadler and Cuthbertson, 1980). The potential implication is that widespread loss of CA3 cells in pups may increase the likelihood of producing alterations in behavioral inhibition that are not altogether different from those occurring after intrahippocampal colchicine infusions. In support of this view, studies demonstrate that after KA treatment, adult rats exhibited deficits in the Morris water maze (Sutherland et al., 1983) as well as in the passive avoidance test (Munoz and Grossman, 1981). Similar alterations were also evident after colchicine-induced damage to dentate granule cells (Sutherland et al., 1983; Walsh et al, 1986). These data implicate a role of CA3 cells in behavioral functions and suggest they may have an important, but as yet undefined role, in modulating mossy fiber inputs activated by appropriate stimuli.

Nonetheless, the loss of CA3 cells in the region of the hilus, where damage was most extensive, produced no significant change in behavioral inhibition. This result suggests that mossy fiber inputs to these cells do not contribute importantly to the expression of behavioral inhibition. In addition, the resultant loss of CA3 fibers originating in the region of the hilus and projecting to field CA1 (Swanson et al., 1978; Ishizuka et al., 1990) also appears to have minimal effects on the occurrence of behavioral inhibition. At the very least, these results serve to clarify the nature of the colchicine-induced damage and its behavioral consequences. That is, any incidental damage to CA3 hilus cells arising from infusion of colchicine was probably not a significant factor in the production of behavioral inhibitory deficits.

*CORT action in the HC and behavioral inhibition*

Bilateral CORT-filled cannulae located in the dorsal dentate gyrus of ADX pups were effective in promoting freezing. This result extends considerably previous work conducted in this laboratory documenting the reinstating effects of CORT on behavioral inhibition after systemic (Takahashi and Rubin, 1993; Takahashi, 1994c) or intraventricular administration of exogenous CORT (Takahashi and Kim, 1994). Furthermore, in the majority of CORT-implanted pups, plasma concentrations of CORT were nondetectable, which suggest that the action of CORT did not extend appreciably beyond the brain. Thus, central actions of CORT appear sufficient to facilitate the occurrence of freezing in the ADX rat pup. Whether other brain sites that bind CORT (Reul and de Kloet, 1985; Rosenfeld et al., 1990) will also produce behavioral effects similar to those occurring after implantation in the HC remains to be answered.

It should be indicated that the HC manipulations used in this study, i.e., electrolytic lesions, selective neurotoxins, CORT implants, had dramatic effects on freezing, whereas no significant changes were detected in ultrasonic vocalizations. These results suggest that the action of CORT in the HC may not have a major role in regulating ultrasound production. Furthermore, examination of ultrasound data presented in Tables 1 to 4 suggest, instead, that the action of exogenous CORT in extrahippocampal regions may be involved in the suppression of ultrasounds. When the action of CORT is limited to the HC region (Table 4), ultrasound production does not appear to be as effectively suppressed (Tables 1 to 3). The putative extrahippocampal suppressive effects of CORT on ultrasound production may account for the higher production of ultrasounds made by ADX pups in comparison to intact animals (Takahashi and Rubin, 1993).

Studies indicate that HC dentate granule cells bind and are developmentally regulated by adrenal steroids (Gould et al., 1991a,b). Because in these previous studies the source of exogenous CORT was from the periphery, it is unclear whether the effects of CORT on dentate granule cell development were due to hormone action occurring specifically in the HC. In the current study, exogenous CORT was delivered via cannulae located in the dorsal dentate gyrus. Although the amount of hormone diffusion from the 30 gauge cannula tip is not known, the amount of CORT released (i.e., approximately 8 μg) during the 5 d implantation period probably did not diffuse considerably beyond the HC. Other investigators who implanted larger 24 gauge cannula into the medial prefrontal cortex reported that after a 4 d implantation period approximately 30 μg of CORT was released and the amount of diffusion was confined to a 1 mm region surrounding the cannula tip (Diorio et al., 1993). Hence, the action of CORT may be confined largely to the region of the dorsal HC dentate granule cells. This local hormone action appears sufficient to maintain the developmental regulation of dentate granule cells important in facilitating the onset of behavioral inhibition.

Although results of this study emphasize the developmental actions of CORT on HC dentate gyrus development and behavior, previous studies showed that glucocorticoids are capable of producing toxic effects on the adult and developing HC (Sapolsky et al., 1985, Sapolsky et al., 1990, Uno et al., 1990). These studies showed that in rodents and primates, CA3 cells are most vulnerable to high doses of glucocorticoids, whereas other pyramidal cells and dentate granule neurons are relatively resistant. It is unlikely, however, that the occurrence of freezing observed

after implantation of CORT into the HC was a result of glucocorticoid-induced damage to CA3 cells. In the present study, KA-induced damage to CA3 cells in proximity to the hilus produced no significant change in the propensity of rats to exhibit behavioral inhibition. Another possibility is that behavioral inhibition was facilitated by CORT-induced destruction of HC cells other than CA3 cells. This scenario, however, is highly unlikely because CA3 cells are most susceptible to the damaging effects of glucocorticoids.

Another possible interpretation that may explain the induction of freezing after dorsal HC implantation of CORT is that negative feedback effects of the CORT implant contributed to a normalization of pituitary ACTH and its secretagogues that were elevated after ADX. In adult rats, elevated secretion of pituitary hormones was implicated in the production of deficits in avoidance behavior (Weiss et al., 1970). In addition, dorsal hippocampectomy (Feldman and Conforti, 1980) or dorsal dentate gyrus lesions (Johnson and Moberg, 1980) alter the negative feedback effects of glucocorticoids. Implantation of CORT into the dorsal HC was also more effective than cholesterol implants in attenuating the ADX-induced elevation of ACTH (Kovacs and Makara, 1988), especially when placed in CA2 and CA3 fields (Kawakami et al., 1968). Nevertheless, previous studies revealed that hypophysectomized-ADX pups continued to exhibit deficits in behavioral inhibition, which suggest that reduced secretion of pituitary hormones does not ameliorate the ADX-induced deficits in behavioral inhibition (Takahashi and Kim, 1995). Normalization of feedback effects produced by bilateral HC implants of CORT is unlikely to be a factor that contributes importantly to the reinstatement of freezing after ADX.

The HC not only binds CORT but is also a site of action of varied neurotransmitters including norepinephrine (Moore and Bloom, 1979), serotonin (Moore and Halaris, 1975), acetylcholine (Lewis and Shute, 1967; Matthews et al., 1987), and glutamate (Cotman et al., 1981; Storm-Mathisen, 1981; Storm-Mathisen and Iversen, 1979). Moreover, studies demonstrate that glucocorticoids influence second-messenger systems (Mobley and Sulser, 1980) and neurotransmitter receptor binding (Biegon et al., 1985). During development, glucocorticoids facilitate the increase in tryptophan hydroxylase (Sze et al., 1976) thereby contributing to the development of the serotonin system. It is possible that ADX severely disrupted the varied effects of glucocorticoids on neurotransmitter systems (McEwen et al., 1986; De Kloet, 1991), which resulted in freezing deficits. It should be emphasized that ADX is most effective in producing deficits in freezing when conducted prior to postnatal day 14 (Takahashi, 1994c). Furthermore, exogenous CORT is highly effective in reinstating freezing in the ADX pup only when administered on days immediately following ADX. After an ADX-induced period of absence of CORT, subsequent administration of exogenous CORT failed to facilitate the occurrence of freezing. Therefore, any behavioral inhibitory alterations produced by effects of ADX and CORT on neurotransmitter systems must be demonstrated to occur developmentally or prior to the appearance of behavioral inhibition. Neurotransmitter alterations induced by an acute presence or absence of CORT without developmental significance may not be of relevance to an understanding of mechanisms underlying the current behavioral responses.

Long-term ADX produces marked degeneration of HC dentate granule cells (Sloviter et al., 1989; Sapolsky et al., 1991), and loss of these cells may underlie the deficits in freezing. It is particularly notable that the ADX-induced degeneration of dentate granule cells is more severe in younger than in older rats (Jaarsma et al., 1992). Although during the inspection of cholesterol implantation sites there was no dramatic absence of dentate granule cells similar to that occurring after colchicine infusion, it is highly possible that ADX-induced alterations were already present. Studies have indicated that in adult rats the appearance of pyknotic granule cells was evident by 3 to 7 d after ADX (Gould et al., 1990). In contrast, cells in CA fields were not altered. Furthermore, death of dentate granule cells in ADX rats was prevented by administration of exogenous CORT. These data reveal the specificity of the ADX-induced loss of cells in the HC formation and the importance of corticosteroids in maintaining survival of dentate granule neurons. An implication of this research is that alterations in endogenous corticosteroid secretion occurring during the period of rapid HC dentate granule cell development may alter HC functional development in such a way that individuals are at risk or predisposed to perform poorly in situations requiring a degree of attentiveness to relevant stimuli and rapid cessation of overt movements. That effects of early ADX are long lasting is evident from studies showing that in adulthood, rats that were ADX on day 11 had reduced latencies to leave a start box and exhibited high levels of running wheel activity (Yehuda et al., 1988). These findings further suggest that even in adulthood, rats that were ADX in early life are less behaviorally inhibited.

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