

Gene × Environment Effects: Stress and Memory Dysfunctions Caused by Stress and Gonadal Factor Irregularities during Puberty in Control and TGF- α Hypomorphic Mice

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The maturation of many neural functions occurs during puberty. An abnormal development of these processes, in the context of genetic vulnerability, may result in sex- and age-dependent penetrance of neuropsychiatric disorders. Reduced transforming growth factors- α (TGF- α) expression in *Waved-1* (*Wa-1*) mice impairs the stress response and fear memory in adult males, but are absent or far less prominent in adult females and in pubertal males. Gonadectomy around the onset of puberty, when the mutant anatomical and behavioral phenotypes are undetectable, results in significant gene × environment effects. Adult control males show reduced physiological stress response as a result of gonadectomy, but not adult *Wa-1* males. In females, pubertal gonadectomy elevates specific anxiety parameters only in adult control mice. There also are general sex-specific effects of pubertal gonadectomy on adult stress and fear memory. Surgical stress alone also induces sex- and genotype-dependent effects, albeit in different behavioral parameters than those affected by gonadectomy. We conclude that normal development of stress and memory processes is reliant on the levels of stress and gonadal factors during puberty, the effects of which are modulated by genetic factors and sex.

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INTRODUCTION

The sexual differentiation of the central nervous system (CNS) is determined by the levels of gonadal factors during development (Goy and McEwen, 1980). Although the perinatal gonadal influence is enduring (Stewart and Kolb, 1994; Kolb and Stewart, 1995; Kolb *et al*, 2000; Mong *et al*, 2001), the CNS remains malleable in response to circulating gonadal hormones throughout life, establishing further sexual dimorphism (Giedd *et al*, 1997). In particular, sexual differentiation of neural networks involved in stress homeostasis and memory continue to develop throughout puberty. The sex differences in the corticosterone response to stress begin to emerge late in adolescence, resulting in the highest corticosterone levels in adult female rats compared to males or prepubescent females (Ramaley, 1972; Gabriel

et al, 1992; Cirulli *et al*, 1996). Similarly, sexual dimorphism in the HPA axis response to neuroendocrine stress is detected around the onset of puberty or later in rodents (Spinedi *et al*, 1997). The adult-level proficiency in cognitive abilities, such as working memory and spatial ability (eg mental rotation), also is not achieved until the onset of sexual maturation or early adulthood in primates and humans (Chelune and Baer, 1986; reviewed in Woo *et al*, 1997; Fitch and Denenberg, 1998). In rodents, sex differences in maze learning and avoidance learning also emerge around the time of puberty, the effect of which can be altered by both neonatal and postpubertal gonadectomy (reviewed in Fitch and Denenberg, 1998).

The sexual differentiation of brain is strongly regulated by gonadal hormones, which increase dramatically during puberty in both males and females. Gonadal hormones are necessary to cope normally with adverse environmental situations. In fact, gonadectomy in adult male and female rodents can increase the behavioral stress response (Bernardi *et al*, 1989) and release of ACTH and corticosterone (Handa *et al*, 1994), which can be reversed by subcutaneous injection of testosterone or estradiol/progesterone (Gaskin and Kitay, 1971; Bernardi *et al*, 1989). Estrogen administration alone can increase basal corticosterone secretion and ACTH and corticosterone responses to physical and psychological stressors in both males and females (Burgess and Handa, 1992; Handa *et al*, 1994) and

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enhance memory performance on the radial arm maze, T-maze, and passive avoidance conditioning (Dohanich *et al*, 1994; Luine and Rodriguez, 1994; Singh *et al*, 1994; O'Neal *et al*, 1996; Daniel *et al*, 1997; Luine *et al*, 1998; Sandstrom and Williams, 2001). These studies indicate that the sex differences in stress and memory processes may be partly due to the sex-dependent gonadal hormone availability in the brain even after the normal developmental milestones.

Surprisingly, although many studies have shown the effects of altered levels of gonadal hormones during adulthood, few studies have investigated the long-term effects of pubertal gonadal factor changes on adulthood behavior and resulting vulnerability to a specific genetic challenge, which in combination may lead to neuropsychiatric disorders. Our recent studies have shown that a spontaneous hypomorphic mutation(s) of transforming growth factor- α (TGF- α) in *Waved-1* (*Wa-1*) mice exhibit sex-dependent, post-pubertal onset of anatomical and behavioral abnormalities. TGF- α is a regulator of numerous early developmental processes (reviewed in Junier, 2000). Its expression is developmentally regulated, sensitive to gonadal steroids, and increases around puberty in female hypothalamus (Ma *et al*, 1994a,b), pituitary and adrenal glands (Koshibu *et al*, 2005). In *Wa-1* mice, the lateral ventricles are enlarged and stress response and fear memory are compromised in adult males, but not in pubertal males or female mice (Burrows *et al*, 2000; Koshibu *et al*, 2005). Similarly, the overexpression of TGF- α results in sex-dependent disruptions in stress behavior (Hilakivi-Clarke *et al*, 1992, 1993). These results suggest that an unrecognized progression of abnormal development of stress and memory neural networks during puberty may result in sex-specific penetration of mutant phenotypes.

To investigate the role of gonadal factors in establishing the stress and memory processes during puberty, in the context of genetic vulnerability, male and female mice were gonadectomized around the onset of puberty. We used the paradigm in male and female *Wa-1* mice to determine whether sex-dependent penetrance of a genetic challenge can be modulated by gonadal hormone level changes during puberty, when the mutant phenotype is not detectable. The behavior of these mice was then assessed in adulthood. The results suggest that the physical stress and modulating levels of gonadal factors during puberty have enduring long-term effects on stress and memory functions in sex- and genotype-dependent manner.

MATERIALS AND METHODS

Animals

Male and female, control and *Wa-1* C57Bl/6^J mice were used. The control group included both homozygous and heterozygous littermates of *Wa-1* mice, because the heterozygotes did not show any abnormal phenotype in the parameters studied. Heterozygous mating pairs were purchased from Jackson Laboratory (Bar Harbor, ME) to establish the colony. All experiments utilized animals obtained through timed mating. Simple sequence repeat PCR was used to genotype the animals as described previously (Koshibu *et al*, 2005). Because *Wa-1* has naturally wavy hair and whiskers from birth to adulthood,

the genotype of *Wa-1* homozygotes was visually confirmed by this phenotype.

All mice were group-housed and maintained in a central facility at University of Pittsburgh School of Medicine in a controlled environment with a 12 h light/dark cycle and free access to food and water. Experiments were performed in accordance with the guidelines provided by University of Pittsburgh Institutional Animal Care and Use Committees (IACUC) and the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Surgical Treatments

Male and female, control and *Wa-1* C57Bl/6^J mice were gonadectomized before or around the onset of puberty (P26 to 30). The secondary sexual characteristic of the onset of puberty includes descending of testis in males (Clermont and Perey, 1957) and vaginal opening in females (Crane *et al*, 1972; Falconer, 1984). Even in inbred mouse strains, there is individual variability in the actual age at which puberty begins. In our study, the secondary sexual traits begin to appear around or after the generally accepted times of P26 to 30. We use this period to estimate the approximate age when puberty begins in mice (Clermont and Perey, 1957; Crane *et al*, 1972; Falconer, 1984; Omoto *et al*, 2005). The gonadectomy in males and females were conducted as described in Hogan *et al* (1994) and Fugger *et al* (2000), respectively. For males, a 1-cm-transverse incision was made close to the ventral midline. The testicular fat pad and testis were exposed. The vas deferens were isolated, tied with a piece of 5-0 surgical silk suture (Henry Schein, Melville, NY), then cut to completely remove the testis. For females, two small vertical incisions were made on either sides of the spinal cord. The ovarian fat pad, ovary, and oviduct were exposed. The oviduct was isolated, tied with a 5-0 surgical silk suture, and then cut to remove the ovary. The uterine horn was placed back into the abdomen. Sham surgery involved similar incisions of the skin and peritoneal wall. The procedures were conducted using sterile instruments under aseptic conditions. For anesthetic, 0.015 ml per gram body weight of 2.5% Avertin (2,2,2-tribromoethanol; Aldrich Chemicals Co., Milwaukee, WI) was injected into peritoneal cavity. Mice were allowed to recover on a heating pad after surgery. Ten control and eight *Wa-1* males were gonadectomized. Ten control and seven *Wa-1* males were sham-operated. Thirteen control and nine *Wa-1* females were gonadectomized. Ten control and six *Wa-1* females were sham-operated.

Behavior

All behavioral experiments were conducted in adult mice (3–4.5 months). The protocols have been described previously in detail (Koshibu *et al*, 2005). Briefly, general activity and anxiety were tested by open-field activity test. Mice were placed in a TruScan Photobeam Activity System (Coulbourn Instrument, Allentown, PA) for 10 min and the total movement time and distance, marginal zone time, stereotypy, and number of rears were determined. The stereotypic activity included repetitive behaviors, such as

head bobbing, grooming, and paw-licking. Light/dark choice test was used as an additional test to determine the anxiety level. The TruScan Photobeam Activity System was divided into light and dark areas and the total time spent on the dark side of the arena over 10 min were calculated. Stress-induced hyperthermia was conducted to determine the stress-associated physiological changes (Zethof *et al*, 1994). In brief, mice were held in one hand and its rectal temperature was measured for 20 s (T1) using a Thermalert TH-5 apparatus (PhysiTemp, Clifton, NJ). The mouse was then returned to the home cage for 10 min, after which, rectal temperature was measured again for 20 s. The change in temperature (dT) was calculated to determine the stress response. As originally described, the acute stressor in this paradigm is the brief restraint and insertion of the rectal probe.

Delay fear conditioning consisted of three pairs of tone (30 s continuous, 80 dB, 2000 Hz) and foot shock (−0.2 mA for 2 s, overlapping the last 2 s of tone) with inter-trial interval of 1 min. Memory performance was assessed by determining the freezing response of mice during training and context and tone tests, executed approximately 24 h after the training. A lack of movement lasting longer than 1 s was considered as a freezing response (freeze time/total test time × 100%). The shock sensitivity also was assessed by measuring the speed of movement during foot shocks (distance moved divided by 2 s). The freezing response during the 1 min inter-trial interval will be referred to as unconditioned response context (UR context) and the tone during the training as UR tone.

We used vaginal color and opening as reliable and accurate measures of estrous cycle (Hogan *et al*, 1994). We did not utilize vaginal smears, to avoid any possible acute or chronic stress caused by this mode of sampling. We note that in all postpubertal females, none of the parameters was affected by the estrous cycle (data not shown). The onset of puberty was not altered in *Wa-1* males or females as determined by the physiological changes in the secondary sex properties described in Hogan *et al* (1994).

Corticosterone Radioimmuno Assay (RIA)

Trunk blood samples (~200–600 µl) were collected in microcentrifuge tubes with 20 µl 1000 USP U/ml heparine sodium (American Pharmaceutical Partners, Inc., Los Angeles, CA) and placed on ice. Plasma was collected by spinning the samples at approximately 1500 × g for 15 min at 4°C and stored at −80°C. The corticosterone levels were determined using Coat-A-Count Rat Corticosterone kit (Diagnostic Products Corporation, Los Angeles, CA) accordingly to the manufacturer's protocol. For the baseline level, blood was collected after an overnight individual housing with free access to food and water. For the stressed level, blood was collected immediately after the stress-induced hyperthermia test. The animals were terminated by decapitation. All samples were collected in the morning during the light-phase of the light/dark cycle, between 0830 and 1030 h.

Statistics

A multivariate generalized linear model (GLM) and type III Sum of Squares were conducted to assess main effects of

genotype and treatment and their interactions for males and females. Where appropriate, Tukey HSD *posthoc* was applied. The treatment groups included non-surgical (ns), sham (s), and gonadectomized (gx) groups. For fear conditioning training responses, repeated measures GLM was used. The context and pre-tone freezing responses were assessed by using the baseline freezing response during training as a covariant to normalize for any general activity differences. For tone test, the training baseline and pre-tone responses were used as covariant. Similarly, the stressed level of corticosterone was normalized to baseline corticosterone level. Any result indicating $p < 0.05$ was considered statistically significant. All data are presented as estimated marginal means ± SEM.

RESULTS

Open-Field Activity

In males, the number of rears was decreased in sham group compared to non-surgical group ($F(2,62) = 10.934$, $p < 0.001$) (Table 1). No differences between genotypes or treatment groups were detected for other parameters for males. In females, total move time ($F(1,54) = 12.209$, $p = 0.001$), total move distance ($F(1,54) = 14.489$, $p < 0.001$), and rear number ($F(1,54) = 5.291$, $p = 0.026$) were elevated in *Wa-1* compared to controls (Table 1). The stereotypy was reduced in *Wa-1* compared to control females ($F(1,54) = 32.317$, $p < 0.001$). When the treatment groups were compared, the total move distance was reduced ($F(2,54) = 6.563$, $p = 0.003$, Tukey HSD $p = 0.001$) while the marginal zone time and stereotypy were increased ($F(2,54) = 4.569$, $p = 0.015$, Tukey HSD $p = 0.012$; $F(2,54) = 10.426$, $p < 0.001$, Tukey HSD $p < 0.001$) in gonadectomized females compared to sham surgery females (Table 1). Neither males nor females exhibited a genotype-dependent treatment effect.

Light/Dark Choice Test

The gonadectomized males spent more time in the dark side than non-surgical males, but not compared to sham operated males ($F(2,52) = 3.545$, $p = 0.037$, Tukey HSD $p = 0.04$) (Table 1; Supplementary Figure 1a). In females, there was a genotype-dependent treatment effect ($F(2,52) = 3.912$, $p = 0.027$). Gonadectomized control females showed increased total dark side time compared to sham-operated control females (419.4 ± 20.4 vs 334.2 ± 22.3 , respectively) (Supplementary Figure 1b), where as *Wa-1* females were not affected by gonadectomy. This change in the behavior of gonadectomized control females resulted in a genotype difference, where gonadectomized controls spent more time in dark side than gonadectomized *Wa-1* females (419.4 ± 20.4 vs 325.0 ± 22.3).

Stress-Induced Hyperthermia Test

In males, there were no genotype- or treatment-dependent changes in the baseline temperature, T1 (Figure 1a). When the stress-induced temperature change, dT, levels were compared, genotype-dependent treatment effect was detected ($F(2,55) = 6.846$, $p = 0.002$) (Figure 1b). Sham surgery

Table 1 Open Field Activity and Light/Dark Choice Test

		Move time (s)	Move distance (cm)	MZ time (s)	Rears	Stereotypy	Dark time (s)
<i>Males</i>							
Genotype	c	439.5±5.0	2309.0±69.5	299.7±9.2	78.5±3.5	37.4±1.1	361.8±13.2
	w	449.2±6.3	2399.9±86.3	315.6±11.4	69.9±4.4	37.2±1.4	325.3±14.2
Treatment	ns	454.7±5.7	2479.1±78.3	294.3±10.4	89.8±4.0	36.6±1.3	318.6±15.9
	s	440.8±7.9	2376.6±109.2	296.4±14.4	71.8±5.5*	35.9±1.8	332.7±17.1
	gx	437.7±7.1	2207.6±97.8	332.3±12.9	60.9±5.0	39.4±1.6	379.3±17.3†
<i>Females</i>							
Genotype	c	414.6±4.9	2037.8±62.6	340.9±10.6	53.8±2.9	43.4±1.1	355.0±12.8
	w	440.3±5.5*	2397.1±70.6*	323.8±12.0	64.0±3.3*	34.2±1.2*	333.7±14.7
Treatment	ns	429.3±6.0	2170.3±77.7	332.8±13.2	64.4±3.6	38.6±1.3	318.2±17.1
	s	433.0±7.1	2454.8±91.5	302.0±15.5	61.8±4.3	34.4±1.6	342.6±18.2
	gx	419.9±5.8	2027.3±75.1*	362.3±12.7*	50.5±3.5†	43.5±1.3*	372.2±15.1

* $p < 0.05$ comparing c and w, ns and s, or s and gx.

† $p < 0.05$ comparing ns and gx.

All data are indicated in mean±SEM.

caused elevation of dT in *Wa-1* males (1.9 ± 0.2 s, 1.2 ± 0.1 ns) but not in control males (1.8 ± 0.2 s, 1.8 ± 0.1 ns). In contrast, gonadectomy reduced dT response in control males (1.5 ± 0.2 gx, 1.8 ± 0.2 s) but not in *Wa-1* males (1.8 ± 0.2 gx, 1.9 ± 0.2 s). The non-surgical *Wa-1* males showed reduced dT, whereas the gonadectomized *Wa-1* males showed elevated dT compared to respective controls (Figure 1b).

In females, sham operation increased the T1 level compared to the non-surgical group (37.0 ± 0.2 s, 35.8 ± 0.1 ns; $F(2,62) = 21.824$, $p < 0.001$, Tukey HSD $p < 0.001$) (Figure 1c). Interestingly, gonadectomy caused a significant reduction in T1 (Tukey HSD $p < 0.001$) to the level same as the non-surgical groups (35.8 ± 0.1 gx). When the stress response was compared, the dT was greater in gonadectomized females than in sham-operated females (1.8 ± 0.1 gx, 0.8 ± 0.1 s; $F(2,62) = 11.151$, $p < 0.003$) (Figure 1d). Increased stress response in gonadectomized females are in agreement with the open field activity and light dark choice test findings, which suggests that female gonadal factors are important in stress regulation for both control and mutant females. The genotype difference was detected only for the non-surgical group, where control females showed a higher dT level than *Wa-1* females (Figure 1d).

Fear Conditioning Training

In males, *Wa-1* males responded less to the foot shocks ($F(1,55) = 10.501$, $p = 0.002$) and unconditioned context ($F(1,58) = 4.755$, $p = 0.034$) than control males, independent of the treatment groups (Table 2). Both unconditioned tone and context responses were increased in gonadectomized males compared to sham-surgery males ($F(2,58) = 8.204$, $p = 0.001$ UR tone, $F(2,58) = 5.855$, $p = 0.005$ UR context) (Table 2).

Similar to *Wa-1* males, *Wa-1* females responded less to the foot shocks ($F(1,52) = 10.626$, $p = 0.002$) and unconditioned context ($F(1,51) = 5.131$, $p = 0.028$) than control females across all treatment groups (Table 2). Gonadectomy caused an increased foot shock response ($F(1,52) = 7.467$,

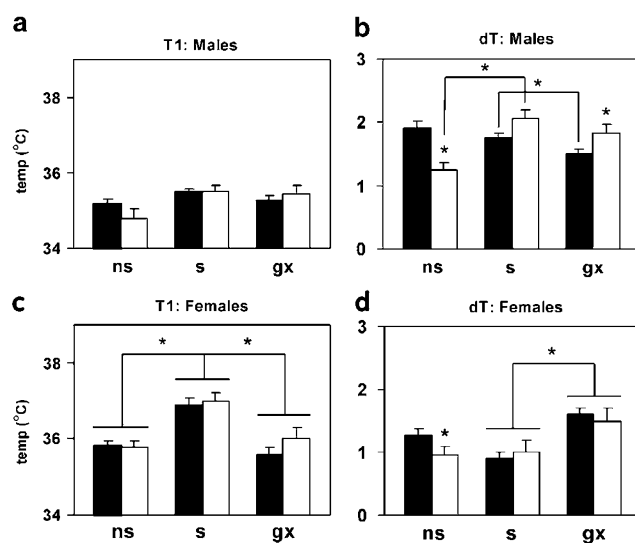


Figure 1 Physiological stress response. The baseline temperature (T1) and stress-induced change in temperature (dT) are indicated for males (a and b) and females (c and d). The genotype and treatment specific effects are detected for dT in males. Gonadectomy reduces the dT level in control males but not in *Wa-1* males. The dT level is increased for both control and *Wa-1* gonadectomized females. Sham surgery alone eliminates the genotype differences originally observed for the non-surgical groups. Control groups are presented as black bars and *Wa-1* groups are presented as white bars. Statistical significant changes ($p < 0.05$) are marked with asterisks. ns = non-surgery group, s = sham-surgery group, gx = gonadectomy group.

$p = 0.002$) and unconditioned context response ($F(2,51) = 8.242$, $p = 0.001$) compared to sham-operated females.

Fear Memory

In general, *Wa-1* males exhibited reduced context memory compared to control males ($F(1,58) = 14.698$, $p < 0.001$). The adult control males showed reduced context memory as a result of surgery around the onset of puberty, but not in adult *Wa-1* males. Gonadectomy caused enhanced context

memory compared to sham surgery, irrespective of genotype ($F(2,58) = 5.443$, $p = 0.007$) (Figure 2a). Perhaps, as a generalized effect of the context memory, pretone response also was reduced in *Wa-1* compared to control males in general ($F(1,58) = 9.891$, $p = 0.003$) and sham-surgery resulted in a reduction in pretone response ($F(2,58) = 5.452$, $p = 0.007$) (Figure 2b). In contrast, sham-surgery enhanced the tone memory ($F(2,54) = 5.398$, $p = 0.008$) (Figure 2b). The only genotype difference in tone memory was for the non-surgical groups where *Wa-1* males exhibited reduced tone memory compared to control males.

In females, neither context or tone memory were affected by the genotype or treatments. The only change was in the pretone response, where sham surgery caused a reduction in the pretone response compared to the non-surgical females, regardless of the genotype ($F(2,58) = 5.083$, $p = 0.01$).

Corticosterone

In males, both baseline and stressed levels of plasma corticosterone were elevated in the gonadectomized groups compared to the sham-surgery groups in general ($F(2,42) = 11.625$, $p < 0.001$, Tukey HSD $p = 0.008$ baseline, $F(2,36) = 144.616$, $p < 0.001$ stressed) (Figure 3a). In addition, *Wa-1* males showed a higher stressed level of corticosterone compared to control males in general ($F(1,36) = 13.753$, $p = 0.001$). In females, sham surgery elevated the stressed level of corticosterone compared to non-surgical levels ($F(2,40) = 9.096$, $p = 0.001$) (Figure 3b).

DISCUSSION

The regulation of neural functions by gonadal and stress factors are interdependent. In this study, we aimed to determine, in a genetically vulnerable state, the effect of altered levels of gonadal factors during puberty on adult stress and memory functions. Thus, the studies were designed to compare the effects of such manipulation in

animals carrying a genetic mutation, which causes sex-specific, adult-onset anatomical, behavioral, and cognitive phenotypes that are reminiscent of certain human psychiatric disorders.

One of the most important findings of our studies to emphasize is that the effects of gonadectomy around the onset of puberty are genotype and sex dependent. Specifically, the alterations in gonadal factor milieu can change the regulatory functions of TGF- α on the maturation of stress, anxiety, and memory-related processes. For example, pubertal gonadectomy resulted in reduced stress-related responses and contextual memory in adult control males, but not in adult *Wa-1* males compared to their respective sham groups (Supplementary Table 1). In fact, the physiological stress response, measured using stress-induced hyperthermia, in *Wa-1* males was significantly elevated compared to controls as a result of pubertal gonadectomy. Interestingly, pubertal gonadectomy in females seem to affect the baseline anxiety/stress behavior in adult females more than in adult males, while the memory functions in adult females seem to remain intact but not in males (Supplementary Tables 1 and 2). This trend of greater anxiety/stress parameters being affected in females compared to males is reminiscent of our previous findings that the female *Wa-1*, in particular, showed greater number of hypothalamic neurochemical changes compared to male *Wa-1* (Koshibu and Levitt, 2006), suggesting that the stress homeostasis are more readily regulated by female hormones. Therefore, the study in this model system demonstrates important gene \times environment interactions regarding the expression of atypical cognition and behavior after puberty. Thus, the model system we utilized here may be useful for investigating further the underlying neurobiological basis of neuropsychiatric disorders.

The integral effects of gonadal steroid hormones and altered TGF- α expression on stress behavior have been reported previously in TGF- α -overexpressing transgenic mice. Gonadectomy of TGF- α transgenic male and female mice cause reduced immobility during Porsolt's swim test, whereas it increased the immobility duration in CD-1

Table 2 Fear Training (% Freezing Response)

		Baseline	UR tone	UR context	Shock response
<i>Males</i>					
Genotype	C	4.4 \pm 0.5	19.4 \pm 1.6	35.8 \pm 1.7	14.4 \pm 0.5
	w	2.9 \pm 0.6	18.2 \pm 2.0	29.5 \pm 2.2*	11.9 \pm 0.6*
Treatment	ns	4.2 \pm 0.6	15.4 \pm 1.9	30.6 \pm 2.1	12.6 \pm 0.6
	S	3.2 \pm 0.8	15.1 \pm 2.4	28.1 \pm 2.6	13.2 \pm 0.7
	gx	3.5 \pm 0.7	25.9 \pm 2.2*	39.3 \pm 2.4*	13.6 \pm 0.7
<i>Females</i>					
Genotype	C	3.8 \pm 0.3	26.5 \pm 1.6	46.5 \pm 1.7	15.7 \pm 0.6
	w	4.0 \pm 0.4	26.7 \pm 2.4	40.3 \pm 2.2*	12.7 \pm 0.7*
Treatment	ns	4.3 \pm 0.4	26.4 \pm 2.3	40.4 \pm 2.2	12.9 \pm 0.8
	S	3.3 \pm 0.5	24.4 \pm 2.8	38.7 \pm 2.7	13.1 \pm 0.9
	gx	4.1 \pm 0.4	29.0 \pm 2.3	51.2 \pm 2.3*	16.7 \pm 0.8*

* $p < 0.05$ comparing c and w, ns and s, or s and gx.

All data are indicated in mean \pm SEM.

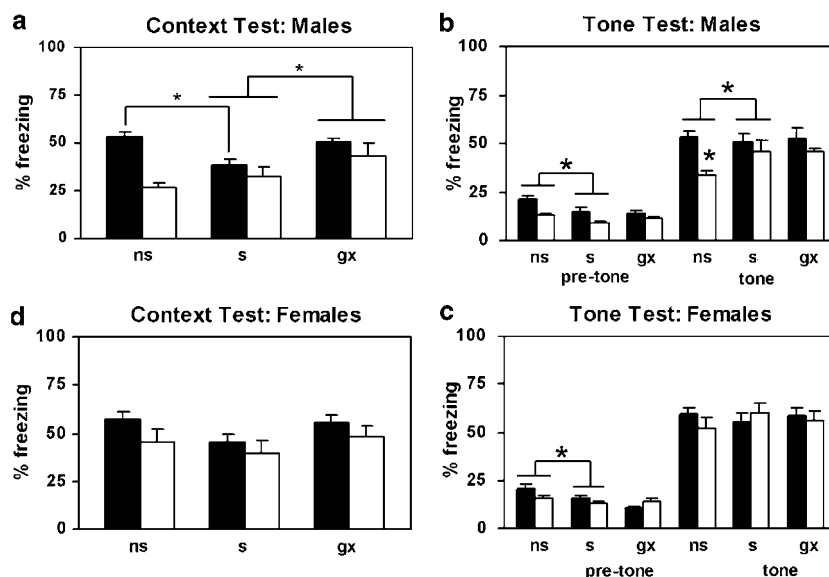


Figure 2 Context and tone fear memory. The context and tone memory performance are presented for males (a and b) and females (c and d). Gonadectomy improves memory for context in males in general. Sham surgery has sex- and genotype dependent effect on both context and tone memory. The context memory and pretone response are lower in *Wa-1* males compared to control males independent of the treatment (Supplementary Table 1). Control groups are presented as black bars and *Wa-1* groups are presented as white bars. Statistical significant changes ($p < 0.05$) are marked with asterisks. ns = non-surgery group, s = sham-surgery group, gx = gonadectomy group.

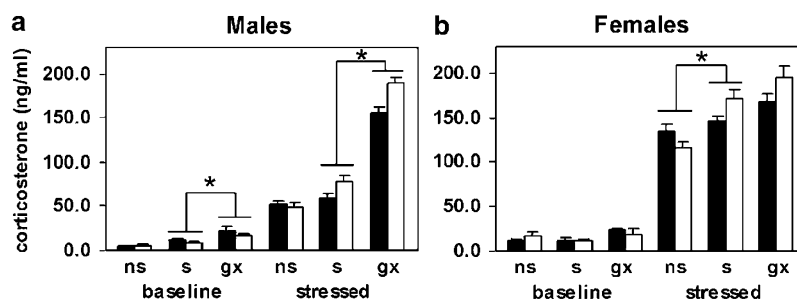


Figure 3 Baseline and stressed corticosterone levels. The corticosterone levels for males (a) and females (b) are indicated. Gonadectomy increases the baseline and stressed levels of corticosterone in males. Sham surgery elevates stressed level of corticosterone in females. No genotype differences are detected. Control groups are presented as black bars and *Wa-1* groups are presented as white bars. Statistical significant changes ($p < 0.05$) are marked with asterisks. ns = non-surgery group, s = sham-surgery group, gx = gonadectomy group.

control males (Hilakivi-Clarke, 1994). However, the genotype difference was altered only in males and not in females (Hilakivi-Clarke, 1994). In addition, there are sex-dependent tumor formation differences in TGF- α transgenic mice that suggest gonadal hormone involvement in TGF- α -dependent stress regulation (Takagi *et al*, 1992). These results are consistent with an interaction between gonadal steroid hormones and TGF- α in sex-specific stress regulation and memory performance.

Gonadectomy, in general, elevated specific anxiety and stress-response parameters particularly in females in our study. In females, the effect of pubertal gonadectomy on stress and anxiety are prominent in behavioral parameters but not in corticosterone levels. We hypothesize that the lack of significant change in corticosterone levels may be due to a ceiling effect of the corticosterone response. Alternatively, it is possible that other parameters of the corticosterone response might be altered, such as the time of onset or duration of the increase. A full analysis of the corticosterone response over time would be necessary

to determine such changes. In contrast, in males, the behavioral anxiety effect of gonadectomy is less pronounced, whereas the corticosterone levels are significantly elevated by gonadectomy. These results indicate a possible dissociation between the corticosterone level and physiological and behavioral stress levels in sex-dependent manner. The observed elevation in stress related behavioral and biochemical changes as a result of gonadectomy are in agreement with previous reports in adult rodents (Ray and Hansen, 2004; Seale *et al*, 2004).

The memory performance is also affected as a result of gonadectomy in males but not in females, reflecting the sex specific effect of pubertal gonadal factors on adult memory performance. The fact that only context memory and not the tone memory was affected indicates that this effect is memory type specific. The contextual memory is reverted to the non-surgical levels in control males after gonadectomy, indicating that the male gonads may hinder stress responses but facilitate contextual memory. However, the fact that the auditory memory is not affected by sham

surgery or gonadectomy treatment suggests that the tone memory is less sensitive to male gonadal factors. Other studies have shown that castration in adult rats can reduce inhibitory avoidance task learning and increase anxiety (Bitran *et al*, 1993; Vazquez-Pereyra *et al*, 1995; Frye, 2001; Frye and Seliga, 2001). The reasons for the discrepancies between previously reported studies and our study may be due to the timing of gonadal factor manipulation. The surgical manipulations in other studies are conducted in adulthood whereas our manipulations were performed around the onset of puberty. Thus, the effect of peripubertal gonadal manipulations may be compensated differently in adulthood.

The findings on gonadectomy in our model were confounded by the pubertal surgical stress (sham) effects, which alone induced sex- and genotype-dependent behavioral alterations in adult mice (summarized in Supplementary Tables 1 and 2). The specific mechanisms responsible for the long-term effects of stressful events experienced during adolescence are unknown. During adolescence, many systems, including glutamatergic (Huttenlocher, 1984; Insel *et al*, 1990), serotonergic (Dinopoulos *et al*, 1997), dopaminergic (Seeman *et al*, 1987; Lidow *et al*, 1991; Lidow and Rakic, 1992; Teicher *et al*, 1995; Tarazi *et al*, 1998, 1999), and GABAergic systems (Lidow and Rakic, 1992; Kellogg *et al*, 1993; Nurse and Lacaille, 1999), undergo synaptic pruning. Furthermore, prominent developmental anatomical transformations are evident in the prefrontal cortex and limbic brain regions of adolescents across species (reviewed in Spear, 2000). Interestingly, forebrain structures essential for cognitive functions and stress homeostasis, including prefrontal cortex, hippocampus, amygdala, and hypothalamus, possess estrogen as well as androgen receptors (reviewed in Janowsky, 2006). These receptors allow the structures to be responsive to both estrogen and testosterone. For instance, cyclical changes in dendritic spine morphology can be observed under the control of circulating estrogen levels (Woolley *et al*, 1996) and this can result in sex-dependent differences in spine density (Shors *et al*, 2001) in hippocampus. In addition, there are indications that the manipulation of gonadal hormones can result in altered levels of NE, DA, 5-HT, glutamate and GABA in prefrontal cortex and hippocampus in a gonadal hormone- and structure-dependent manner (Luine *et al*, 1998; Bowman *et al*, 2004). Given these significant effects of gonadal hormones, there are likely to be many neural changes that can occur in these brain structures, in addition to the gonadal-independent developmental changes. Thus, these maturation processes of neural systems during the time of significant gonadal hormone flux in adolescence may, in part, underlie the sensitivity and permanency of the environmental influences during this period of development on adult behavior.

We also noted the novel finding that the effect of stress at puberty differs depending on genetic background, indicating that the environmental factors and genetic disposition are both crucial for the development and detection of abnormal neural functions. For some parameters, pubertal stress appears to ameliorate the mutant phenotype of the *Wa-1* genetic challenge in adulthood. For example, the sham surgery in *Wa-1* males elevated their physiological stress response compared to non-surgical *Wa-1* males, but this did not occur in control males. In addition, context memory

is specifically reduced as a result of pubertal surgical stress in control males, but not in *Wa-1* males. The results are in agreement with the previous studies indicating emotional learning and memory are modulated by the stress level of animals (Maier, 1990; Shors and Servatius, 1995; Kirschbaum *et al*, 1996; Roozendaal and McGaugh, 1996; Sandi *et al*, 1997; Shors and Servatius, 1997; de Quervain *et al*, 1998; Buchanan and Lovallo, 2001). Moreover, differential pathophysiological effects of environmental factors can be dependent on the genetic predisposition for some human psychiatric disorders, such as depression (Kramer, 2005; Rutter, 2005; reviewed in Rutter *et al*, 2003, 2006a,b). We suggest that the *Wa-1* mutant mouse will serve as an important model for understanding the developmental and gene \times environment-interaction mechanisms that result in increased disease vulnerability in adulthood.

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DISCLOSURE/CONFLICT OF INTEREST

We declare that, except for income received from our primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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