

# Handling, genetic and housing effects on the mouse stress system, dopamine function, and behavior

Jean-Louis Gariépy<sup>a,\*</sup>, Ramona Marie Rodriguiz<sup>a</sup>, Byron C. Jones<sup>b</sup>

<sup>a</sup>Center for Developmental Science, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-8115, USA

<sup>b</sup>Program in Biobehavioral Health, Pennsylvania State University, University Park, PA, USA

Received 24 July 2001; received in revised form 25 February 2002; accepted 2 March 2002

## Abstract

This research was designed to examine how early stimulation (i.e., handling), subsequent housing conditions and genetic factors interact to produce adult differences in stress regulation. High-aggressive (NC900) and low-aggressive (NC100) mice were handled for 3 weeks postpartum and were subsequently isolated or grouped until observed as adults in an open field or a dyadic test. In NC100, handling abolished the temporal variations seen in open-field activity among the nonhandled subjects and reduced corticosterone (CORT) activation. In NC900, these two measures were unaffected by handling. Only among handled NC100 did subsequent group rearing further reduce CORT activation. By contrast, handling caused an up-regulation of D1 dopamine receptors in both lines, and, in NC100, this effect was increased by group rearing. In a dyadic encounter with another male mouse, subjects of both lines showed handling effects. NC100 froze less rapidly and NC900 attacked more rapidly. This multifactorial design showed that the systemic effects of handling are modulated by genetic background, and that measures of these effects are affected by experience beyond infancy. Our findings also showed that the effects of handling vary when assessed across different physiological systems and across social and nonsocial testing conditions. © 2002 Elsevier Science Inc. All rights reserved.

**Keywords:** Mice; Handling; Selective breeding; Isolation; Group rearing; Social behavior; Aggression; Freezing; Corticosterone; Dopamine; D1 receptor

## 1. Introduction

It is well documented that stimulative events early in development have pervasive and long-lasting effects on subsequent responses to environmental stress. One method frequently used to study this phenomenon in rodents consists of removing whole litters from their home cage, placing the pups in a small container and returning them to the dam after a few minutes. Maximum effects are obtained when newborn subjects are handled in this way once a day over the first 3 weeks of life. Several investigators have reported that adult rats and mice that have been so treated in infancy are less fearful in novel environments and show a decreased corticosterone (CORT) response to stress as compared to non-handled controls (Levine, 1957; Denenberg and Morton, 1962; Meaney et al., 1996; Pfeifer et al., 1976). In the decades following Levine's initial reports on this phenom-

enon, the experiential and physiological pathways of handling effects have been mapped out in detail. On the experiential side, it has been shown that early handling owes its beneficial effects to the increase in maternal care that the procedure induces. The physiological pathways of these effects were later shown to involve an up-regulation of glucocorticoid receptors in the hippocampus and frontal cortex, a system implicated in the negative feedback control of hypothalamo-pituitary–adrenal (HPA) activity (Meaney and Aitken, 1985). One of the authors of this discovery referred to the chain of events causing the up-regulation of this receptor as a form of environmental programming that reflects the unique openness of early development to modification by experience (Meaney et al., 1996).

The clarification of these pathways, experiential and physiological, is now regarded as providing a model of the development of stress regulation that may be generalizable to the human species. The progress achieved on these issues during the last decades required to (1) confine research to a few rodent strains where the effects of handling were readily observed, (2) contrast experimental groups that only differed in postnatal experience (i.e.,

\* Corresponding author. Department of Psychology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3270, USA. Tel.: +1-919-962-2392; fax: +1-919-962-2537.

E-mail address: jlgariépy@mindspring.com (J.-L. Gariépy).

handled vs. undisturbed), (3) evaluate adult outcomes by means of the same types of tests (e.g., foot shock, restraint, cold stress) and, until recently, (4) look primarily for handling effects on the HPA axis. Although these limits were set for purposes of experimental control, they inevitably forced the postponement of a number of equally important developmental questions. These questions, addressing, as they do, the issues of gene–environment interactions in handling effects, of the contribution of stimulation beyond the early stages, of the possibility of multisystemic effects and of assessment under social conditions (as opposed to nonsocial ones), are just as important as the initial ones to the formulation of a model generalizable to the human species. That the long-term effects of early handling may vary as function of the various factors implicated by these issues has been known for a while. Our survey of the handling literature, however, suggest that, with a few exceptions, this knowledge has not, as of yet, generated systematic investigation, and that it still remains poorly integrated in the models currently offered to describe the development of the stress response (e.g., Anisman et al., 1998; Meaney et al., 1988; Sapolsky, 1997).

By the 1960s, a substantial number of studies had shown that various forms of postnatal stimulation do not affect in the same way animals that differ in genetic background. The evidence was especially striking in comparisons of different breeds of the same species. In dogs, for example, terriers showed less fearfulness when introduced to a novel environment following isolation and showed less pronounced effects of early separation than more emotionally responsive breeds, such as the beagle (Fuller, 1967; see also Scott, 1970). Similar differences in the effects of early experience have been noted among primate species (Seay et al., 1972; Rosenblum and Kaufman, 1968). In one of the first comparative studies of handling effects, King and Eleftheriou (1959) demonstrated that two subspecies of deer mice were differentially affected by the procedure (see also Levine and Broadhurst, 1963). With the exception of a few reports in more recent literature, little work has been conducted to determine how genetic differences may modulate the effects of handling. In one report, Fernandez-Teruel et al. (1992) showed that adult CORT activation values were low in the Roman Low-Avoidance strain whether the subjects were handled as pups or not. By contrast, the higher values measured in the Roman High-Avoidance strain were substantially reduced by handling. A similar pattern of handling effects was reported by Anisman et al. (1998). This group showed that mice of the C57BL/6byJ strain naturally exhibit shorter escape latencies in a Morris water-maze and less failures to escape in repeated testing than mice of the BALB strain. Again, in this case, the performance of BALB was significantly improved by handling while that of C57BL/6byJ was not. The authors of this research called, as we do, for more information on the effects of handling among animals that differ in genetic background (Anisman et al., 1998).

Given that in most investigations, the experimental subjects were kept after handling under uniformly controlled laboratory conditions until tested in adulthood, it is somewhat surprising that the effects of this form of early stimulation be regarded as permanently modifying the capacity to regulate stress (Meaney et al., 1996; Francis and Meaney, 1999). We concur with Levine that: “There is no reason to assume that any given function can be modified only at one critical period . . .” (Levine, 1969, p. 16–17). In support of this view, Ader and Grotta (1969) showed that the magnitude of the stress response was reduced among rats handled during the postweaning period (see also Hughes, 1971). More recently, Mohammed et al. (1993) demonstrated that environmental enrichment during the same period results in increased glucocorticoid receptor mRNA expression. In the light of this evidence, it would be informative to determine whether the effects of early experience can be either consolidated or attenuated by subsequent experience or, more in line with the assertions made in the current literature, whether early experience modulates the effects of subsequent stimulation.

During the last decade, three lines of evidence have prompted researchers to examine potential effects of early handling on the dopaminergic system. The first was the demonstration that exposure to stress in adult life also activates the dopaminergic system (see Puglisi-Allegra et al., 1990 for a review) and that this system, just like the HPA axis, undergoes significant developmental changes postnatally (e.g., Foster et al., 1988). Another was the discovery that its functional organization remains sensitive to changes in stimulative conditions throughout development (e.g., Feldon and Wiener, 1992). The third line of evidence was the demonstration that there are bidirectional relationships between the activity of the HPA system and that of the dopaminergic system. These relations were identified when chronic injections of CORT at doses comparable to stress activation levels were shown to cause an augmentation of dopamine release in the nucleus accumbens (Cabib and Puglisi-Allegra, 1994). Casolini et al. (1993) also showed that the relationship between these two systems is mediated by the D1 dopamine receptor. One of the first demonstrations that early handling affects the development of the dopaminergic system was provided by Cabib et al. (1993). Their research showed that neonates removed daily from their home cages and exposed to clean unfamiliar bedding in the absence of the mother explored more and were less fearful as adults as compared to a control group removed from the home cage but exposed to familiar bedding. Moreover, nucleus accumbens stress-induced dopamine release in the experimental group was significantly reduced as compared to controls. Since low fearfulness in adult life is typically associated with attenuated CORT activation, reduced dopaminergic release was consistent with the bidirectional relationship shown to exist between the two systems. As mentioned earlier for the HPA system, little is currently known concerning the joint effects of early handling, genetic

background and stimulation beyond infancy on the adult organization of the dopaminergic system.

The last point made earlier with reference to the formulation of a generalizable model of the development of the stress response system concerned the types of testing conditions that have been used in handling research to elicit measurable outcomes. Denenberg (1964) initially suggested the open field as a standard procedure for obtaining estimates of HPA activation, but in subsequent research, investigators have more often used foot shock, cold stress or forced restraint to elicit this response (see Meaney et al., 1996 for a review). More recently, the Morris water maze has been used to evaluate relationships between handling, improved stress regulation and memory development (Anisman et al., 1998; Meaney et al., 1988; Sapolsky, 1996). The need for uniform and well-calibrated stressors may explain why so little research has been conducted to document the effects of handling on social behaviors. Although social encounters can be as good elicitors of HPA activation as nonsocial elicitors (Henry and Stephens, 1977), the fact that they involve at least two animals introduces a problematic source of variability in stimulation. On the other hand, it is also the case that among rodents and other mammalian species, including our own, interactions with other members of the group are often the most important source of stress (Sapolsky, 1993). While it is well documented that handling reduces fear in novel environments and facilitates their exploration, its effects on the regulation of social interactions remain, as of yet, poorly documented. The authors of the present article are aware of only one series of studies that reported handling effects on the social behaviors of mice (Ginsberg, 1966, 1969). This work showed that handling significantly reduced latencies to attack, and that the magnitude of this effect varied significantly among different mouse strains.

In the light of the above considerations, the present research was designed to determine the effects of postnatal handling and subsequent rearing conditions on the stress response of mice that have been selectively bred over 30 consecutive generations for high and low aggression. Whereas the high-aggressive line animals attack rapidly and fiercely in a dyadic test following isolation rearing, animals in the low-aggressive line do not attack but freeze upon social contact (Cairns et al., 1983; Gariépy et al., 2001). To assess the effects of neonatal stimulation on these animals, males of each line were either handled or left undisturbed with their mother from Day 3 postpartum until weaning at 21 days of age. At this point, subjects of each neonatal condition were placed either in social isolation or in groups of four males until they reached adulthood. The addition of this variable permitted us to verify if social stimulation during the prepubertal period would affect the organization of the adult response to stress in these selected lines, either independently or as a function of early handling. When the subjects assigned to these four experimental conditions reached 56 days of age, they were exposed to an open-field

arena, *CORT* activation was measured and circulating levels were compared across selected lines, postnatal experience and rearing conditions. In addition to these measures, the effects of early handling and subsequent rearing conditions (i.e., isolation vs. group housing) on measures of adult densities of the D1 dopamine receptor were also evaluated. The rationale for including this measure was the evidence presented earlier that handling reduced the release of nucleus accumbens dopamine (Cabib et al., 1993). Given that there is often a negative relationship between catecholamine content in the synapse and the density of postsynaptic receptors (Hess et al., 1988) handling was expected to cause an up-regulation of this receptor. The D1 receptor, as opposed to D2-like receptors, was targeted for investigation because of its known activational role (see Missale et al., 1998 for a review).

The open-field test was used in the present research to elicit *CORT* activation instead of one of the tests more commonly used in contemporary research so that behavioral effects of early handling could be assessed. Obtaining such measures also permitted us to determine whether any line difference in the effects of handling revealed under this testing condition, including the direction of these differences, would be preserved or differ under social testing conditions. For the purpose of this comparison, another group of high- and low-aggressive males were randomly assigned to the handling or control conditions as described above and were observed as young adults in a brief social interaction test. All subjects assigned to this condition were reared in individual cages following weaning. The joint effects of handling and subsequent rearing conditions were not assessed in this case because previous research has shown that group rearing strongly reduces the propensity to attack in the high line and the propensity to freeze in the low line (Gariépy et al., 1995). Accordingly, comparisons of handling and line effects on observable behaviors in the open field and the dyadic test were only conducted among subjects isolated after weaning.

## 2. Methods

### 2.1. Subjects

Three sets of animals randomly selected from different litters born in the 30th and the 31st generations of our selective breeding program were used for the present research. The details of the selective breeding procedures have been given in previous publications (e.g., Gariépy et al., 2001). The determination of line, handling and rearing effects on *CORT* measures and D1 receptor densities required a total of 88 male mice, 43 low-aggressive (NC100) and 45 high-aggressive (NC900). Baseline measures of *CORT* levels were obtained using an additional set of 35 male subjects (18 NC100 and 17 NC900). The effects of line and handling on social behaviors were determined

using a third set of animals comprised of 80 male subjects (40 NC100 and 40 NC900). All animals had access to food and water ad libitum and were kept on a reverse light cycle (12 h light/12 h relative darkness). The experiments reported here were approved by the Animal Review Committee of the University of North Carolina at Chapel Hill, which is in compliance with the AAALAC Council on Accreditation as described in the Guide for the Care and Use of Laboratory Animals (NRC 1996).

## 2.2. Neonatal handling

Seventy-two hours after parturition, all litters were culled to consist of four females and four males. In the first set of animals, 19 NC100 and 21 NC900 litters were handled approximately 7 h into the dark cycle. A control group consisting of 24 NC100 and 24 NC900 was also constituted. The animals assigned to this condition were left undisturbed in their home cage until weaning. The subjects assigned to the second set were also left undisturbed until weaning. Finally, among the subjects used in dyadic tests (3rd set), half were handled (20 NC100 and 20 NC900) and half were left undisturbed (20 NC100 and 20 NC900). Handling consisted of placing an entire litter in a 500-ml opaque plastic beaker for 60 s once every 48 h from Day 3 postpartum until weaning at 21 days.

## 2.3. Postweaning rearing conditions

At 21 days of age, all male pups were weaned and assigned to either relative social isolation or group rearing. In the first condition, they were placed singly in standard mouse compartments and had no social contact other than exposure to the noises and odors in the colony room. Group rearing consisted of housing four males of the same line, age and neonatal experience together (i.e., handled or non-handled) in a standard opaque mouse compartment. Among the subjects assigned to the first set, in each line, half of the handled and half of the nonhandled subjects were placed in isolation after weaning, while the other half was placed in groups. The subjects assigned to the second and the third sets were all reared in isolation. The animals remained in their respective rearing conditions until they were tested.

## 2.4. Open-field testing

All tests were conducted within the first 5 h of the dark cycle (09:00–14:00 h) when the animals were approximately 56 days of age ( $\pm 4$  days). Mice were singly placed in a large Plexiglas chamber (60 cm<sup>2</sup>  $\times$  30 cm) that was indirectly illuminated with 15 W of fluorescent light. On every day of testing, handled male mice were matched with a nonhandled male of the same genetic background. Pairs of mice were randomized across testing, and observers were blind as to the genetic and experiential background of each subject. To allow enough time for blood collection, each test

was separated by 5 min. The test chambers were cleaned between tests with a 70% ethanol solution, rinsed with tap water and dried.

The floor of the arena was divided in squares (5  $\times$  5 cm) sequentially numbered 1–144 to record the location of the animal. The location of the subject (front paws on square no.) was noted every 5 s on specially prepared sheets containing 120 five-second blocks. Interobserver agreement on location exceeded 90%. Using this information, a measure of arena crossings was derived, which was defined as the number of times an animal completely crossed the open field, moving from one wall to the adjacent or the opposite one. To be counted as crossing, the midpoint of the straight path joining the initial and the final location had to be situated within at least half the distance to the center of the arena. In addition to location, rearing behavior was also recorded. Supported rearing was coded when the subject was in a full upright posture with at least one forepaw in contact with the vertical sides of the testing chamber. Unsupported rearing was defined as an upright posture in which the forepaws were lifted from the floor and not in contact with the wall of the arena. The interobserver agreement for these behaviors exceeded 90%. The coding system used in this test did not permit accurate measurements of locomotor activity.

## 2.5. Collection of serum

The subjects exposed to the open field were anesthetized 20 min after completion of the test by halothane inhalation (Halocarbon Laboratories, River Ridge, NJ), and blood was collected by cardiac puncture. The subjects used to obtain baseline measures of CORT were brought from the colony room to the laboratory around 1000 h. About 1 h later, blood was obtained from them following the same procedures as those used for the animals exposed to the open-field. Blood samples were placed into 10-ml serum gel separator (Microtainer, Becton Dickinson, Rutherford, NJ) and were centrifuged at room temperature (25.5 °C). The serum was stored at  $-72$  °C until assayed.

## 2.6. Determination of CORT concentrations

Serum samples were assayed in duplicate for CORT concentration using the Immuchem Double Antibody Corticosterone <sup>125</sup>I Kit (ICN Biomedicals, Costa Mesa, CA). The amount of [<sup>125</sup>I]-CORT bound for each sample was determined using a LKB gamma counter (Model 1272, CliniGamma). Concentrations were expressed as nanograms per milliliter. Data are expressed as means ( $\pm$  S.E.M.).

## 2.7. Tissue dissection

Immediately after blood collection, the animals exposed to the open field were decapitated, and their brains were rapidly removed, with care taken to keep the olfactory bulbs

intact. Mouse brains were dissected on an ice-cold aluminum block, according to the atlas of Stolnick and Leonard (1975). Tissue samples were taken from two brain regions: the nucleus accumbens and the caudate–putamen. These areas were obtained by removing the olfactory bulbs, making a coronal cut 2 mm posterior to the frontal pole, approximately 0.5 mm anterior to the optic chiasma and making a second cut to obtain a slice 1 mm thick. For the caudate–putamen, bilateral punches (1000  $\mu\text{m}$  diameter) were taken on this slice just below the corpus callosum. The nucleus accumbens was taken by bilateral punches (750  $\mu\text{m}$  in diameter) on the same slice but medial and ventral to the anterior commissure.

### 2.8. Receptor binding

Saturation binding of [3H]-SCH 23390, a highly specific ligand for the  $D_1$  receptor, was carried in the caudate–putamen and nucleus accumbens. Tissue preparation used whole membrane fragments. Individual tissue samples were homogenized and centrifuged (18,000 rpm) at 4 °C for 20 min in 0.9% isotonic saline. Pellets were resuspended in 600  $\mu\text{l}$  of cold saline (4 °C), homogenized and centrifuged for a total of three additional washes. The final pellet was resuspended in saline and was incubated in triplicate in BSA coated microtiter plates (96-well plates, Corning), in the presence of [3H]-SCH 23390 at concentrations ranging from 0.03 to 10 nM, for a total assay volume of 100  $\mu\text{l}$ . A comparable set of samples was incubated in the presence of 100 nM Butaclamol for nonspecific binding. Following 2 h of incubation at room temperature (25 °C), samples from the titer plates were passed through Brandel filters pretreated with 0.2% polyethyleneamine. Sample sites from the filters were punched out and placed into 10-ml scintillation vials and were counted for 5 min in a gamma counter. Specific binding was determined for each sample by subtracting nonspecific binding from total binding of [3H]-SCH 23390. The amount of protein per milliliter necessary for the binding experiment was determined by the method of Lowery et al. (1951). Densities are reported as femtomoles per milligram of protein.

### 2.9. Social interaction test

In the social interaction test, the subject was placed alone for 5 min in one side of a Plexiglas compartment (20  $\times$  21  $\times$  31 cm) in order to habituate to the test environment. A sliding sheet-metal panel was then removed, exposing the subject to a same-age, group-reared male (marked for identification), which had been placed in the other half of the compartment. In the succeeding 10 min, social interactions were recorded after which both animals were weighed and returned to their home cages. These dyadic tests were conducted between 14:00 and 16:00 h in a dimly illuminated room.

Behavioral observations consisted of recording latencies and frequencies of “attack” (a vigorous lunge toward the

other animal, with biting or slashing) and “freeze” (rigid immobility upon social contact). For the several generations in which these categories have been used, interobserver agreement has always exceeded 90%. Given the short duration of this test, instances when animals were wounded have been extremely rare across the several generations produced by this selective breeding program. When this happened, the test was terminated before completion, and the wounded animal was immediately euthanized. Instances of this nature did not occur in the series of dyadic tests reported in this article.

## 3. Results

### 3.1. Handling effects on open-field behavior

NC900 mice explored the arena more than NC100 mice did as reflected by a higher frequency of arena crossings [ $F(1,36)=6.48$ ,  $P<.02$ ; Fig. 1]. Overall, this form of activity decreased from the first 5-min to the second 5-min block [ $F(1,36)=32.48$ ,  $P<.001$ ]. As indicated by the significant Line  $\times$  Handling interaction, this temporal change occurred in all conditions, except in the handled NC100 group, where a constant rate was observed [ $F(1,36)=4.69$ ,  $P<.05$ ].

Supported rears (data not shown) were fairly frequent but were observed at similar rates across the four experimental conditions without appreciable change over time. Accordingly, the analyses indicated no main effects of either line or handling for this measure. Although less frequent, the rates of unsupported rears were globally higher in NC900 [ $F(1,36)=4.34$ ,  $P<.05$ ; Fig. 1]. This behavior was more frequent during the second half of the test [ $F(1,22)=6.38$ ,  $P<.01$ ], except among handled NC100 subjects who maintained a constant rate over the two periods [Line  $\times$  Handling interaction;  $F(1,36)=8.40$ ,  $P<.01$ ].

### 3.2. Handling effect on CORT activation in the open field

The CORT valued measured in the present experiment are presented in Fig. 2. The left panel presents the baseline values measured among nonhandled NC100 and NC900 subjects reared in isolation. The right panel presents the CORT activation values measured as a function of line, handling, and rearing conditions. As seen by a comparison of the two panels, baseline measures were, on average, twice as low as those obtained following exposure to the open field. The only exception to this are the CORT values obtained in the NC100 line among the handled subjects reared in groups that were in the range of the baseline values estimated for this line. There was no reliable difference in CORT baseline estimates between the lines [ $F(1,33)=2.27$ ,  $P=.14$ ].

Although blood was always collected between 15 and 20 min after exposure to the open field, the number of animals to test required samples to be taken throughout the

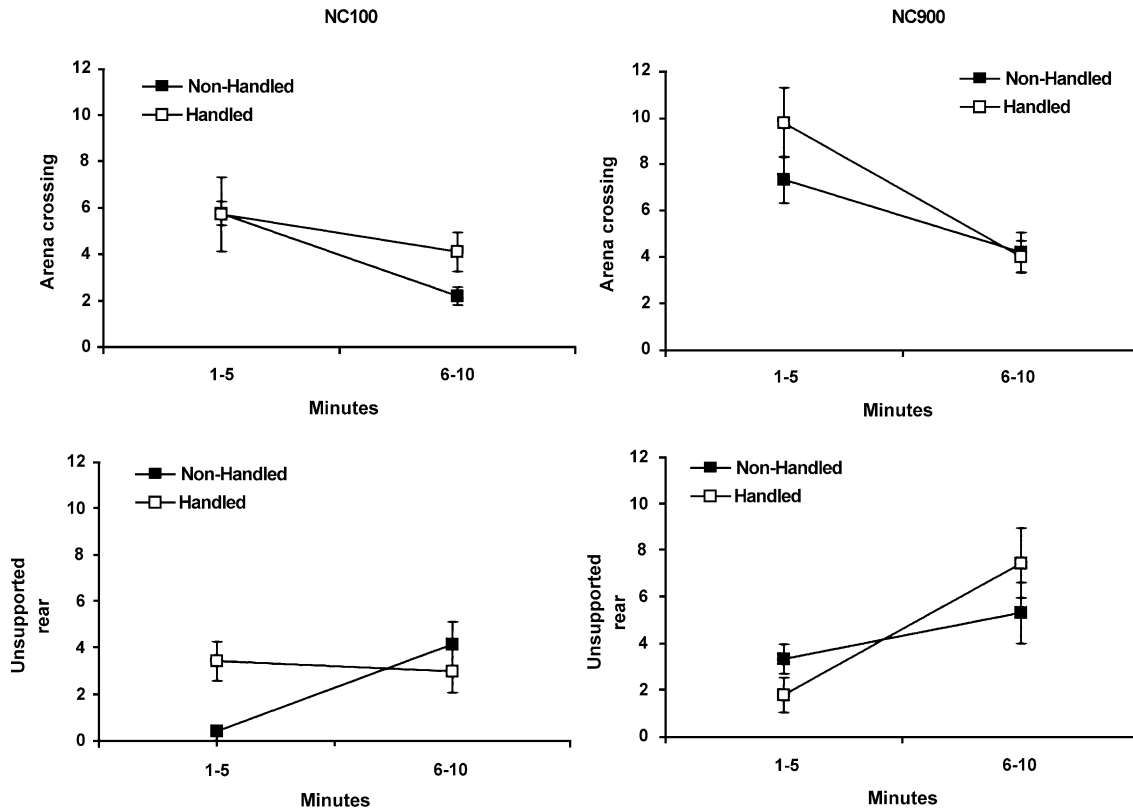


Fig. 1. Frequencies of arena crossing and unsupported rears during the first and last 5 min of exposure to the open field. Results are presented as a function of neonatal treatment and line (left panels, NC100; right panels, NC900).

morning hours. Because this is a period in which sharp variations in CORT secretion have been noted (Cheifetz, 1971), the collection time of the blood samples was used as a covariate in the analysis of these data. The effect of time of sampling on measured concentrations of CORT approached significance [ $F(1,86)=2.96, P=.09$ ]. Accordingly, main effects of line and handling were examined with time of sampling as a covariate. Overall, no main effect of selected line was detected in CORT activation in the open field. However, analysis of variance revealed a significant inter-

action between line and housing [ $F(1,86)=4.25, P<.04$ ] and a nearly significant interaction between line and handling [ $F(1,86)=3.74, P=.056$ ]. Because the NC900 animals appeared refractory to both handling and housing conditions, we examined the NC100 data for simple main effects. As illustrated in Fig. 2, we observed significant effects of handling and housing in this line [ $F(1,42)=6.02, P<.02$  and  $F(1,42)=11.12, P<.01$ , respectively]. What is also evident from inspection of Fig. 2 is that in the NC100 line, handling and housing had apparent additive effects on CORT response to novelty.

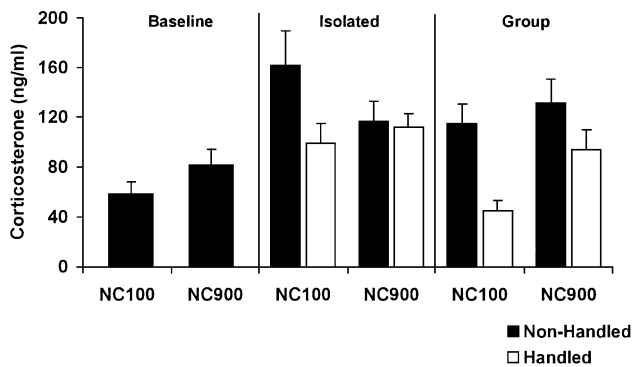


Fig. 2. Left panel: Baseline measures of plasma CORT concentration as a function of selected line. Middle and right panels: CORT activation in the open field as a function of selected line and neonatal treatment (handled/undisturbed) and rearing condition (isolation/group).

### 3.3. Handling and rearing effects on D1 dopamine receptor densities

No appreciable effects of handling or housing conditions were measured on the density of the D1 dopamine receptors in the caudate–putamen of either NC100 or NC900 mice. The D<sub>1</sub> dopamine receptor densities measured in this nucleus were about the same in the two lines, around 15 fmol/mg<sup>-1</sup>. By contrast, the factors line, neonatal treatment and housing had strong effects on measures obtained for the nucleus accumbens density values of the same receptor. These results are presented in Fig. 3 (left panel: isolation; right panel: group).

D1 receptor density values were significantly higher among handled than among nonhandled subjects [ $F(1,86)=$

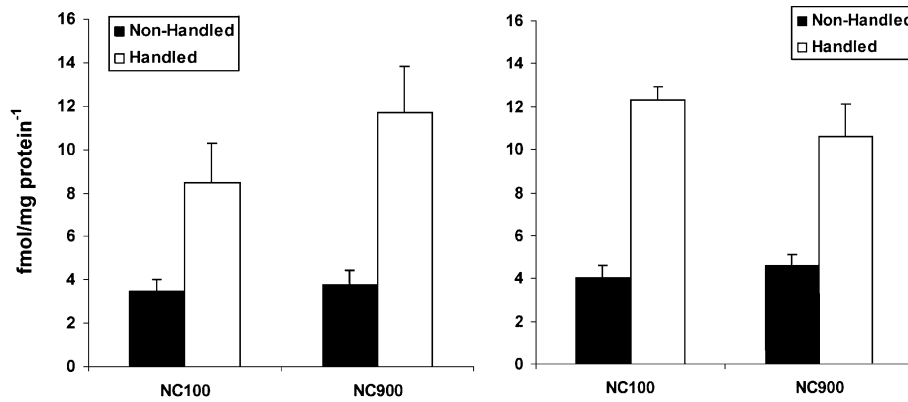


Fig. 3. D1 dopamine receptor density measured in the nucleus accumbens. Data are presented as a function of line, neonatal treatment and housing condition (left panel, isolation; right panel, group).

52.49,  $P < .001$ ]. This effect was large, observable in both lines and under both housing conditions. A nearly significant three-way interaction [ $F(1,86) = 3.67$ ,  $P = .058$ ] was obtained, which indicated that the joint effects of handling and subsequent housing conditions were line specific. Fig. 3 shows that NC100 D<sub>1</sub> dopamine receptor densities were highest when the subjects had been handled and subse-

quently housed with other males [ $F(1,42) = 4.41$ ,  $P < .05$ ]. This interaction was not observed in NC900. In this line, group rearing did not significantly augment nor did it reduce the handling effects on D1 dopamine receptor densities.

Given the hypothesis that a bidirectional relationship may exist between HPA activation and dopaminergic release in the nucleus accumbens, partial correlations con-

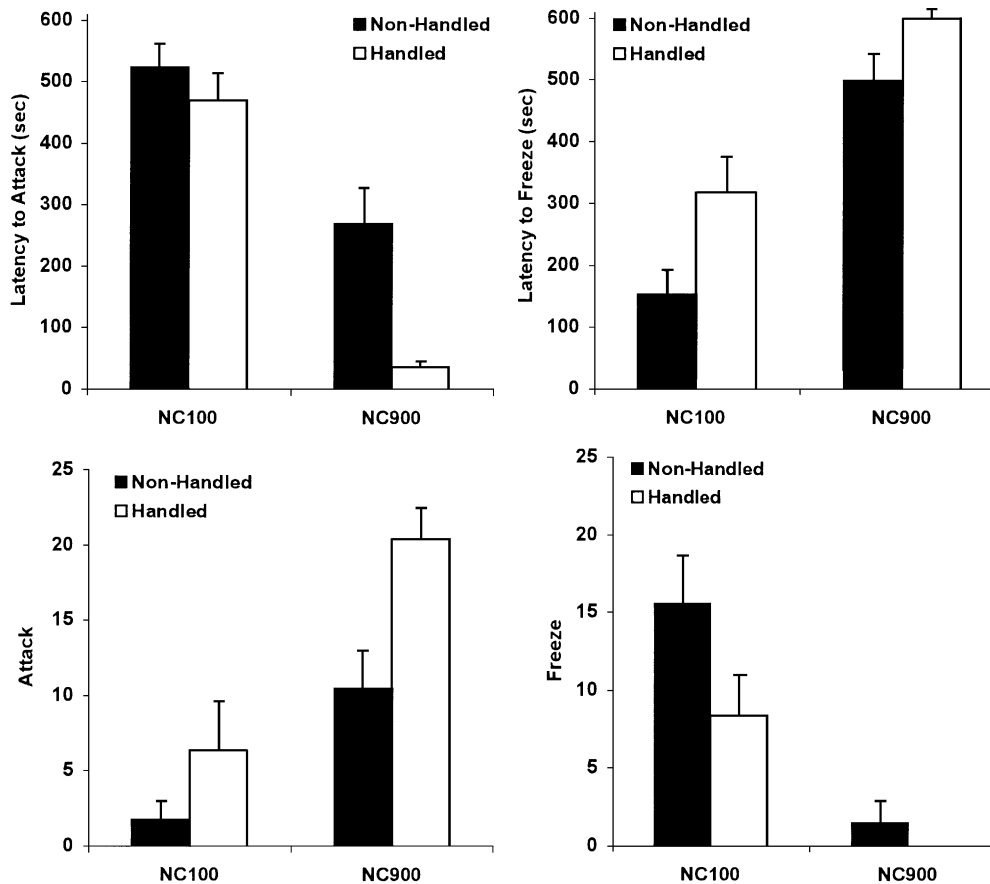


Fig. 4. Top left panel: Latency to attack. Top right panel: Latency to freeze. If no attacks or freezing were observed during the 10-min test, latencies were recorded as 600 s. Bottom left panel: Number of 5-s blocks during dyadic tests where attacks were observed. Bottom right panel: Number of 5-s blocks during dyadic tests where freezing was observed. Data are presented as a function of handling condition and selected lines.

trolling for circadian changes in HPA activation and D1 dopamine receptor densities were conducted. This statistic revealed a significant negative relationship between these two measures among NC100 animals [ $r(36) = -.41$ ,  $P < .005$ ]. The direction of this correlation was consistent with the negative relationship known to exist between dopamine release and its postsynaptic receptors. In the high-aggressive line, this correlation was not significant [ $r(36) = -.1337$ ,  $P = .21$ ].

### 3.4. Handling effects on social interactions

Consistent with previous reports, NC100 subjects, irrespective of early experience, exhibited significantly shorter latencies to freeze in a dyadic test than NC900 subjects [ $F(1,80) = 56.52$ ,  $P < .0001$ ] (Fig. 4, top right panel). The same line difference was also observed for freezing frequencies [ $F(1,80) = 24.40$ ,  $P < .0001$ ] (Fig. 4, bottom right panel). Similarly, NC900 showed a greater readiness to attack [ $F(1,80) = 68.84$ ,  $P < .0001$ ] and attacked the partner animal significantly more than NC100 subjects [ $F(1,80) = 47.68$ ,  $P < .0001$ ]. Again, these differences were evident irrespective of neonatal treatment. The line differences on all four measures remained large and robust when estimated within experimental groups (all  $P$ 's  $< .01$ ).

While preserving line differences, early handling strongly affected most of the behavioral measures obtained in dyadic testing. Specifically, handled subjects took significantly longer to enter a freezing mode in the dyadic tests than undisturbed subjects [ $F(1,80) = 9.97$ ,  $P < .005$ ]. Independent  $t$  tests further showed that this effect was significant in both lines [NC100:  $t(1,20) = -2.365$ ,  $P < .05$ ; NC900:  $t(1,20) = -2.15$ ,  $P < .05$ ]. Although freezing frequencies were globally reduced among handled subjects, as seen in Fig. 4, no significant effects of neonatal treatment on this measure were noted in either line. The analyses further indicated a strong effect of neonatal treatment on latencies to attack [ $F(1, 80) = 11.95$ ,  $P < .001$ ]. This effect reached significance, however, only in the NC900 line. In this case, handled subjects attacked their partner almost immediately, on average, five times faster than control subjects [ $t(1,20) = 3.92$ ,  $P < .001$ ]. Analyses of variance conducted on frequency measures revealed a significant line by neonatal treatment interaction [ $F(1,80) = 5.14$ ,  $P < .05$ ]. As seen in Fig. 4, the frequency of attacks was higher among handled NC900 but not among NC100 subjects similarly treated.

## 4. Discussion

In the decades following Levine's (1957) demonstration that early handling had positive rather negative effects on the development of the stress response, the physiological pathways mediating this paradoxical effect have been the object of systematic analysis. Advancement on this question required tight experimental controls that forced postponing

equally important questions regarding this phenomenon. Accordingly, the present research was designed to examine how early stimulation (i.e., handling), subsequent housing conditions and genetic factors interact to produce adult differences in stress regulation. The effects of these factors and their interactions were examined in two physiological systems, the HPA axis and the dopaminergic system, and behavioral effects were examined under both nonsocial (open field) and social (dyadic encounter) testing conditions. To this end, high-aggressive (NC900) and low-aggressive (NC100) mice produced by selective breeding in our laboratory were handled for 3 weeks postpartum, subsequently isolated or grouped, and relevant measures were taken when the subjects reached young adulthood.

The behaviors measured in the open field suggest that when away from the walls of the arena, the animals began exploring the surface of the novel environment first and gradually shifted to the exploration of its third dimension. This is based on the observation of a gradual decline in arena crossings over time and a corresponding augmentation of unsupported rears, a posture in which the animal was typically looking up. By these two measures, NC900 were globally more active than NC100. The relative frequencies of these behaviors over the first and the last 5 min of the test were not affected by handling in the NC900 line. Overall, the nonhandled NC100 subject exhibited a pattern of temporal variation in open-field behaviors that closely matched that seen in the high-aggressive line. However, activity rates among the handled low-aggressive subjects were, by comparison, more constant across the first and the last part of the test. This lack of temporal change would be consistent with Levine's (1969) observation that handled animals habituate more rapidly to novel situations, although the present results offer no basis to support this interpretation. At the very least, these observations indicate that the effects of early handling on open-field behaviors may not be the same among animals that differ in genetic background.

Compared to the baseline values of plasma CORT obtained among nonhandled, naive subjects, the measures of this steroid obtained following exposure to the open-field were substantially higher and consistent with the common view that this test is a reliable elicitor of HPA activation in rodents (Denenberg, 1964). Although questions have been raised concerning possible effects of sacrifice by halothane inhalation on measured CORT activation values, Carlberg et al. (1995) found no evidence that blood concentrations of CORT obtained via cardiac puncture a few minutes following halothane inhalation differed from those obtained from animals sacrificed by rapid decapitation. Nonetheless, care was taken in the present experiment to expose all subjects to the same treatment prior to collecting blood from them.

The plasma CORT concentration values measured across lines and postnatal treatments conditions showed that neonatal handling failed to produce any adult differences in stress regulation in the high-aggressive line. These animals exhibited moderate elevations of CORT in the open-field as



compared to NC100 undisturbed as pups, and these levels were virtually the same irrespective of neonatal treatment. By contrast, early experience significantly affected CORT activation in the NC100 line. The main effect of handling in this line was to reduce CORT activation to levels comparable to those measured in the high line in the absence of handling. The line specificity of handling effects obtained in the present experiment closely replicated those reported by Fernandez-Teruel et al. (1992) and Anisman et al. (1998) who conducted similar experiments on the genetic modulation of handling effects. Both groups observed, as reported here, that CORT values among strains that naturally exhibit lower responses to stressors were unaffected by handling, and that the usual effects of this manipulation, namely, a reduction in CORT activation under stress, was only observed among those strains in which this response was naturally elevated.

This finding naturally begs the question: “How did differences in genetic background interact with early experience to produce the line-specific effects that we observed?” It has been demonstrated that the most likely experiential mediator of handling effects is the augmentation of maternal care following the return of stressed pups to the nest (Smotherman, 1983; Liu et al., 1999). On the basis of this maternal mediation hypothesis, we have recently observed mother–pup interactions in our selected lines and showed that maternal care was unaffected by the daily experience of handling in the high-aggressive line but was significantly augmented in the low line. We further determined that this augmentation in NC100 coincided with a tendency, more pronounced among pups of this line, to emit ultrasonic distress calls (known to elicit maternal behaviors) when returned to the nest (Rodríguez et al., in preparation). Results such as these are interpreted in the current literature as evidence that maternal care is a fundamental contributor to adult differences in stress regulation, providing as it does an essential environmental input at a time when the functional organization of the HPA axis is highly programmable by such stimulation (Meaney et al., 1996; Liu et al., 1999). A strong interpretation of these findings would hold that early experience creates stable adult differences. A weaker interpretation of the same findings would argue that because development is a cumulative process, early stimulation affects how subsequent stimulation contributes to further development. In order to shed light on these considerations, we included in our research design a second condition in which handled and nonhandled subjects were exposed or not exposed to social stimulation from the time they were weaned to the time they were tested as young adults.

This addition to the traditional design where the experimental subjects and their controls are reared under uniform conditions after weaning yielded compelling evidence against the strong interpretation. Among our low-aggressive animals, group rearing by itself was almost as effective as postnatal handling in reducing subsequent CORT response

to the open-field arena as compared to the social isolation condition. Our results also suggested that group rearing and postnatal handling had additive effects in that CORT activation was reduced further among those subjects that experienced both the early and the later forms of stimulation. The fact that high-aggressive animals were as refractory to differences in rearing conditions as they were to postnatal handling during CORT activation, again, necessitates to factor in differences in genetic background in the analyses of interactions between early and subsequent experience. While maternal behavior has been clearly linked to the effects of handling on HPA regulation (Liu et al., 1999), further research will be necessary to identify specific mechanisms whereby interactions with peers during adolescence may produce similar effects. In the context of the present study, a potentially promising avenue of research would be to begin with the fact that, by contrast to that of the high-aggressive line, the social ecology of our low-aggressive males is characterized by a high frequency of low-intensity social contacts and the apparent absence of dominance hierarchies (Gariépy, 1994). In comparisons of low-aggressive males exposed to peers of their own selected line or to peers of the high-aggressive line, one could determine whether the social ecology of adolescence and early adulthood conditions the release of serotonin, a substance linked in earlier research to the expression of glucocorticoid mRNA expression (Meaney et al., 1996). Such a study would either demonstrate that the openness of the HPA axis to experiential modification during this later period of development is mediated by the same pathways as those implicated in the effects of early stimulation, or it would indicate that alternative pathways, yet to be identified, may bring about similar effects.

Consistent with previous studies on the effects of early handling on the dopaminergic system, the findings reported here showed profound effects of this manipulation on the density of nucleus accumbens D1 dopamine receptor. That this effect consisted of a significant up-regulation of this receptor is in good agreement with the demonstration by Cabib et al. (1993) that handling reduced the release of dopamine in the same nucleus. Here, it is worth emphasizing that in contrast to the line specificity of handling effects on CORT activation, the adult densities of this receptor were augmented as a result of handling in both the high- and low-aggressive lines. This finding raises important questions concerning the experiential mediation of handling effects. Given that maternal behavior was not affected by handling in the high-aggressive line (see above), it would seem that the experiential mediation of the changes induced by handling on the dopaminergic system follows a different pathway that does not involve maternal stimulation. As recently suggested by Denenberg (1999), maternal stimulation may not be the only mediator of handling effects in infancy, and, as suggested by the present results, maternal stimulation may not account for all systemic changes induced by handling. In his recent commentary, Denenberg (1999)

reminds us of the direct action hypothesis—that the stress experienced by handled pups, in and of itself may also account, at least in part, for the handling effects, and suggests that any form of social interaction, including pup–pup interactions, may also have similar effects. This last possibility finds some support in the present results, at least with our low-aggressive line, where D1 receptor densities were highest when, in addition to handling, the subjects were reared in groups during adolescence.

Another goal of the present research was to begin the investigation of handling effects under social conditions as opposed to nonsocial ones. Our aim in this study was not to evaluate the stress response elicited by social encounters, as this situation introduces several confounding factors. Instead, we wanted to determine whether the direction of handling effects on the behavior of our high- and low-aggressive animals would be preserved or differ when evaluated under the conditions created by open-field testing and those generated by a social encounter. The rationale for this comparison was that different testing conditions may mobilize different adaptive systems (Thelen and Smith, 1994) and may potentially reveal different aspects of handling effects and their interaction with genetic background. As seen in Section 4, dyadic testing reduced the propensity to freeze in the low-aggressive line and shortened latencies to attack in the high-aggressive line. Although these effects may appear to be line specific, the global effect may be interpreted as shifting both groups from a more defensive or less offensive toward a less defensive or more offensive interaction style. This would be consistent with the view that handling reduces fearfulness. On this point, it should be noted that open-field behaviors revealed handling effects in the low line only and that, by this test, animals of the high line appeared refractory to handling effects. As suggested by the literature, it is likely that such a change in social interactive style involves a change in dopaminergic functions. Indeed, the dopaminergic system has been shown to be an important mediator in the initiation of action such as responses to novelty in the environment (Cigrang et al., 1986; Thullier et al., 1997). Moreover, it has been suggested that optimal activation of this system promotes a rapid attentional shift to unexpected stimuli and the selection of appropriate behavioral responses to biologically significant events (Redgrave et al., 1999; Feldon and Wiener, 1992). It will remain in future research to determine, by means of appropriate dopamine receptor antagonists, whether the handling-induced up-regulation of the D1 dopamine receptor reported here actually mediates this change in interactive style.

### Acknowledgments

This work was partially supported by NIMH Grant IP50MH52429 (J.-L.G., co-PI) and a NSF predoctoral Ford Foundation Fellowship (R.M.R.).

### References

- Ader R, Grotta LJ. Effects of early experience on adrenocortical reactivity. *Physiol Behav* 1969;4:303–5.
- Anisman H, Zaharia MD, Meaney MJ, Merali Z. Do early life events permanently alter behavioral and hormonal responses to stressors? *Int J Dev Neurosci* 1998;16:149–64.
- Cabib S, Puglisi-Allegra S. Opposite responses of mesolimbic dopamine system to controllable and uncontrollable aversive experiences. *J Neurosci* 1994;14:3333–40.
- Cabib S, Puglisi-Allegra S, D'Amato FR. Effects of postnatal stress on dopamine mesolimbic system responses to aversive experiences in adult life. *Brain Res* 1993;232–9.
- Cairns RB, MacCombie D, Hood KE. A developmental-genetic analysis of aggressive behavior in mice: I. Behavioral outcomes. *J Comp Psychol* 1983;97:69–89.
- Carlberg KA, Gwosdow AR, Alvin BL. Effects of anesthesia with halothane and methoxyflurane on plasma corticosterone concentration in rats at rest and after exercise. *Lab Anim Sci* 1995;45:487–584.
- Casolini P, Kabbaj M, Leprat F, Piazza PV, Rouge-Pont F, Angelucci L, Simon H, LeMoal M, Maccari S. Basal and stress induced corticosterone secretion is decreased by lesion of mesencephalic dopaminergic neurons. *Brain Res* 1993;622:311–4.
- Cheifetz PN. The daily rhythm of the secretion of corticotrophin and corticosterone in rats and mice. *J Endo* 1971;49:9–12.
- Cigrang M, Vogel E, Misslin R. Reduction of neophobia in mice following lesions of the caudate–putamen. *Physiology* 1986;36:25–8.
- Denenberg VH. Critical periods, stimuli input and emotional reactivity: a theory of infantile stimulation. *Psychol Rev* 1964;71:335–51.
- Denenberg VH. Commentary: is maternal stimulation the mediator of the handling effect in infancy? *Dev Psychobiol* 1999;34:1–3.
- Denenberg VH, Morton JRC. Effects of environmental complexity and social hypotheses concerning infantile stimulation. *J Comp Physiol Psychol* 1962;55:242–6.
- Feldon J, Wiener I. From an animal model of an attentional deficit towards new insights into the pathophysiology of schizophrenia. *J Psychiatr Res* 1992;26:345–66.
- Fernandez-Teruel A, Echoriuela RM, Nunez JF, Goma M, Driscoll P, Tobena A. Early stimulation effects on novelty-induced behavior in two psycho-genetically selected rat lines with divergent emotionality profiles. *Neurosci Lett* 1992;137:185–8.
- Foster GA, Schultzberg M, Kokfelt T, Goldstein M, Hemmings HC, Ouitmet CC, Walass SI, Greengard P. Ontogeny of the dopamine and cyclic adenosine 3':5'-monophosphate-regulated phosphoprotein (DARPP-32) in the pre- and postnatal mouse central nervous system. *Int J Dev Neurosci* 1988;6:367–86.
- Francis DD, Meaney MJ. Maternal care and the development of stress responses. *Curr Opin Neurobiol* 1999;9:128–34.
- Fuller JL. Experiential deprivation and later behavior. *Science* 1967;158:1645–52.
- Gariépy J-L. The mediation of aggressive behavior in mice: a discussion of approach–withdrawal processes in social adaptations. In: Greenberg G, Hood KE, Tobach E, editors. *Behavioral development in comparative perspective: the approach–withdrawal theory of T.C. Schneirla*. New York: Garland, 1994. p. 231–84.
- Gariépy J-L, Gendreau PL, Mailman R, Tancer M, Lewis MH. Rearing conditions alter social reactivity and D1 dopamine receptors in high- and low-aggressive mice. *Psychopharmacol Biochem Behav* 1995;51:767–73.
- Gariépy J-L, Bauer D, Cairns RB. Selective breeding for differential aggression in mice provides evidence for heterochrony in social behaviours. *Anim Behav* 2001;61:1–15.
- Ginsberg BE. Genetic variables affecting responses to postnatal stimulation. In: Ambrose A, editor. *Stimulation in early infancy*. New York: Academic Press, 1969. p. 73–96.

- Ginsberg BE. All mice are not created equal—recent findings on genes and behavior. *Soc Serv Rev* 1966;40:121–34.
- Henry JP, Stephens PM. Stress, health and the social environment: a socio-biologic approach to medicine. New York: Springer, 1977.
- Hess EJ, Norman AB, Creese I. Chronic treatment with dopamine receptor antagonists: behavioral and pharmacological effects on D1 and D2 dopamine receptors. *J Neurosci* 1988;8:2361–70.
- Hughes RN. Reactions to novelty following differential postweaning rearing and handling. *Percept Mot Skills* 1971;32:883–6.
- King JA, Eleftheriou BE. Effects of early handling upon adult behavior in two subspecies of deermice, *Peromyscus maniculatus*. *J Comp Physiol Psychol* 1959;52:82–8.
- Levine S. Infantile experience and resistance to physiological stress. *Science* 1957;126:405–6.
- Levine S. Infantile stimulation. In: Ambrose A, editor. Stimulation in early infancy. New York: Academic Press, 1969. p. 1–19.
- Levine S, Broadhurst PL. Genetic and ontogenetic determinants of adult behavior in rats. *J Comp Physiol Psychol* 1963;56:423–8.
- Liu D, Diorio J, Tannerbaum B, Caldji C, Francis D, Freedman A, Sharma S, Pearson D, Plotsky P, Meaney M. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic–pituitary–adrenal responses to stress. *Science* 1999;277:1659–62.
- Lowery OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265–75.
- Meaney MJ, Aitken DH. The effects of early postnatal handling on the development of hippocampal glucocorticoid receptors: temporal parameters. *Dev Brain Res* 1985;22:301–4.
- Meaney MJ, Aitken DH, van Berkel C, Bhatnagar S, Sapolsky RM. Effect of neonatal handling on age-related impairments associated with the hippocampus. *Science* 1988;239:765–8.
- Meaney MJ, Diorio J, Francis D, Widdowson J, LaPlante P, Caldji C, Sharma S, Seckl JR, Plotsky PM. Early environmental regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress. *Dev Neurosci* 1996;18:49–72.
- Missale C, Nash SR, Robinson SW, Jaber M, Caron MC. Dopamine receptors: from structure to function. *Physiol Rev* 1998;78:189–225.
- Mohammed AH, Henriksson BG, Soderstrom S, Ebendal T, Olsson T, Seckl JR. Environmental influences on the central nervous system and their influences for the aging rat. *Behav Brain Res* 1993;57:183–91.
- Pfeifer WD, Rotundo R, Myers M, Denenberg VH. Stimulation in infancy: unique effects of handling. *Physiol Behav* 1976;17:781–4.
- Puglisi-Allegra S, Kempf E, Cabib S. Role of genotype in the adaptation of brain dopamine system to stress. *Neurosci Biobehav Rev* 1990;14:523–8.
- Redgrave P, Prescott TJ, Gurney K. Is the short-latency dopamine response too short to signal reward error? *Trends Neurosci* 1999;22:146–51.
- Rodriguez RM, Gariépy J-L, Mills-Koonce R, Nehrenberg D. Differential effects of handling in mouse lines selected for high and low aggression and the maternal mediation hypothesis (in preparation).
- Rosenblum LA, Kaufman IC. Variations in infant development and response to maternal loss in monkeys. *Am J Orthopsychiatr* 1968;38:418–26.
- Sapolsky RM. The physiology of dominance in stable vs. unstable social hierarchies. In: Mason W, Mendoza S, editors. Primate social conflict. New York: SUNY Press, 1993. p. 171–204.
- Sapolsky RM. Stress, glucocorticoids, and damage to the nervous system: the current state of confusion. *Stress* 1996;1:1–19.
- Sapolsky RM. The importance of a well-groomed child. *Science* 1997;277:1620–1.
- Scott JP. Critical periods for the development of social behavior in dogs. In: Kazda S, Denenberg VH, editors. The postnatal development of phenotype. New York: Academic Press, 1970. p. 420–9.
- Seay BM, Schlottmann RS, Gandolfo R. Early social interaction in two monkey species. *J Gen Psychol* 1972;87:37–43.
- Smotherman WP. Mother–infant interaction and the modulation of pituitary–adrenal activity in rat pups after early stimulation. *Dev Psychobiol* 1983;16:169–76.
- Stolnick BM, Leonard CM. A stereotaxic atlas of the albino mouse forebrain. Rockville, MD: US Department of Health, Education and Welfare, 1975.
- Thelen E, Smith LB. A dynamic systems approach to cognition and action. Cambridge: MIT Press, 1994.
- Thullier F, Lalonde R, Mahler P, Joyal CC, Lestienne F. Dorsal striatal lesions in rats: effects on exploration and motor coordination. *Arch Physiol Biochem* 1997;104:300–6.