



Research report

Developmental changes in *c-fos* expression to an age-specific social stressor in infant ratsChristoph P. Wiedenmayer^{a,b,*}, Gordon A. Barr^{a,b,c}^a Department of Psychiatry, Columbia University College of Physicians and Surgeons, 1051 Riverside Drive, Unit 40, New York, NY 10032, USA^b Division of Dev. Psychobiology, NY State Psychiatric Institute, New York, NY 10032, USA^c Department of Psychology, Hunter College, City University of New York, New York, NY 10021, USA

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Abstract

Young rats become immobile when exposed to a potentially infanticidal adult male rat. Male-induced immobility declines during the preweaning period, paralleling the decrease in infanticidal threat. To investigate the neural substrates underlying the developmental change in immobility, male-induced expression of the immediate-early gene *c-fos* was assessed on postnatal days 7, 14 and 21. A huddle of three young rats was exposed to an adult male behind a screen. As control, three littermates were put in the testing chamber but not exposed to the male. On day 7, male exposed and control pups were immobile most of the time and *c-fos* expression did not differ between conditions. On day 14, rats in the presence of the male stopped ongoing behaviors and became immobile. They had significantly higher *c-fos* expression in the paraventricular nucleus of the hypothalamus, the amygdala, the periaqueductal gray, and the locus ceruleus. On day 21, the male-exposed rats that were immobile had elevated *c-fos* expression in a similar pattern as on day 14, however, different nuclei of the amygdala were activated. In contrast, male-exposed 21-day-old rats that showed control levels of immobility did not have elevated *c-fos* expression in these areas. These results demonstrate that male exposure induced *c-fos* expression in brain areas of young rats in an age-specific pattern. Some of the activated brain areas seem to have contributed to immobility. Differential activation of neuronal populations may underlie developmental changes in defensive immobility during early ontogeny. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

When an animal perceives a potentially harmful stimulus, it responds defensively to counteract the threat. Immobility, or freezing, is a prominent defensive response in a variety of animal species [27,31,35,43]. However, a stimulus may be harmful and threaten an animal's growth and survival only during specific ontogenetic periods. Therefore, it depends on the age of the individual whether an animal responds with immobility

to a particular stimulus. Adult male rats may kill unrelated pups [61]. Infanticide increases the male's reproductive success because it terminates lactation and induces estrus in the female that lost her litter, thereby allowing the male to mate with her [40,87]. Therefore, infanticide disappears after weaning when the female resumes cycling [68]. Young rats have the ability to respond to the male threat. When exposed to male cues, they have elevated corticosterone levels [83], suppress nociception and become immobile, which may conceal them from the male [81,88]. However, they respond selectively to male cues in an age-specific way that parallels the changes in infanticidal threat [88,89]. On postnatal day 7, pups are quiescent and immobile in the

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huddle almost all of the time, and they do not need to change this behavior when a male is in close proximity to the nest. On day 14, pups are more active in the nest area, and when exposed to the male they stop ongoing behaviors and become immobile. This male-induced immobility decreases on day 21 and disappears by day 26, around weaning. On day 21, the response to the male varies between animals. The majority of the young rats still become immobile but some rats show almost no immobility and instead approach the male, which indicates a transitional period in the development of this defensive response.

Changes in responsivity during the life span of an animal are mediated by changes in the underlying neural substrate. Changes in the neural substrate could occur at various sites within the stimulus-response system [44]. Peripheral sensory filters could assess the salience of a stimulus differently at different ages; central brain areas could process a stimulus differently during different developmental periods; or developmental changes in the effectors could contribute to changes in the response. The aim of the present study was to determine how male exposure activates brain areas that may contribute to immobility and how such activation changes during the preweaning period in young rats.

In the adult rat, forebrain structures, in particular the hippocampus [8], subnuclei of the hypothalamus [12], the bed nucleus of the stria terminalis [74] and the amygdala [53], and brainstem structures such as the periaqueductal gray [4,29,65] and the locus ceruleus [80] mediate stress-induced immobility. During aversive stimulation, these structures act in concert, making up a neural circuit, the fear pathway [30,53], which sequentially processes aversive stimuli and generates defensive behaviors [17,18,45]. To identify the brain areas that are activated by male cues and are involved in male-induced immobility in the infant rat, we used a marker for neuronal activation, the expression of the immediate early gene *c-fos*. Aversive stimulation rapidly induces the expression of the *c-fos* gene and its product, the Fos protein, functions as a heterodimeric transcription factor involved in genomic regulation, [19,64,75] and its expression may contribute to long-term alterations of the defensive response [63,66].

To investigate how male-induced neuronal activation changes during early development, we exposed preweaning rats at one of three different ages, on day 7, 14, or 21, to a male and assessed *c-fos* expression in multiple brain areas associated with stress-induced immobility. We hypothesized that on day 7, when huddled pups do not respond to male proximity, male exposure would not induce *c-fos* expression. For day 14, when rats display male-induced immobility, we hypothesized that *c-fos* is expressed in those brain areas that mediate stress-induced immobility in the adult rat. Finally, we hypothesized that 21-day-old rats that become immo-

ble in the presence of the male show a similar *c-fos* expression pattern as 14-day-old rats, whereas 21-day-old rats that are more active during male exposure show diminished expression or a different pattern of expression.

2. Materials and methods

2.1. Animals

Long-Evans hooded rats were housed in standard laboratory cages in a colony room maintained at 22–24 °C with a 12 h light/dark cycle with light onset at 07:00 h. Cages were checked twice daily, at approximately 09:00 and 18:00 h. Pups found at either time were designated as 0 days of age. Young rats were tested on postnatal days 7, 14, and 21. Treatments were administered according to the guidelines of the Institutional Animal Care and Use Committee.

2.2. Procedure and behavioral measurements

Rats were tested in small huddles to decrease isolation-induced stress. On the day of testing, three male rats were taken randomly from a litter, marked with a nontoxic marker (Sharpie, VWR, Bridgeport, NJ) on their fur, and placed in one compartment of the testing cage. The testing cage (46 × 25 × 21 cm) was subdivided by a wire-mesh partition positioned in the middle of the cage, thereby forming two equal compartments. The compartment into which the young rats were placed contained home cage bedding to simulate the nest area. The other compartment of the testing cage was empty. The testing cage stood on a heating pad maintained at 30 °C when 7-day-old pups were tested. The rats were allowed to acclimate for 15 min. Afterward, a sexually experienced, unfamiliar, unrelated adult male was placed in the adjacent compartment for 5 min, and the behavior of the young rats was recorded by scan sampling. Every 15 s the behavior of each of the three rats was recorded on a checklist of behavioral categories including 'immobile'. 'Immobile' was defined as any posture in which the animal did not exhibit any movement except that necessary for respiration, and was expressed as a percentage of the scans. After removal of the male, the young rats spent another 5 min in the testing cage and were then put back into the home cage in the colony room. Three young male rats from the same litter were used as control animals. They were submitted to the same procedure, but without the unfamiliar male in the adjacent compartment. One male exposed pup and one control pup in each of the two conditions were used for the assessment of *c-fos* expression. The order of testing male exposed and control animals was alternated. All tests were conducted in the

first half of the light cycle. On day 7 animals from six litters (six exposed to the male, six controls) were used, on day 14 animals from nine litters (nine exposed to the male, nine controls), and on day 21 animals from sixteen litters (16 exposed to the male, 16 controls). These 16 male-exposed rats were selected after exposure according to a criterion defined before the experiment started: half of the animals were immobile more than 50% of the observation time (eight freezers), and the other half was immobile 50% or less of the observation time (eight non-freezers).

2.3. Immunocytochemistry

c-fos expression was determined by immunocytochemistry. Two hours after testing, when *c-fos* expression peaks [47,64], rats were removed from the home cage, injected intraperitoneally with an overdose of sodium pentobarbital and perfused transcardially with cold 0.9% saline, followed by cold 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). The brains were removed and stored in 4% paraformaldehyde for 48 h followed by 30% sucrose solution for cyroprotection. The brains were frozen and sectioned coronally. Brains of 7-day-old pups were sectioned in a microtome (50 μ m in thickness) and brains of 14- and 21-day-old rats were sectioned in a cryostat (30 μ m in thickness). In 7-day-old pups, neuronal myelination is not completed. Thicker sections were cut to prevent them from breaking. Two out of every six sections were collected in PBS. One section was placed on a microscope slide for cresyl violet staining to locate the brain areas and one was used for immunocytochemical processing. Sections of male-exposed and control animals were assayed together. Sections were preincubated in 3% hydrogen peroxide for 10 min in order to quench endogenous peroxidase activity. Sections were processed using a modified protocol for a commercially available antibody staining kit (ABC kit, Vectastain Elite, Vector Laboratories, Burlingame, CA) that uses the diaminobenzidine-peroxidase method of visualizing antigen binding sites [46]. The sections were incubated for 48 h at 4 °C in the primary antibody, rabbit anti-Fos (*c-fos* Ab-2, Oncogene Research Products, Cambridge, MA), diluted 1:2000 in PBS with Triton-X and 1% goat serum. Afterwards, they were rinsed, incubated in the secondary antibody (goat anti-rabbit, Vector Laboratories, Burlingame, CA) for 1 hour, and processed using the ABC kit protocol. Stained sections were mounted on gelatin-covered slides, dehydrated in alcohol and xylene, and coverslipped.

2.4. Data acquisition and analysis

Positively labeled Fos-like immunoreactive cells were visualized using a microscope (Leitz with a 50 \times objec-

tive) equipped with a drawing tube, which provides sufficient magnification to identify all Fos-positive cells. Brain nuclei were outlined with the cresyl violet stained sections using an atlas of the rat brain [69]. Immunocytochemistry sections were superimposed on these drawings and all Fos labeled cells were counted bilaterally in the outlined brain nuclei by a person unaware of the treatment groups. For a cell to be considered expressing Fos-like immunoreactivity, it had to be distinct from the background regardless of the intensity of staining. Sections of male-exposed and control animals were matched for corresponding neuroanatomical levels. For each animal the mean number of cell counts per brain area was calculated by averaging counts from all sections. Number of sections varied across areas: bed nucleus of stria terminalis (BNST) medial division anterior part, 2 sections; BNST lateral division, 2 sections; BNST medial division ventral part, 2 sections; paraventricular nucleus of hypothalamus (PVN), 1 section; hippocampus, 6 sections; lateral amygdala, 5 sections; basolateral amygdala, 5 sections; central amygdala, 4 sections; medial amygdala, 3 sections; cortical amygdala, 5 sections; lateral periaqueductal gray (IPAG), 8 sections; ventrolateral periaqueductal gray (vIPAG), 6 sections; locus ceruleus, 2 sections.

Comparisons were only made within age groups because *c-fos* expression changes during ontogeny [48,50]. For 7-, 14- and 21-day-old rats, cell counts of control and male-exposed animals were compared with paired *t*-tests to compare each brain area. In addition, for 21-day-old animals, a one-way ANOVA was conducted to compare control animals, non-freezers and freezers for each area. Fisher's Protected LSD test was used for post-hoc analysis.

Immobility was analyzed with paired *t*-tests at all three ages. In addition, for 21-day-old animals, a one-way ANOVA was used to compare control animals, non-freezers and freezers. To explore associations between brain areas and immobility, Pearson correlations between number of Fos-like immunoreactive cells in all examined areas and levels of immobility were carried out.

3. Results

3.1. Day 7

The rat pups were immobile in the huddle most of the observation time (Fig. 1). There was no difference in immobility between male-exposed and control pups. Fos-like immunoreactive cells were found in all examined areas (Fig. 2) but the number of cells did not differ between male-exposed and control animals.

3.2. Day 14

When the young rats were exposed to the adult male rat, they stopped ongoing behaviors and became immobile. Immobility of male-exposed animals was significantly higher than in control animals (Fig. 1,

t -value = -10.1 , $P < 0.001$). Fos-like immunoreactivity was found in all examined areas (Fig. 3). Significantly more Fos-like immunoreactive cells were found in the following areas of male-exposed rats: the paraventricular nucleus of the hypothalamus (Fig. 4, t -value = -2.5 , $P < 0.05$), the lateral nucleus of the

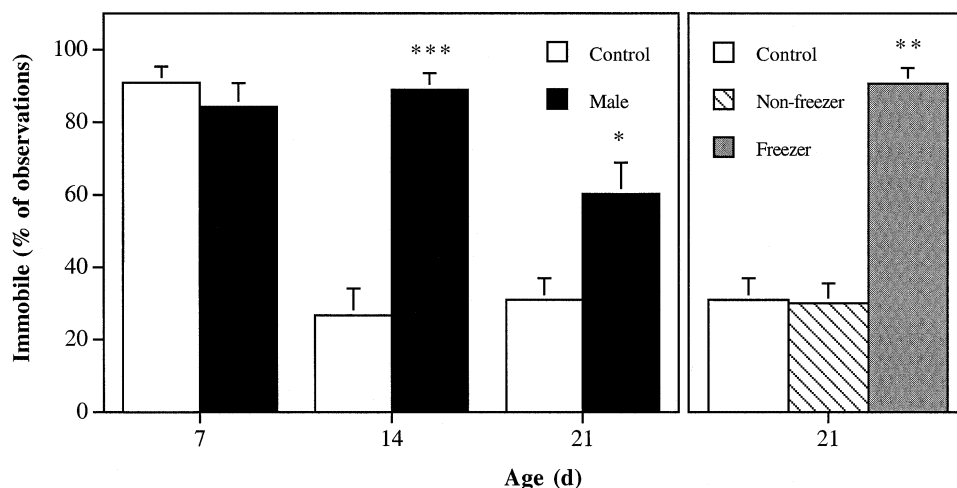


Fig. 1. Development of male-induced immobility in rats during the preweaning period. Columns depict immobility of non-exposed controls and rats that were exposed to an adult male. On day 7, six animals were exposed to the male and six were controls; on day 14, nine each; on day 21, 16 each. On day 21, it was distinguished between rats that did not become immobile (non-freezers, eight animals) and rats that became immobile (freezers, eight animals) in the presence of the male (mean \pm S.E., * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

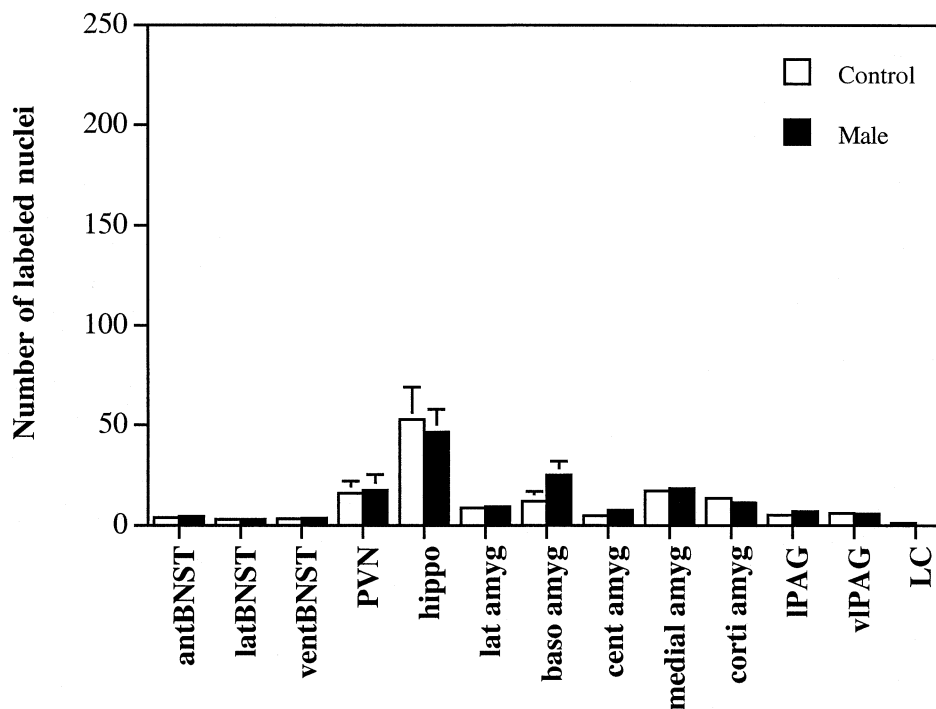


Fig. 2. Number of Fos-like immunoreactive cells in brain areas of rats that were exposed to an adult male and in control rats on postnatal day 7 ($N = 6$; mean \pm S.E.). Numbers represent cell count per area. Abbreviations: antBNST, bed nucleus of the stria terminalis, medial division anterior part; latBNST, bed nucleus of the stria terminalis, lateral division; ventBNST, bed nucleus of the stria terminalis, medial division ventral part; PVN, paraventricular nucleus of hypothalamus; hippo, hippocampus; lat amyg, lateral nucleus of amygdala; baso amyg, basolateral nucleus of amygdala; cent amyg, central nucleus of amygdala; medial amyg, medial nucleus of amygdala; corti amyg, cortical nucleus of amygdala; IPAG, lateral periaqueductal gray; vIPAG, ventrolateral periaqueductal gray; LC, locus ceruleus.

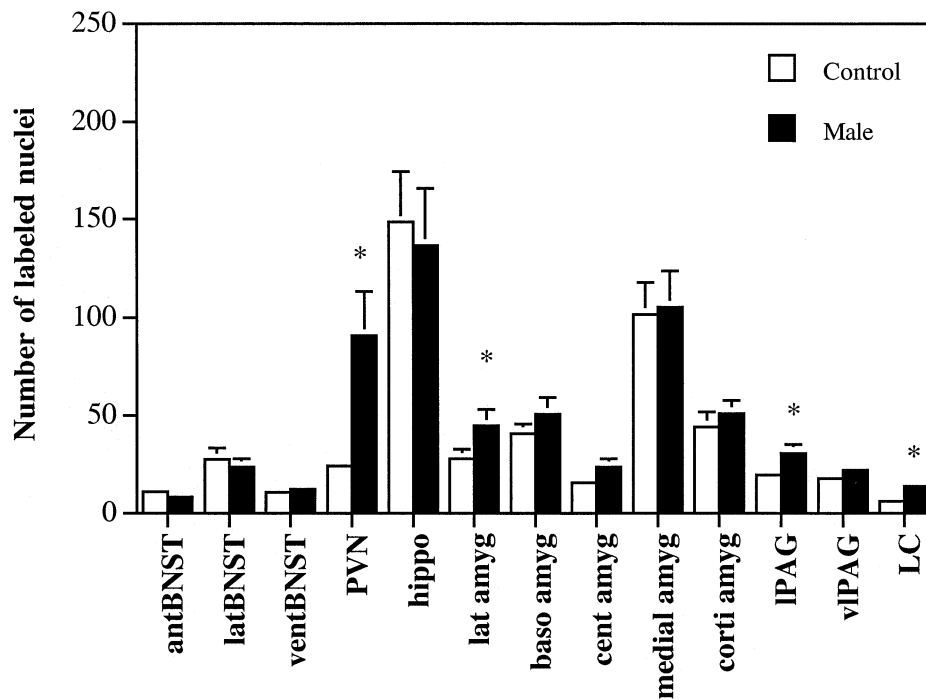


Fig. 3. Number of Fos-like immunoreactive cells in brain areas of rats that were exposed to an adult male and in control rats on postnatal day 14 ($N=9$; mean \pm S.E., * $P < 0.05$). Abbreviations: see Fig. 2.

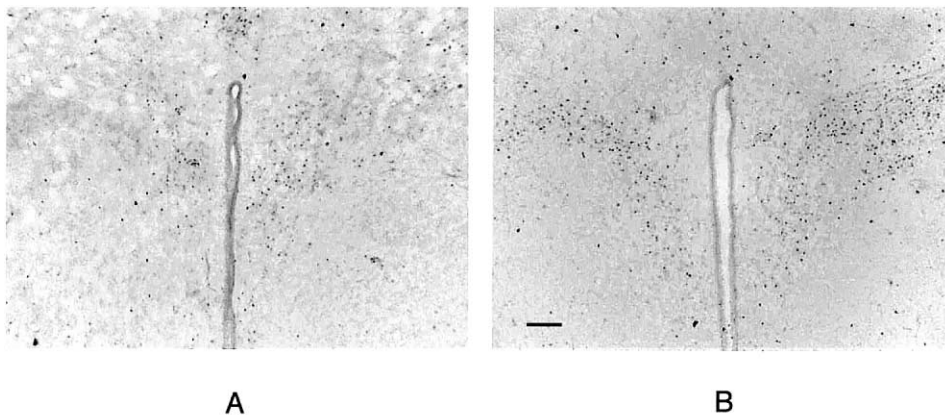


Fig. 4. Photomicrographs showing the distribution of Fos-immunoreactive cells in coronal sections of the paraventricular nucleus of the hypothalamus in 14-day-old rats: a non-exposed control animal (A) and an animal that was exposed to an adult male (B). Bar indicates 200 μ m.

amygdala (t -value = -2.4 , $P < 0.05$), the lateral PAG (t -value = -2.8 , $P < 0.05$), and the locus ceruleus (t -value = -2.7 , $P < 0.05$). Number of Fos-like immunoreactive cells in the lateral PAG were positively correlated with immobility ($r = 0.68$, $P < 0.05$).

3.3. Day 21

Male-exposed rats were significantly more immobile than control rats (Fig. 1, t -value = -2.9 , $P < 0.05$).

When male-exposed animals were divided into non-freezers and freezers, then the three groups differed significantly ($F(2,29) = 26.6$, $P < 0.001$). The non-freezers showed similar immobility levels as the control animals and both were significantly less immobile than the freezers ($P < 0.01$). Fos-like immunoreactivity was first compared between all male-exposed and control rats (Fig. 5). Male-exposed rats had significantly higher Fos-like immunoreactivity in the bed nucleus of the stria terminalis, medial division anterior part (t -value =

– 3.1, $P < 0.01$), the paraventricular nucleus of the hypothalamus (t -value = – 2.1, $P = 0.05$), the medial (t -value = – 3.8, $P < 0.01$) and cortical (t -value = – 2.2, $P < 0.05$) nuclei of the amygdala, and the lateral PAG (t -value = – 2.4, $P < 0.05$). When male-exposed rats were divided into non-freezers and freezers, Fos-like immunoreactivity differed significantly between the three groups for the bed nucleus of the stria terminalis, medial division anterior part ($F(2,23) = 7.3$, $P < 0.01$), the medial nucleus of the amygdala ($F(2,29) = 7.4$, $P < 0.01$) and the locus ceruleus ($F(2,22) = 4.0$, $P < 0.05$), and approached but did not reach statistical significance for the lateral PAG ($F(2,29) = 3.0$, $P < 0.06$). Freezers had significantly more Fos-like immunoreactive cells than control animals in the medial nucleus of the amygdala (Fig. 6, $P < 0.01$), and had more than controls and non-freezers in the bed nucleus of the stria terminalis, medial division anterior part ($P < 0.01$) and the locus ceruleus ($P < 0.05$). Number of Fos-like immunoreactive cells in the locus ceruleus were positively correlated with immobility ($r = 0.73$, $P < 0.01$).

4. Discussion

In preweaning rats, short exposure to an ecologically relevant social stimulus, an adult male rat, elicited immobility and induced *c-fos* expression, indicative for neuronal activation, in an age-specific pattern. Male exposure did not have an effect on 7-day-old pups, but

activated several brain areas in 14- and 21-day-old rats. Although during early ontogeny the growing brain undergoes rapid changes [78], male exposure consistently activated the PVN, the amygdala, the PAG and the locus ceruleus at both ages. However, in 21-day-old rats different nuclei of the amygdala and the BNST were activated. The 21-day-old rats that did not become immobile in the presence of the male did not show such neuronal activation. Therefore, differential activation of neuronal populations may underlie developmental changes in the immobility response.

Basal and stimulus-induced *c-fos* expression in young rats is subject to developmental changes [36,48]. For example, basal expression in the hypothalamus peaks on postnatal day 28 when assessed between days 3 and 35 [47]. Stimulus-induced expression, on the other hand, increases during development. Noxious stimulation and seizure induced higher levels of *c-fos* expression in older than in younger rats [73,91]. Differences in *c-fos* expression across age groups indicate maturational changes in synaptic connections or in stimulus-transcription coupling. Therefore, absolute levels of *c-fos* expression can not be compared across ages. In the following, each brain area is discussed for its possible role in immobility.

Stressors are relayed to the PVN, which activates the hypothalamic-pituitary-adrenal (HPA) stress axis [41]. In young rats, age-specific stressors such as cold stress [5] or maternal separation [55,71,86] activate the HPA axis, which is associated with the induction of *c-fos*

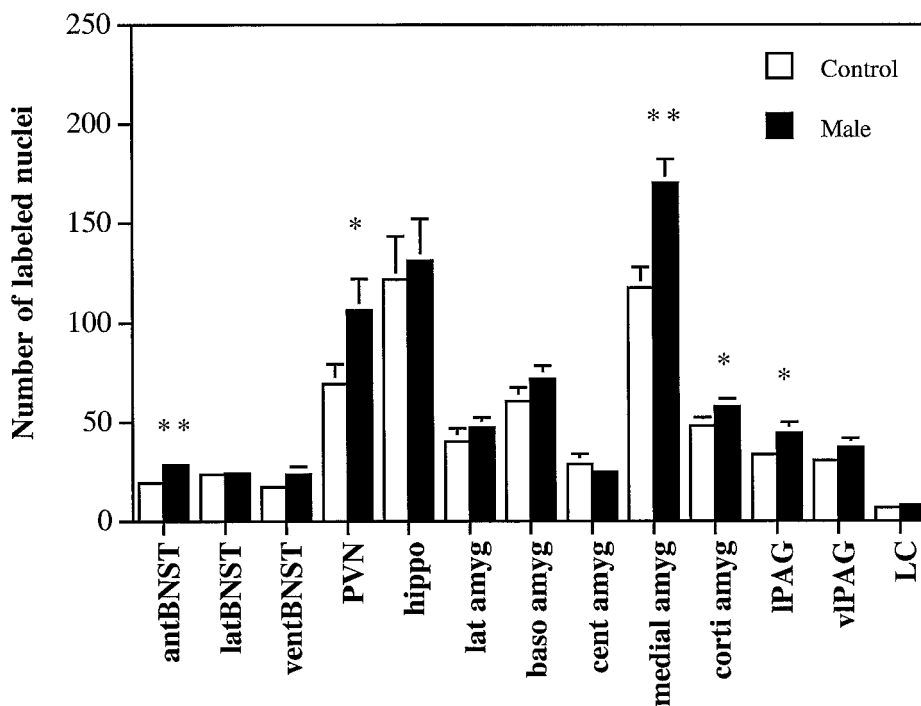


Fig. 5. Number of Fos-like immunoreactive cells in brain areas of rats that were exposed to an adult male and in control rats on postnatal day 21 ($N = 16$; mean \pm S.E., * $P < 0.05$, ** $P < 0.01$). Abbreviations: see Fig. 2.

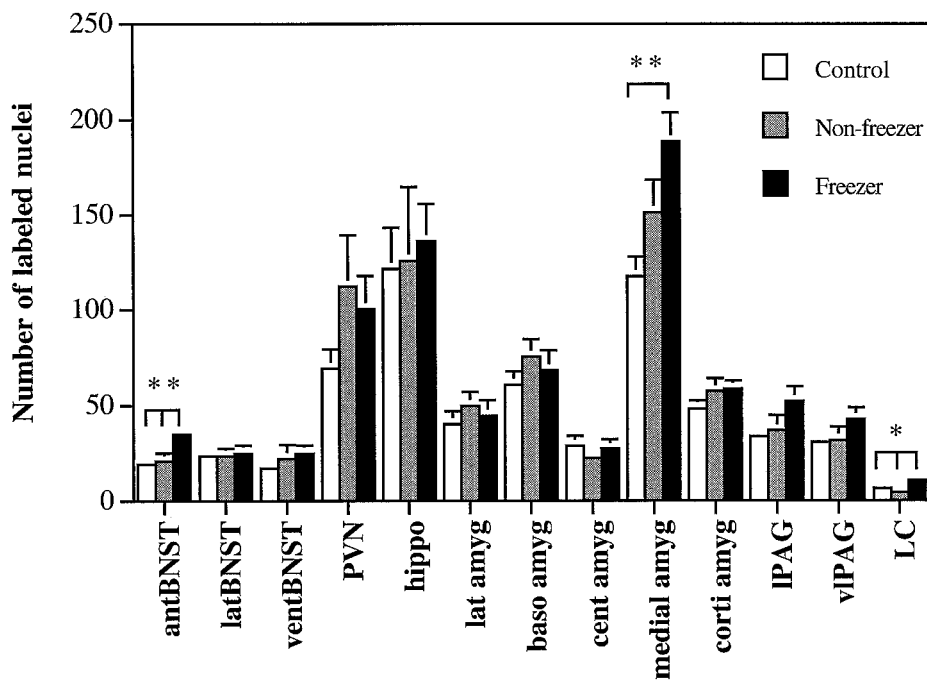


Fig. 6. Number of Fos-like immunoreactive cells in brain areas of rats that were exposed to an adult male and of control rats ($N=16$) on postnatal day 21. Male-exposed rats are divided into animals that were immobile 50% or less of the exposure time (non-freezers, $N=8$) and animals that were immobile more than 50% of the exposure time (freezers, $N=8$; mean \pm S.E., * $P < 0.05$, ** $P < 0.01$). Abbreviations: see Fig. 2.

expression in the PVN [77]. In the present study, 14- and 21-day-old rats exposed to the male had elevated *c-fos* expression in the PVN, which may indicate an activation of the HPA axis at both ages. Seven-day-old pups did not show increased *c-fos* expression in the PVN. Male cues can be effective, however, in inducing stress responses even at a younger age when in closer proximity to rat pups. Five-day-old pups had increased plasma corticosterone levels when placed directly on male-soiled bedding [83]. In adult rats, HPA axis activation and behavioral response to stress occur simultaneously, but the endocrine and behavioral response seem to be mediated by two separate systems [62,70]. It remains to be investigated how PVN activation relates to male-induced immobility in young rats.

The amygdala is crucial for assessing the biological significance of stimuli and in regulating autonomic, endocrine, motor responses, and memory functions [1,10]. Anatomically, the amygdala is organized into several nuclei that play distinctive functional roles [72]. Sensory information converges in the lateral and basolateral nuclei, where synaptic changes encode fear memory [58]. Information is then sent to the central nucleus, which is the major output nucleus for the execution of defensive responses such as immobility [45]. The medial and cortical nuclei receive sensory information from the olfactory pathway and send projections to the hypothalamus and to the PAG [14,60]. Such anatomical and functional distinction of amygdala nuclei is supported

by *c-fos* studies in adult rats. Swim stress, restraint, novelty, foot shock and social defeat all induced *c-fos* expression in the medial amygdala, and to a lesser degree in the central nucleus [20,23,26,28,59]. In contrast to these unconditioned stressors, re-exposure to or avoidance of an environment, in which the animal received foot shocks, induced *c-fos* expression not only in medial but also in lateral, basolateral and, in some cases, central amygdala [6,9,33].

In the present study, male exposure induced *c-fos* expression in the amygdala in an age-specific way. On day 7, *c-fos* expression was not increased, whereas 14-day-old animals had more expression in the lateral amygdala, and 21-day-old rats more in the medial and cortical nuclei than control animals. Male cues seem therefore to activate different amygdala nuclei at different ages. Two maturational processes could contribute to such developmental change. The different nuclei of the amygdala and their connections may become functional in a successive way during the postnatal period. Indeed, it has been shown that functional maturation of the amygdala may underlie developmental changes in aversion learning [79]. According to this hypothesis, the lateral nucleus matures and processes aversive stimuli earlier in ontogeny than the medial and cortical nuclei. One of the rare studies on the early development of *c-fos* expression demonstrated an age-dependent increase but not a change in amygdala activation. When adolescent 28-day-old rats were restrained, they ex-

pressed *c-fos* in the medial and cortical amygdala but at lower levels than young adult rats [49]. The authors interpreted this developmental increase with maturation of interconnections between various brain areas. A similar argument was made for developmental changes in *c-fos* expression to injections of FG-7142, a drug that induces stress-like responses [57]. Therefore, maturational changes in the organization of brain areas and their connections with other areas [78] may underlie developmental differences in *c-fos* activation by male cues. Alternatively, different sensory channels may transmit male cues at different ages. On day 14, the pups have their eyes still closed. It is very likely that they perceive the male by odor cues, because 12-day-old rats made anosmic did not become immobile to a male [76]. On day 21, however, perception of the male could also have involved the visual senses. Therefore, the change from the lateral to the corticomедial component of the amygdala could have been produced either by a change from olfactory to visual input or by a change from olfactory to multiple sensory inputs. Findings in adult rats, however, argue against this interpretation. The main olfactory bulb projects monosynaptically to the corticomедial amygdala [67] and olfactory cues activate mainly the medial nucleus [34]. Also, neurons that receive multiple sensory modalities are located in the lateral and basolateral amygdala [85], which is inverse to the male-induced activation pattern in the present study. How changes in sensory channels relate to changes in the activation of different amygdala nuclei remains to be investigated. Although the amygdala was activated by male cues, the roles of individual subnuclei mediating immobility are unclear. Lesioning or antagonism of neurotransmitter function, which reduce unconditioned and conditioned immobility in the adult rat [30], could provide insight in the role of the amygdala in male-induced immobility in infant rats.

Recently, it has been proposed that the BNST, in contrast to the amygdala, may mediate responses to unlearned aversive stimuli [22]. In support of this view, lesions of the BNST decreased immobility in response to unconditioned loud tones [74]. In another study, however, BNST lesions did neither affect unconditioned behavior in the plus-maze nor conditioned shock-probe burying [84]. In the present study, exposure to an unconditioned stimulus, the male, induced *c-fos* expression in the medial division, anterior part, of the BNST but only in 21-day-old pups that were immobile in the presence of the male. Co-activation of the BNST and the amygdala by male cues is consistent with the view that the BNST is an extension of the amygdala [1,38] and that both structures have a similar role in integrating limbic information [42].

The amygdala and the BNST link sensory systems with brainstem areas such as the PAG [14,21,39], which is involved in executing responses to threat [4]. Cardio-

vascular changes, pain modulation and defensive behaviors are mediated by anatomically distinct neuronal columns of the PAG that extend along the midbrain aqueduct [4]. In adult rats, aversive stimuli induce *c-fos* expression in the ventrolateral and the lateral columns (vlPAG and lPAG). Swim stress, restraint stress and re-exposure to an aversive environment induced *c-fos* expression in the vlPAG [7,15,16,20]. Cat exposure induced expression in the vlPAG and the lPAG [13]. In the present study, male exposure induced *c-fos* expression in the lPAG in both 14- and 21-day-old animals. This activation seems to contribute to male-induced immobility. Number of labeled cells in the lPAG were positively correlated with immobility on day 14, and on day 21 only animals that were immobile in the presence of the male had elevated *c-fos* expression. The role of the different PAG columns in immobility, however, is still controversial. When pharmacologically stimulated, both the lPAG and the vlPAG produce immobility [25,65]. Lesions, instead, are only effective in suppressing immobility in the vlPAG [24,51,56,90], whereas lesions to the dorsal PAG (including the lPAG) are ineffective [51,90] or even increase immobility [24]. Some authors argued that the vlPAG mediates only conditioned immobility because, when rats are re-exposed to an environment previously paired with a shock, they become immobile and express *c-fos* in the vlPAG only [16]. Selective antagonism of neurotransmission within discrete PAG columns may reconcile these contradictory findings.

The locus ceruleus plays a major role in the integration of responses to stressful stimuli [54]. Areas involved in the processing of aversive stimuli such as the BNST, the amygdala and the PAG project to the locus ceruleus [3,11,52]. Accordingly, stressful situations such as forced swimming, restraint or foot shock activated locus ceruleus neurons [20,26]. Besides sending projections to forebrain areas that are involved in attentional processes [2], the locus ceruleus projects to the spinal cord and modulates motoneurons in the ventral horn [32]. These noradrenergic projections have been suggested to mediate behavioral responses to stress, such as immobility, by inhibiting motoneuron excitability [3]. Accordingly, receptor antagonism, which is supposed to block locus ceruleus output, decreased shock-induced immobility [80]. Our findings indicate that male-induced locus ceruleus activation may contribute to immobility. On day 21, the number of Fos labeled cells correlated positively with immobility. In addition, only animals becoming immobile in the presence of the male had elevated *c-fos* expression in the locus ceruleus. As the animals that did not become immobile also responded to the male by approaching him, locus ceruleus activation can not be explained by attentional processes alone.

Neurons in the hippocampus did not express elevated *c-fos* levels. In general, the absence of *c-fos* expression does not necessarily indicate that the neurons have not been activated, because only a subset of activated neurons express a particular type of immediate early genes such as *c-fos* [19,75]. Nevertheless, the hippocampus unexpectedly did not show male-induced *c-fos* expression, although it has been implicated in modulating male-induced immobility in young rats [37,82]. The discrepancy to the present study could be explained by differences in our experimental design, as we tested a huddle of pups and not a single isolated pup. Alternatively, the hippocampus may play a role in immobility, but male exposure may not induce *c-fos* expression.

Taken together, male exposure resulted in an age-specific activation of several brain areas, of which some may have assessed the salience of male cues and others may have contributed to immobility. Male cues may be processed by the olfactory and visual systems and transmitted to the amygdala. Amygdala and BNST output may activate, via the HPA axis, endocrine responses and, via PAG and locus ceruleus, defensive immobility. In some 21-day-old animals, however, male cues did not activate these structures and the animals did not become immobile. At that age, other brain areas may be involved in assessing the salience of male cues and in inducing other behavioral responses such as approaching the male. In conclusion, differential signal processing may underlie changes in a defensive response during early ontogeny.

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