Anterior and Posterior, but not Cheek, Intraoral Cannulation Procedures Elevate Serum Corticosterone Levels in Neonatal Rat Pups

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Implantation of intraoral cannulas is a procedure that has been typically assumed to be relatively unstressful in neonatal rat pups. To test this assumption, endocrine responses to such implantations were compared with those of other standard procedures. In Experiment 1, corticosterone and growth hormone (GH) levels were assessed in 4-day-old rat pups placed in an incubator for 15 or 60 min following either: no treatment, subcutaneous (sc) injection of 0.9% NaCl, anterior or posterior intraoral cannulation, ice anesthesia or ether anesthesia. Corticosterone levels were elevated relative to nontreated controls 15 min after all treatments except sc injection. These levels remained elevated after 60 min in both cannulation groups and the ice anesthesia group. In Experiment 2, the ability of ether anesthesia to reduce the hormonal response to the cannulation procedures was assessed in addition to examining the hormonal response to intraoral cannulations through the cheek in 4-day-old rat pups. Ether did not attenuate the corticosterone response to either anterior or posterior

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cannulations. Pups subjected to the cheek cannulation procedure did not exhibit any significant alterations in serum corticosterone levels when compared with nontreated control pups. GH levels were found to differentiate less among the various procedures than corticosterone levels, with GH levels generally being low in all groups, including nontreated control animals. These data suggest that a cheek placement is less stressful than anterior and posterior placements and may provide a viable alternative in studies necessitating the implantation of a cannula into the buccal cavity during the early postnatal period.

There have been a number of technical advances during the last decade that have proved to be very useful for researchers in the field of developmental psychobiology. Among the more notable of these advances has been the development of the technique for implantation of anterior and posterior intraoral cannulas, developed and described in detail by Hall (1979a,b). Implantation of cannulas into the buccal cavity has been used extensively in studies investigating behavioral activation, ingestion independent of the dam, intraoral pressure during suckling, and appetitive conditioning in neonatal rat pups (Hall, 1979b; Johanson & Hall, 1979; Caza & Spear, 1982; Johanson, Hall & Polefrone, 1984; Brake, Tavana, & Myers, 1986). These cannulation procedures require minimal time to administer, and pups appear to fully "recover" from the cannulation procedure within minutes after implantation. The behavioral reaction elicited by the cannulation process in nonanesthetized pups appears to be no more extensive than that associated with a subcutaneous injection at that age. With increasing concern about the humane treatment of animals, there has been some question regarding the use of these implantation procedures in nonanesthetized pups. The purpose of the present study was to assess whether these manipulations elicit hormonal signs of a stress response in neonatal rat pups and to compare these responses with those of another type of intraoral cannulation procedure, the cheek cannulation procedure (developed by Rudy & Hyson, 1982) as weli as with several other common experimental procedures used in neonates (i.e., subcutaneous injection, anesthetization with ether or hypothermia).

Serum levels of corticosterone and growth hormone were used as endocrine indices of a stress response. Whereas some laboratories have reported that there is little evidence of a pituitary-adrenocortical response to stress in rat pups tested from about postnatal Day 2 until well into the second postnatal week (e.g., Shapiro, Geller, & Eiduson 1962; Sapolsky & Meaney, 1986), other researchers have documented that rat pups throughout this period exhibit significant increases in serum corticosterone in response to a number of stressors such as heat, electric footshock, and exposure to ether (e.g., Haltmeyer, Denenberg, Thatcher, & Zarrow, 1966; Zarrow, Haltmeyer, Denenberg, & Thatcher, 1966; Schoenfeld, Leathem, & Rabii, 1980). Although the magnitude of this corticosterone response observed early in life is relatively blunted compared with that observed in older preweanling and adult animals, these stress-induced increases are readily evident using radioimmunoassay (RIA), a procedure which is substantially more sensitive than the fluorometric analyses used in early work (see Schoenfeld et al., 1980, for discussion). Serum growth hormone levels also appear to be highly sensitive to experimental manipulation early in life, with growth hormone levels declining rapidly following exposure to ether (Strosser & Mialhe, 1975; Rieutort & Jost, 1976), and footshock (Strosser & Mialhe, 1975; Percile, Ferrario, Falconi, & Muller, 1967) during the early postnatal and even fetal (Rieutort & Jost, 1976) age

periods. Even short-term deprivation from the dam has been reported to reduce growth hormone levels dramatically in neonatal rat pups (Schanberg, Evoniuk, & Kuhn, 1984). As these endocrine measures respond markedly and rapidly to various manipulations during the early postnatal period, they have the potential to provide a roughly quantifiable measure of the "stressfulness" of the test procedures. Circulating corticosterone has proved especially useful in this regard, and has been used frequently in both developing and adult animals to assess the stressfulness of various experimental procedures.

General Methods

Animals

A total of 246 male and female 4-day-old rat pups derived from established Sprague–Dawley breeding pairs at SUNY-Binghamton were used in these experiments. Litters were culled to 10 pups on the day following parturition, with the day of birth being designated postnatal Day 0. Litters containing less than eight pups were not used in these experiments. Pups were housed with their parents in standard translucent plastic maternity cages within a temperature and humidity controlled colony room maintained on a 12 : 12 hr light/dark cycle with lights on at 0700 hr. All experimental manipulations were conducted within 2 hr of the midpoint of the light phase of the diurnal cycle. No more than one pup of each sex per litter was placed into a given test group, with pups from a minimum of 5–6 litters being represented in each test condition.

Procedure

Pups were sacrificed for assay of serum levels of corticosterone and growth hormone either immediately upon removal from the home cage, or 15 or 60 min after exposure to the various experimental manipulations. Unless otherwise specified, pups were communally placed in a temperature- and humidity-controlled incubator (33 ± 1°C) following the experimental manipulations until the time of sacrifice. At the time of sacrifice, each rat pup was quickly decapitated using stainless-steel surgical scissors and trunk blood was collected on ice. Samples were allowed to coagulate at 4°C for approximately 45 min, followed by centrifugation under refrigeration at 10,000 rpm for 15 min. Blood serum was then withdrawn and kept frozen until the time of corticosterone and growth hormone assay. Coded serum samples were analyzed for growth hormone and corticosterone by RIA using procedures previously reported (Bero, Lurie, & Kuhn, 1987).

Experiment 1

The purpose of this experiment was to compare the serum levels of growth hormone and corticosterone of pups cannulated intraorally via anterior or posterior placements with hormone levels in pups given a subcutaneous injection or not manipulated prior to sacrifice. In addition, the hormonal response to two common anesthetization procedures used in neonates (ether and ice anesthesia)

were also assessed to provide additional data for comparison with the implantation procedures.

Methods

The design of this experiment was a 6 (treatment) × 2 (sacrifice interval—15 vs. 60 min) factorial, plus one additional group of pups that was sacrificed within 3–4 min upon removal from the maternity cage (IMM). Eight pups, four of each sex, were randomly assigned to each of the 13 experimental groups. The treatment groups consisted of: (1) no treatment (NT); (2) subcutaneous injection (SC); (3) anterior intraoral cannulation (AC); (4) posterior intraoral cannulation (PC); (5) ether anesthesia (ETH); and (6) ice anesthesia (ICE). All pups were weighed to the nearest .01 g. and numbered with a making pen 1 hr prior to the initiation of the experimental procedures.

Pups in the NT group were merely placed in a holding incubator (33 ± 1°C) until the time of sacrifice. Animals in the SC treatment group received an injection of .05 mL 0.9% NaCl solution delivered subcutaneously through a 27G needle at the nape of the neck just anterior to the scapulae. Pups in the AC and PC groups were implanted with oral cannulae (PE-10 polyethylene tubing) using procedures modified slightly from those described by Hall (1979a). Briefly, a piece of PE-10 tubing, 3–4 cm in length, was flanged at one end using a heat source. The nonflanged end of the tubing was tightly fit onto one end of a curved stainless-steel wire (.285 mm diameter, approximately 4 cm in length). This wire was then inserted gently and rapidly through the posterior aspect of the tongue (for PC) or through the soft tissue of the lower jaw just anterior to the tongue and behind the dental ridge (for AC). The PE-10 tubing was then drawn through the tongue or soft tissue and separated from the wire guide, leaving the flanged end of the cannula within the buccal cavity. This cannulation procedure was typically accomplished within 5–10 sec with minimal or no bleeding. Pups in the ETH group were placed in a small, covered glass container lined with a layer of dry surgical gauze placed over a layer of gauze moistened with ether. After the pups were rendered motionless, they were removed from the ether and placed in a holding incubator. Animals in the ICE group were placed in a container of chipped ice for approximately 5 min to produce surgical levels of anesthesia, and were allowed to recover at room temperature until movement was restored prior to being placed in a holding incubator.

Results and Discussion

Hormone data were analyzed by 6 (treatment) × 2 (sacrifice interval) × 2 (sex) analyses of variance (ANOVA). The mean square error term and its associated degrees of freedom for each ANOVA was adjusted for the addition of data from the IMM group prior to Dunnett's analyses comparing the treatment groups with the two control groups—IMM and NT. The serum sample from one male animal in the ETH group was lost during preparation, reducing the sample size in this treatment group to seven.

The ANOVA of the corticosterone data revealed a significant main effect of treatment ($F(5,71) = 11.941$, $p < .001$) and a significant sacrifice interval ×

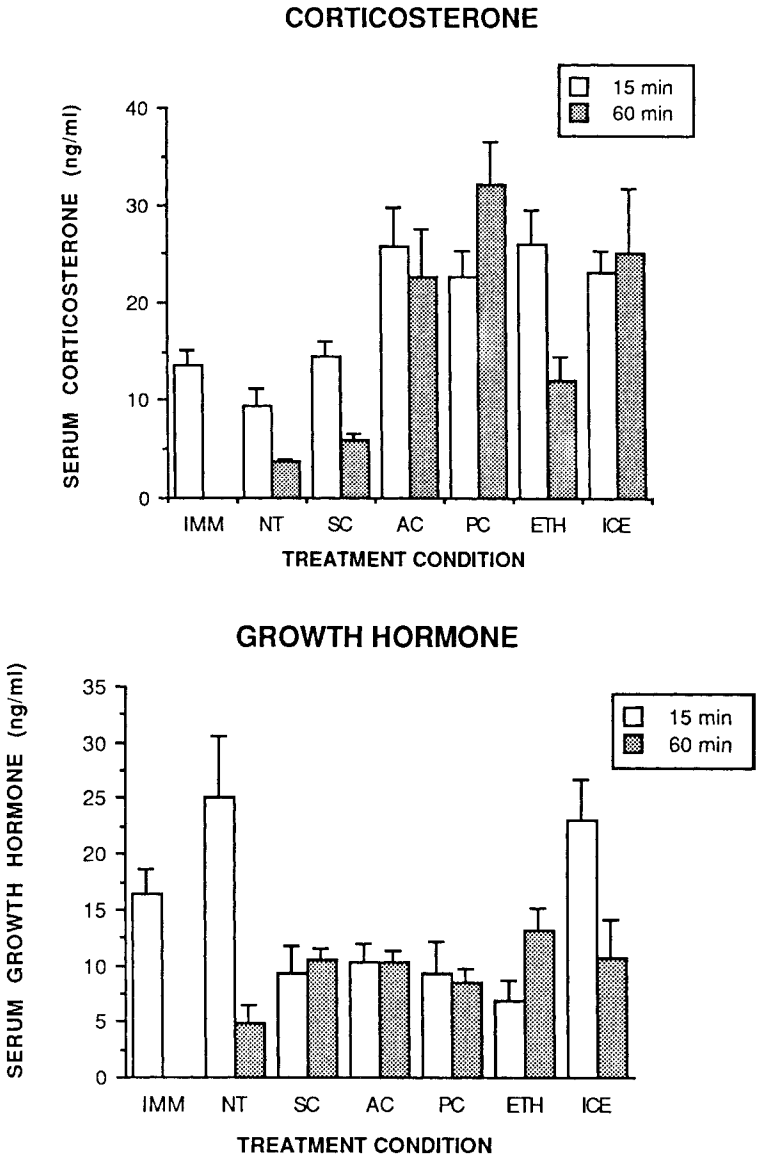

Fig. 1. Serum levels of corticosterone (a) and growth hormone (b) in 4-day-old rat pups sacrificed either immediately (IMM), 15 (clear bars), or 60 (hatched bars) min. following each of the following manipulations: no treatment (NT); subcutaneous injection (SC); anterior (AC) or posterior (PC) intra-oral cannulation, ether exposure (ETH) or ice anesthesia (ICE). S.E.M.'s are indicated as brackets.

treatment interaction ($F(5,71) = 2.879$, $p < .05$). As can be seen in Figure 1 (a), pups in the AC, PC, ETH, and ICE groups exhibited significantly greater levels of corticosterone at 15 min post-treatment than pups in the NT group. At 60 min post-treatment, levels of corticosterone in the AC, PC, and ICE groups were still elevated relative to NT animals, with PC pups also exhibiting greater levels of corticosterone than pups in the IMM group.

The ANOVA on the growth hormone (GH) data revealed significant main effects of sacrifice interval ($F(1,69) = 7.677$, $p < .01$) and treatment ($F(5,69) = 2.571$, $p < .05$) along with a significant interaction of sacrifice interval and treatment ($F(5,69) = 6.653$, $p < .001$). As can be seen in Figure 1(b), at 15 min post-treatment, pups in the SC, AC, PC, and ETH groups exhibited significantly lower GH levels than NT pups. There were no differences among the treatment groups at 60 min post-treatment, although pups in the NT group were observed to exhibit lower levels of GH than IMM pups.

A 6 (treatment) × 2 (sacrifice interval) × 2 (sex) ANOVA on body weights revealed no significant main effects or interactions, suggesting that pups of different body weights were randomly assigned to the different test groups.

The results of the corticosterone analyses suggest that the intraoral cannulation process as well as anesthetization elicits an adrenocortical stress response in neonatal rat pups that is not seen in pups that are merely given a subcutaneous injection. Corticosterone levels remain elevated at 60 min in both cannulation groups as well as the ICE anesthesia group, but not in the ETH group.

Pups in all treatment groups except the ICE group exhibited lower GH levels at 15 min post-treatment when compared to NT animals, suggesting that virtually any manipulation of the pups may be sufficient to result in a rapid reduction in serum GH levels. At 60 min there were no differences among the treatment groups in GH levels, predominantly as a result of a reduction in GH levels in NT pups at this time relative to levels seen either immediately or 15 min after removal from the nest (Fig. 1(b)). Such a time-related depression in GH levels is not surprising given that deprivation from the dam for as little as 1 hr has previously been reported to result in a substantial decrease in serum GH levels in rat pups (e.g., Schanberg et al., 1984). Given that serum levels of growth hormone were observed in this experiment to differentiate less among the treatment groups than corticosterone levels, the following experiment focused on serum corticosterone levels as the dependent measure of interest.

Experiment 2

This experiment was designed to assess whether anesthesia with ether prior to the cannulation procedures would reduce the hormonal response to the cannulation process. In addition, the endocrine response to a third type of intraoral cannulation procedure, cheek cannulation (see Rudy & Hyson, 1982), was also assessed.

In Experiment 1, pups sacrificed "immediately" upon removal from the home nest exhibited slightly higher corticosterone levels than typical basal levels reported in the literature (Bero & Kuhn, 1987). In that experiment, all pups were briefly removed from the dam for weighing 1 hr prior to sacrifice, and pups placed into the "immediate" group were sacrificed up to 4 min following removal from the home nest. Procedures were slightly modified in this experiment in an attempt to produce more accurate basal levels of corticosterone by weighing all pups immediately prior to sacrifice, and sacrificing pups in the "immediate" group within 60–90 sec of removal from the home nest.

Methods

The design of this experiment was a 2 [ether (ETH) vs. no ether (NO ETH)] × 4 [treatment condition] × 2 [sacrifice interval—15 vs. 60 min] factorial, with an additional group of animals that was sacrificed immediately (within 60–90 sec) upon removal from the home nest (IMM). Sample sizes for each of the 17 experimental groups in this experiment ranged from 7–9 pups, with the exception that 12 pups were included in the IMM group. Sex was equated whenever possible within each experimental group. The four treatment conditions consisted of: (1) no treatment (NT); (2) anterior cannulation (AC); (3) posterior cannulation (PC); and (4) cheek cannulation (CHK). Pups in the NT, AC, and PC were treated as detailed in Experiment 1. Animals in the CHK group were cannulated through the cheek immediately caudal to the mystacial pad using cannulation procedures as outlined in Experiment 1 (see also Rudy & Hyson, 1982). Half of the pups in each treatment condition received the experimental manipulations under light ether anesthesia (ETH), while the remaining pups were not exposed to ether (NO ETH). All pups were numbered with a marking pen at the onset of the experimental treatments, and were rapidly weighed to the nearest .01 g immediately (within 5–10 sec) prior to sacrifice. Pups were sacrificed, and serum collected and analyzed for corticosterone as outlined under General Methods.

Results and Discussion

A 2 (sacrifice interval) × 2 (ETH vs. no-ETH) × 2 (sex) × 4 (treatment) ANOVA on serum corticosterone levels revealed significant main effects of sacrifice interval ($F(1,94) = 8.830$, $p < .01$) and treatment ($F(3,94) = 7.682$, $p < .001$). A sacrifice interval × treatment interaction approached but did not reach significance ($F(3,94) = 2.535$, $p < .06$). Corticosterone levels were greater at 60 min than at 15 min, and levels in pups in the AC and PC groups were greater than those of NT pups. As can be seen in Figure 2, both of these main effects appear to be predominantly a result of elevated corticosterone levels in AC and PC pups at 60 min post-treatment. In contrast to the results for the AC and PC groups, corticosterone levels for pups in the CHK groups did not differ significantly from NT animals. Dunnett's tests conducted as outlined in Experiment 1 also showed that levels of corticosterone in the IMM pups were significantly lower than pups in all treatment groups.

ANOVA of the body weight data revealed significant main effects of sex ($F(1,92) = 7.224$, $p < .01$) and sacrifice interval ($F(1,93) = 4.517$, $p < .01$) along with a significant sacrifice interval × time × treatment interaction ($F(3,93) = 3.072$, $p < .05$). In addition to the often reported finding that male pups weigh slightly more than female pups when sufficient sample sizes are examined, there were also slight differences among some of the test groups in body weight due to an inadvertent sampling bias. However, there was not a significant correlation between body weight and serum corticosterone level ($r = 0.03$), suggesting that body weight differences across groups did not contribute to the observed hormonal findings.

In this experiment, as in Experiment 1, the AC and PC implantation procedures were observed to result in an activation of the adrenocortical system that was evident for at least 1 hr post-treatment. There were no main effects or

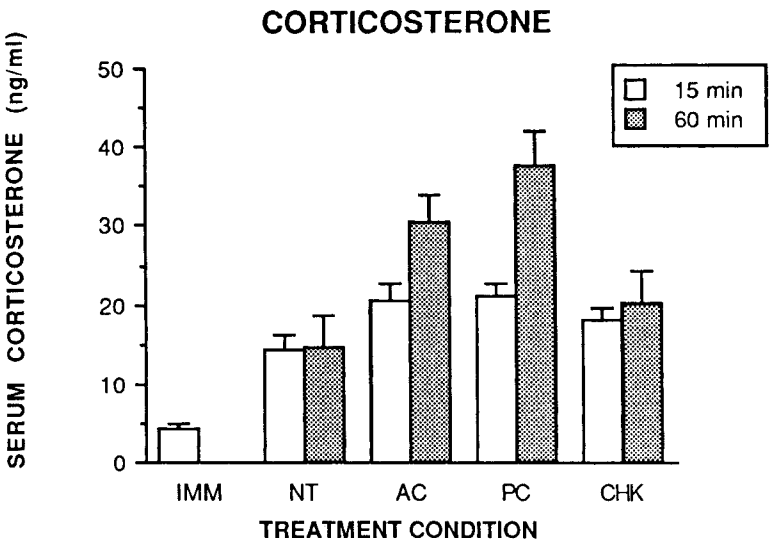

Fig. 2. Serum levels of corticosterone in 4-day-old rat pups sacrificed either immediately (IMM), 15 (clear bars), or 60 (hatched bars) min. following either no treatment (NT), or anterior (AC), posterior (PC), or cheek (CHK) intraoral cannulation. Data are collapsed across the variable of the presence or absence of ether as there were no significant main effects or interactions of this factor in the analysis of variance on these data. S.E.M.'s are indicated as brackets.

interactions involving ether in these analyses, suggesting that light ether anesthesia prior to the cannulation procedure is not sufficient to prevent this apparent stress-induced release of corticosterone. In contrast, pups subjected to the cheek intraoral cannulation procedure did not exhibit any significant alterations in serum corticosterone levels when compared with non-treated control pups. From these data, it appears that this cannulation procedure may not induce significant activation of the adrenocortical system in neonates.

General Discussion

In these experiments, pups subjected to anterior or posterior intraoral cannulation procedures, as well as ice anesthesia, exhibited elevated levels of serum corticosterone when compared with nontreated animals or with pups given a subcutaneous saline injection. This steroid response to cannulation was not attenuated by prior anesthesia with ether, a manipulation which increased corticosterone levels only transiently when administered alone. The observed increase in corticosterone was seen for at least 1 hr following the anterior and posterior implants in contrast to ether-anesthetized pups which exhibited normal corticosterone levels at 1 hr. These data suggest the possibility that the cannulation process itself and/or the presence of the cannula in the buccal cavity through or underneath the tongue may prove to be stressful to neonates.

In contrast to the consequences of the anterior and posterior intraoral cannulation procedures, cannulation through the cheek in either lightly etherized or nonetherized pups did not elicit an adrenocortical response. From these data it appears that the cheek cannulation procedure may be preferable to anterior and

posterior cannulation procedures in studies necessitating implantation of a buccal cannula. It should not be inferred from these data, however, that use of the cheek cannulation procedure will necessarily be devoid of adrenocortical activation in all circumstances. In these experiments the cannulas were carefully implanted by investigators with extensive surgical experience with neonates; such cannulations produced under other circumstances could perhaps elicit significant adrenocortical activation. When using this cannulation procedure it would appear prudent to make every attempt to minimize perturbations of the pups during the implantations. Moreover, these data were collected in rat pups at 4 days postnatally, near the beginning of the "stress-reduced responsiveness" period where the adrenocortical response to stress has been observed to be relatively blunted compared to that observed in older preweanling and adult animals (Schoenfeld et al., 1980). Given the increasing capacity for adrenal responsiveness in older infant and preweanling animals, it is likely that offspring at these older ages may exhibit a more marked adrenocortical response to manipulations such as intraoral cannulation. This ontogenetic increase in responsiveness may prove to be problematic if adrenal steroid secretion is used as the exclusive response measure of the relative "stressfulness" of various manipulations in comparisons directed across age.

In Experiment 1, cold anesthesia was observed to result in significantly elevated levels of corticosterone at 15 and 60 min post-treatment. Although it is possible that changes in steroid metabolism contribute to the observed elevation of corticosterone, studies of both acute and prolonged cold exposure in adults suggest that cold evokes a marked stimulation of the hypothalamo-pituitary-adrenal axis (Brown & Hedge, 1973; Daniels-Severs, Goodwin, Keil, & Vernikos-Danellis, 1973; Mueller, Chen, Dibbet, Chen, & Meites, 1974; Lenox, Kant, Sessions, Pennington, Mougey, & Meyerhoff, 1980) that is not reliably associated with a suppression of GH secretion (cf., Mueller et al., 1974; Lenox et al., 1980). Cold appears to evoke a particular pattern of endocrine response, including a rise in TSH, ACTH, corticosterone, and prolactin, that resembles but is not identical to hormonal alterations typically associated with a stress response. This characteristic pattern of hormonal response to cold exposure may be related in part to the metabolic consequences of hypothermia per se. Consequently, in spite of the elevation in corticosterone in response to cold exposure observed in Experiment 1, it cannot necessarily be concluded that this manipulation resulted in a stress response. Indeed, this method of anesthesia is a standard surgical procedure that has been reported to be effective, safe, and appropriate for use in preweanling rodents (Phifer & Terry, 1986).

In Experiment 1, GH levels were found to differentiate less among the various treatment procedures than corticosterone levels. GH levels were generally low in all groups of animals, with few differences between treatment groups noted. Serum levels of GH appear to rapidly decline with virtually any manipulation of the pups, including mere removal of pups from the dam (e.g., Schanberg et al., 1984), which may obscure any potential differential responsiveness to the various treatments. There are also limitations, however, associated with the use of corticosterone as a hormonal index of a stress response. Although there is evidence that corticosterone release is responsive to differing circumstances which elicit arousal (Hennessey & Levine, 1979), serum corticosterone levels tend to reach maximal levels after relatively mild stressors, at least in adulthood

(e.g., Kant, Bunnell, Mougey, Pennington, & Meyerhoff; 1983). Thus, serum corticosterone appears to be an indicator of the presence of a stress or arousal reaction, but may not necessarily be an adequate index of the intensity of that response (see Kant et al., 1983). Consequently, although both the anterior and posterior cannula implants elicited similar increases in corticosterone in the present experiments, no conclusions can be derived with regard to the relative magnitude of the stress response elicited by these two procedures.

Taken together, the results of the present experiments suggest that careful consideration be given to the routine use of anterior and posterior intraoral cannulation procedures in neonatal rat pups. These procedures induce notable elevations in serum corticosterone that are evident for at least 1 hr following surgery, data suggesting that these manipulations are stressful in the neonate. Although prior anesthesia with ether did not attenuate these surgery-induced elevations in corticosterone, it is possible that other types of anesthetization procedures may prove more effective in this regard. If serum corticosterone levels are to be used as a dependent measure in such experiments, hypothermia should be avoided as an anesthetic agent due to the observed increases in serum corticosterone seen in response to cold anesthesia at both sacrifice intervals in Experiment 1. In contrast to the adrenocortical activation resulting from the anterior and posterior cannula placements, placement of a cheek cannula resulted in no significant alteration in serum corticosterone levels in neonatal rat pups relative to nontreated control animals. It would appear that the cheek placement may provide a viable alternative to the use of anterior and posterior placements in studies necessitating the implantation of a cannula into the buccal cavity during the early postnatal period.

Notes

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