

Social isolation dysregulates endocrine and behavioral stress while increasing malignant burden of spontaneous mammary tumors

Gretchen L. Hermes^{a,b}, Bertha Delgado^{a,c}, Maria Tretiakova^{a,c}, Sonia A. Cavigelli^a, Thomas Krausz^{a,c}, Suzanne D. Conzen^{a,d}, and Martha K. McClintock^{a,b,e,1}

^aInstitute for Mind and Biology and Departments of ^bComparative Human Development, ^cPsychology, ^dPathology, and ^eMedicine, The University of Chicago, Chicago, IL 60637

Communicated by Joseph S. Takahashi, University of Texas Southwestern Medical Center, Dallas, TX, October 14, 2009 (received for review March 1, 2009)

AQ: A

In a life span study, we examined how the social environment regulates naturally occurring tumor development and malignancy in genetically prone Sprague–Dawley rats. We randomly assigned this gregarious species to live either alone or in groups of five female rats. Mammary tumor burden among social isolates increased to 84 times that of age-matched controls, as did malignancy, specifically a 3.3 relative risk for ductal carcinoma in situ and invasive ductal carcinoma, the most common early breast cancers in women. Importantly, isolation did not extend ovarian function in late middle age; in fact, isolated animals were exposed to lower levels of estrogen and progesterone in the middle-age period of mammary tumor growth, with unchanged tumor estrogen and progesterone receptor status. Isolates, however, did develop significant dysregulation of corticosterone responses to everyday stressors manifest in young adulthood, months before tumor development, and persisting into old age. Among isolates, corticosterone response to an acute stressor was enhanced and recovery was markedly delayed, each associated with increased mammary tumor progression. In addition to being stressed and tumor prone, an array of behavioral measures demonstrated that socially isolated females possessed an anxious, fearful, and vigilant phenotype. Our model provides a framework for studying the interaction of social neglect with genetic risk to identify mechanisms whereby psychosocial stressors increase growth and malignancy of breast cancer.

breast cancer | glucocorticoids | physiological stress | psychological stress | social behavior

Fn1

A growing body of basic research and clinical studies suggest that stress and other psychosocial variables including low social support and chronic social isolation contribute to the cancer progression (1–3). Specifically, dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis is linked to breast cancer mortality, predicted by disrupted cortisol diurnal rhythms in women with metastatic disease (4).

AQ: B

Within a conceptualized framework of stress and disease, bio-behavioral factors are understood to influence multiple aspects of tumor growth including apoptosis, angiogenesis, invasion, and immunological escape to the metastatic cascade (5). As one example, transferring laboratory mice from group housing to social isolation accelerates growth of induced tumors and attenuates the effects of chemotherapy (6, 7). Prolonged exposure to the synthetic glucocorticoid dexamethasone has both mutagenic and clastogenic effects (8, 9). In the Sprague–Dawley rat model of naturally occurring breast cancer, the magnitude of the glucocorticoid stress response was associated with mammary tumor onset, whereas time to hormonal recovery from a stressor predicted growth rate of tumors (10).

Cellular mechanisms whereby the HPA axis could regulate cancer growth have been established in human breast cancer cell lines and in xenograft models. Glucocorticoid receptor (GR)-mediated survival mechanisms are induced by prolonged exposure to physiological concentrations of glucocorticoids and

inhibited by GR specific antagonists (11). GR activation and resultant gene expression changes inhibit apoptosis in human breast cancer cell lines treated with clinically appropriate concentrations of a chemotherapeutic agent commonly used for treating human breast cancer—paclitaxel (12).

As in humans, animals living in the wild spontaneously develop benign and malignant tumors (13) and so may serve as a powerful model of the lifelong dynamic interplay between the psychosocial environment, physiology and genetic mechanisms that increase cancer risk. To date, most rodent models have been constrained by lack of facilities to adequately conduct life span studies of spontaneous tumors, and so are typically limited to the effects of acute or artificial stressors on carcinogen-induced tumors. During middle age, Norway rats spontaneously develop mammary tumors with a wide range of pathological diagnoses, ranging from benign fibroadenomas to invasive ductal carcinomas (14). Such rats provide an excellent model for a life span study of the effect of the social environment on stress vulnerability and spontaneous mammary tumor pathology (15).

Like humans, Norway rats are naturally gregarious, spend significant time in physical contact, form social relationships, and rear offspring cooperatively. In naturalistic settings, their burrow systems are a complex web of social interactions, including individuals that live apart from the group (16, 17). In laboratory settings, the costs of social isolation for female rats have proven to be high. Socially isolated female rats have a sustained and dysregulated glucocorticoid response to an acute stressor (18) and dysregulated cardiovascular responses to the everyday stressors of animal husbandry procedures (19). A life span study of sisters living in groups identified two independent and additive psychosocial risk factors associated with subsequent mammary tumor growth and mortality: an anxious temperament and failure to engage in reciprocal social contact during a stressor (20, 21).

The molecular genetics literature using transgenic, knockout and in vitro models points to a variety of gene candidates that could be affected by dysregulated stress responses to cause spontaneous tumors, for example, down-regulation of tumor suppressor genes such as *PTEN* or DNA repair genes such as *BRC1*. Rapid growth could be mediated by up-regulation of genes involved in cell proliferation and/or cell survival, for example, *SGK1*, *MKP1*, *Myc*, and *AKT*. Some of these pathways are also regulated by ovarian steroids, although isolation accelerates reproductive senescence (22, 23), suggesting that estrogen and progesterone receptor status

Author contributions: G.L.H., B.D., M.T., S.A.C., T.K., S.D.C., and M.K.M. designed research; G.L.H., B.D., M.T., S.A.C., T.K., and M.K.M. performed research; G.L.H., B.D., M.T., S.A.C., T.K., S.D.C., and M.K.M. contributed new reagents/analytic tools; G.L.H. and M.K.M. analyzed data; and G.L.H. and M.K.M. wrote the paper.

The authors declare no conflict of interest.

¹To whom correspondence should be addressed. E-mail: mkm1@uchicago.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0910753106/DCSupplemental.

might not be associated with tumor burden in middle-aged and socially isolated female rats.

Keeping potential genetic targets in mind, our goal is to determine whether social isolation dysregulates glucocorticoid stress responses across the life span and increases glucocorticoid receptor activity in nuclei of mammary tumor cells. This would be a potential downward causal pathway along which chronic social stressors increase risk for mammary cancer. Indeed, glucocorticoids have been shown in cell lines to down-regulate expression of the important human tumor suppressor gene, *BRC1A1* in breast cells, potentially leading to increased malignant transformation (24).

Here, we randomly assigned genetically comparable female rats (99% inbred strain) to two social conditions: living in a social group or living alone. We test the hypothesis that social isolation is associated with dysregulation of endocrine and behavioral responses to stress detectable early in adulthood, months before tumorigenesis. We further hypothesize that the accumulated effects of dysregulated stress responses typical of social isolates would affect multiple basic tumor characteristics, namely onset, mass, multiplicity, location, and malignancy.

To verify that lifelong isolation was associated with dysregulated hormonal and behavioral stress, we examined the basal and reactive functions of the adrenal axis at puberty and middle age to determine whether or not rats maintain characteristic differences in the magnitude of stress reactivity throughout adulthood and whether adrenal dysfunction is subsequently associated with development of mammary tumors. We had already established a correlation between animals with fearful temperament and mammary tumor burden and death (20). Here, we establish a causal relationship between affect and disease by manipulating the social context and creating the fearful, anxious, and tumor-prone phenotype through random assignment to isolate housing.

Results

Mammary Tumors. Tumor burden. By middle age (15.1 ± 0.1 months), 74% of female rats, whether group housed or isolated, had developed spontaneous mammary tumors detectable by palpation. Socially isolated females, however, had a tumor burden 84 times that of age matched controls living in groups (isolated = 27.17 ± 14.99 gm vs. grouped = 0.32 ± 0.12 gm; log transformed weights, $P \leq 0.04$; Fig. 1A). Although incidence of developing at least one palpable mass was similar in the two social conditions (relative risk 1.11; 78% isolated rats vs. 70% group housed rats, Fisher Exact, $P = 0.72$), isolation increased the number of discrete tumor masses by 135% (isolated = 4.7 ± 1.4 tumors, grouped = 2.0 ± 0.5 tumors, $t = 2.2$, $P \leq 0.05$). Among isolates, tumors were more widespread, developing in three if not all four mammary quadrants (left and right, thoracic and inguinal; each quadrant contains three glands); the tumors of all group-housed rats were confined to one quadrant (Fisher's Exact, $P = 0.03$). Regardless of tumor burden, imposed social isolation did not affect body weight [social condition $F(1, 32) = 1.34$, NS; tumor burden $F(1, 32) = 0.32$, NS; interaction $F(1, 32) = 1.3$, NS].

Tumor diagnosis. The naturally occurring tumors were diverse histological types (Fig. 1 B–H), including malignant tumors (36%: invasive ductal carcinoma, ductal carcinoma in situ, premalignant intraductal hyperplasia, and fibrosarcoma) and benign tumors (64%: fibroadenoma, intraductal papilloma, and lactating hyperplasia and adenoma). The majority (63%) were epithelial in origin, 26% were stromal and 11% a mixture of hyperplastic epithelial and stromal cells. Ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) were the most prevalent malignant tumors (90%). IDCs were the largest (33.6 ± 16.7 g; all post-hoc P values ≤ 0.01), micromasses the smallest (0.11 ± 0.02 g; all post-hoc P values ≤ 0.01); DCIS and benign tumors were of similar intermediate weight [17.0 ± 11.8 g, 15.7 ± 15.3 g; post-hoc NS; one-way analysis of variance $F(3, 80) = 10.7$, $P \leq 0.0001$].

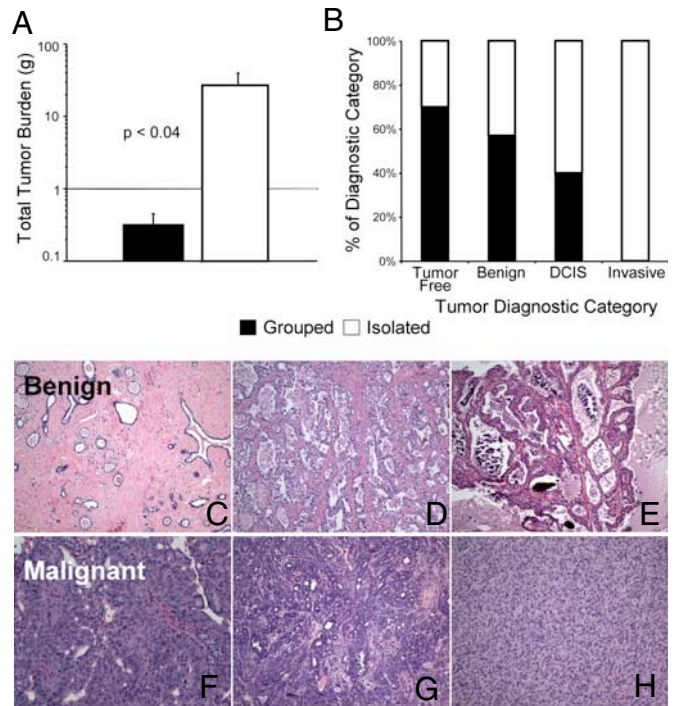


Fig. 1. Effect of long-term social isolation on mammary tumor growth and diagnosis at 15 months. (A) Tumor burden (mean ± SEM) after living alone or in noncrowded social groups. (B) Rats with ductal carcinoma in situ (DCIS) or malignant tumors were primarily socially isolated; those with benign or no tumors lived in groups. (C–H) Wide range of naturally developing mammary tumors: (C) fibroadenoma; (D) lactating adenoma; (E) intraductal papilloma; (F) DCIS; (G) invasive ductal carcinoma; (H) fibrosarcoma.

Isolation and malignancy. Isolation increased the development of malignant tumors (Fig. 1B), which naturally developed in all four mammary gland quadrants [social condition $F(1, 51) = 8.96$, $P \leq 0.01$, quadrant $F(3, 51) = 8.80$, $P \leq 0.0001$; interaction $F(3, 51) = 3.37$, ≤ 0.03 , repeated measures (mammary gland quadrant) ANOVA]. Isolated animals had a 3.3-fold relative risk of developing at least one mammary carcinoma; fully 50.0% had IDC, DCIS, or a pre-DCIS tumor in sharp contrast to group housed females, where their incidence was only 15.4%.

Lifelong Housing Effects on Stress Responses. Response to predator odor at 3 months of age.

Compared to those in stable social groups, female rats that were socially isolated from 1 to 3 months of age had a larger corticosterone response, measured 30 min after exposure to a novel cage scented with fox urine (isolated increased 9.2 ± 2.3 µg/dL, grouped increased 0.9 ± 2.4 µg/dL; $P \leq 0.02$; see Fig. 2A). Thus, random assignment to social isolation, rather than group living, increased the corticosterone responses to a natural psychological stressor (predator odor) by 10-fold.

Response to physical stressor at 13 months of age. After 12 months of isolation and before mammary tumors were palpable, rats developed basal hypocortisolemia, with low baseline levels of corticosterone, in comparison with group-housed rats (see Fig. 2B; $t = 3.38$, $P \leq 0.002$). They also had larger corticosterone response to a 0.5-h of physical restraint, a stressor that simulates a burrow collapsing (see Fig. 2C, reactive corticosterone at 30 min adjusted for baseline levels: isolated = 68.5 ± 5.4 µg/dL change from baseline, grouped = 50.4 ± 6.8 µg/dL; $t = 2.0$, $P \leq 0.05$). From baseline, their corticosterone rose 10.2 ± 2.7-fold within 30 min and continued to rise after the stressor ended, whereas corticosterone in group housed animals rose only 2.6 ± 0.9-fold ($t = 2.8$, $P \leq 0.01$).

OK
AQ: C
F1

COLOR

ital bf

F2

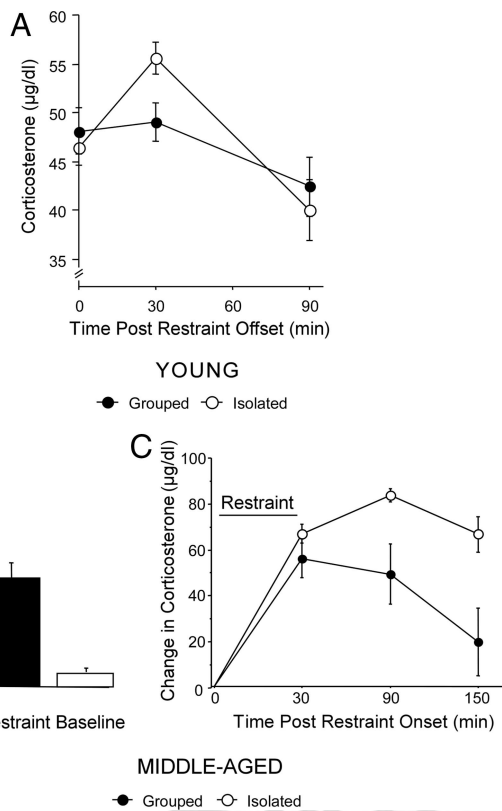


Fig. 2. Corticosterone dysregulation evident in young socially isolated females (3 months old) and severe by middle age (13 months old). (A) High corticosterone response and normal recovery (mean \pm SEM) after an acute stressor, predator odor. (B) Low baseline corticosterone at the diurnal rhythm nadir. (C) A high prolonged corticosterone response, still rising from baseline 1 h poststressor, and delayed recovery 2 h post-stressor.

In addition to differences in basal and reactive corticosterone levels, isolates recovered hormonally from this stressor more slowly (Recovery z Score: isolated = -0.36 ± 0.23 , grouped = $+0.25 \pm .17$, $t = 2.1$, $P \leq 0.04$), demonstrating that isolated animals sustained higher circulating levels of glucocorticoid 2 h after the stressor had ended. Taken together, these data indicate that before tumor development, socially isolated rats were exposed throughout adulthood to higher and more prolonged corticosterone in response to experimental stressors.

The dynamics of the corticosterone response to an acute stressor predicted the mammary tumor burden measured 2 months later [multiple regression $F(4, 22) = 5.89$, $r = 0.75$; $P \leq 0.003$; social condition ($\beta = -2.4$, $t = 2.9$, $P \leq 0.01$)]. Both high corticosterone at the end of the stressor and slow recovery to baseline predicted a larger tumor burden [corticosterone reactivity (log rise as proportion of baseline) $\beta = -2.8$, $t = 2.4$, $P \leq 0.03$; corticosterone recovery (z score) $\beta = -2.0$, $t = 4.0$, $P \leq 0.001$]. Baseline values of stress hormone, in this model, had no significant effect (baseline, $\beta = 0.0$, $t = 0.5$, NS).

Glucocorticoid Receptor Status of Mammary Tumors and Corticosterone Dynamics. To determine whether glucocorticoid receptors (GR) were expressed in mammary gland tumor tissue of rats, we performed immunohistochemistry and found that indeed, rat mammary tumors, including benign fibroadenomas, DCIS, and IDC all expressed the GR (Fig. 3 A–C), demonstrating the capacity of mammary tumor cells to respond to corticosterone.

Mammary gland tumors can arise from ductal epithelial cells, both basal and luminal, as well as from stromal cells, which are

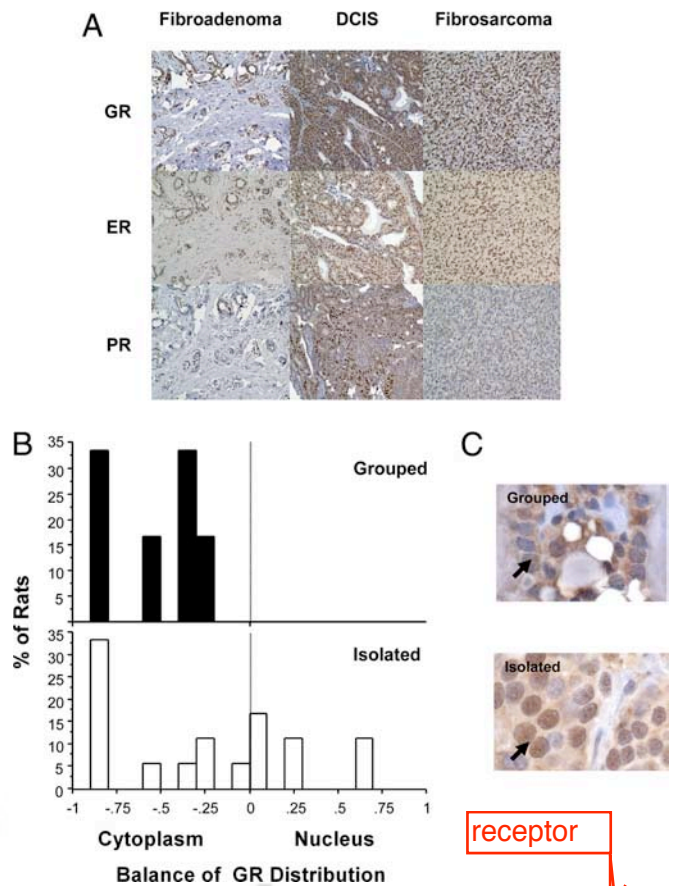


Fig. 3. Glucocorticoid (GR), estrogen (ER), and progesterone (PR) status (brown stain) in different mammary tumor types. (A) Fibroadenoma: GR+ in epithelial and stromal cells, ER+ and PR+ only in epithelial cells. DCIS: GR+, ER+ and PR+. Fibrosarcoma, GR+ and ER+, but PR-. (B) Isolation and the relative distribution of GR (+ = nucleus vs. - = cytoplasm). (C) Exemplars: grouped: more cytoplasmic GR (brown surround-blue center); isolates: more nuclear GR (brown center-blue surround).

found in the **intraductal** connective tissue. First, we assessed distribution of GR within the cells of each type of tissue (i.e., cytoplasmic and nuclear). In the epithelial tumor tissue, GR was found in the nucleus and the cytoplasm, although more cells had GR in the cytoplasm ($75.0 \pm 0.1\%$ of cells) than in the nuclei ($29.2 \pm 0.1\%$ of cells). Mammary stromal cells had much lower levels of GR in both intracellular locations (cytoplasm, 20% of cells; nuclei, 16% of cells).

Among socially isolated animals, the GR was more likely to be found in the nucleus compared to the cytoplasm in the tumor sample (Fig. 3 B and C; 44% of isolated females vs. 0% of grouped females, $X^2 = 4.0$, $P \leq 0.05$), indicative of dynamic translocation rather than steady receptor state. Nuclear translocation is typical of ligand-bound GR and demonstrates the potential for regulation of gene expression.

Lifelong Housing Effects on Ovarian Function and Tumor Status. If the middle-aged ovary mediates the effects of isolation on mammary tumor growth and malignancy via estrogen or progesterone receptor activation (25), then we would expect isolation to be associated with more hormonally active ovaries and/or perhaps, increased ER and PR expression in tumor epithelial cells. In fact, mammary tumors from isolated female rats grew in the milieu of early senescent ovaries, with only secondary and atretic follicles at necropsy, while mammary tissue from group-housed rats continued to be exposed to hormonally active ovaries, including ovulatory

bottom of words in figure legend is cut off

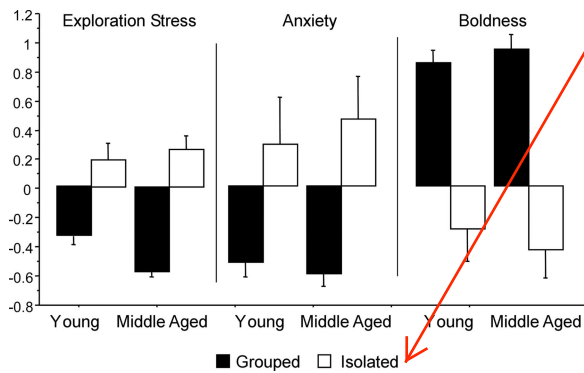


Fig. 4. Social isolation exacerbated three types of behavioral stress responses (age specific z-score mean ± SEM.) assessed in young adulthood (5 months) and repeated in middle age (15 months; see S1 Text).

see pop-up replacement text

follicles (estrogen) and corpora lutea (progesterone) (23). To confirm inferences from these anatomical cross-sectional data, we determined that estrogen exposure throughout the four months before tumor diagnosis did not predict tumor burden ($r = 0.02, P \leq 0.95$; bioassay, estrogenization of vaginal epithelium).

The expression of nuclear ER and PR expression by immunohistochemistry in mammary tumor cells were highly correlated ($r = 0.79, P \leq 0.0001$). Most tumors were ER⁺PR⁺ (60% of cells with nuclear staining (Fig. 3A)). The remainder was ER⁻PR⁻ (30%) or positive for only ER or PR [ER⁺PR⁻ (5%); ER⁻PR⁺ (5%)]. Malignant tumors had more ER-positive cells than did benign tumors ($48.5 \pm 9.5\%$ vs. $11.0 \pm 6.9\%$), and tended to have more PR-positive cells [$27.5\% \pm 8.0\%$ vs. $15.0 \pm 5.5\%$; Diagnosis $F(1, 18) = 5.8, P \leq 0.03$; Receptor type $F(1, 18) = 1.2, P \leq 0.30$, interaction $F(1, 18) = 5.7, P \leq 0.03$, repeated measures (ER and PR in each tumor) ANOVA].

Nonetheless, social condition was not associated with nuclear staining for ER or PR [social condition $F(1, 18) = 0.31, NS$; receptor type $F(1, 18) = 0.26, NS$; interaction of social condition and receptor type $F(1, 18) = 0.93, NS$]. Finally, none of these indicators of ovarian function and ER/PR status were associated with stress reactivity or recovery, measured hormonally or behaviorally, or with GR status ($NS, 0.28 \leq \text{all } P \text{ values} \leq 0.88$).

Lifelong Housing Effects on Behavior: Routine Stressors Assessed at 5, 10, and 15 Months of Age. Social isolation also increased anxiety and reduced boldness, measured during an exploration stressor at both 5 and 15 months of age. When placed in the home corner of this novel exploration arena, isolated females did not move for prolonged times, and then proceeded slowly only in tight contact with the walls, exploring few new areas (Fig. 4). At both ages, isolated female displayed more species-typical anxiety behaviors during exploration: freezing, piloerection, urination, or defecation, which correlated with their level of Exploration Stress (Fig. 4; Anxiety z Score and Exploration Stress z Score: 5 month $r = +0.51, P \leq 0.0001$; 15 month $r = +0.60, P \leq 0.0001$). In contrast, group housed animals, explored more at both 5 and 15 months of age, crossing the open field, the most threatening part of the environment and displaying more boldness [e.g., rearing on their hind legs, standing, a steady, constant gait (Fig. 4); Boldness z and Exploration Stress z: 5 months $r = -0.49, P \leq 0.002$; 15 months $r = -0.78, P \leq 0.0001$].

The emotional effects of social isolation persisted within individual rats between 5 and 15 months of age (Exploration Stress z scores $r = +0.51, P \leq 0.0001$; Anxiety z Scores $r = +0.35, P \leq 0.04$; Boldness z Scores $r = +0.46, P \leq 0.004$). The level of Exploration Stress (ES) also predicted level of vigilance and anxiety after opening the home cage, a routine daily husbandry practice [latency of high ES rats to emerge = 54.2 ± 17.2 s., average to low ES rats = 27.4 ± 14.1 s, Logrank (Mantel-Cox) $X^2 = 4.77, P \leq 0.03$].

Responses to this everyday practice also persisted throughout middle age, even after 300 and 450 repetitions (10 and 16 months of age, $r = +0.51, P \leq 0.0001$; $r = +0.88, P \leq 0.0001$).

Association Among Manipulated Social Environment, Subsequent Psychoendocrine Stress, and Tumor Growth. To assess the coherence of psychoendocrine variables and disease outcomes within individual animals, we conducted a confirmatory factor analysis. As expected, key variables were significantly associated with each other, and contributed to a single factor measured at multiple levels of organization (orthogonal or varimax rotation, Eigen Value = 2.3, 55% of variance, $P < 0.01$; coefficients of shared variance with a single factor: social isolation 0.92, anxiety, fearfulness and vigilance 0.82, prolonged glucocorticoid stress recovery 0.55, and tumor burden 0.63). These associated variables may be candidates for a causal cascade, given their sequential development during a longitudinal experiment: social isolation, dysregulated hormonal and behavioral stress responses, and mammary tumor progression.

Discussion

In these studies, we show that female rats living in social isolation from puberty through late middle age became progressively more reactive to superimposed acute stress, first developing a heightened, and ultimately a prolonged, corticosterone stress response to either brief predatory odor or restraint stress. By randomly assigning female Sprague–Dawley rats to social isolation, we also reveal the importance of psychosocial modulation of a heritable risk for tumor development, because social isolation increased the size, number, distribution, and malignancy of spontaneous mammary tumors.

Interestingly, the magnitude of social isolation's effect on several characteristics of mammary neoplasia—135% increase in number, 8,391% increase in size and a 3.3-fold increase in relative risk of malignancy—is significantly greater than that of unlimited-access to food versus an energy-restricted diet, widely documented as the greatest environmental modulator of mammary tumor development in rodents (26). By comparison, unlimited access to high metabolizable energy food increased tumor incidence by 96%, produced a modest 1.33 relative risk for malignancy, and had no effect on tumor growth (27).

Beginning in early adulthood and continuing throughout midlife of the female rat, we found that the adrenal axis of socially isolated animal was dysregulated, first manifesting as a higher corticosterone response and ultimately as markedly low baseline corticosterone levels indicative of hypocortisolemia (28). This was followed by high and sustained levels of corticosterone in response to a moderate stressor. This last pattern typically reflects damage and aging of the hippocampal system, as well as effects of vasopressin, corticotropin-releasing hormone and pro-opiomelanocortin-derived peptides regulating the adrenal axis (29). Isolation also induced glucocorticoid hyperresponsiveness that developed months before mammary tumors developed. Finally, mammary tumors reduce, rather than augment, glucocorticoid reactivity in rats (30), contravening the converse hypothesis that tumor development was itself stressful and caused the observed hyperreactivity.

Given the mild, yet repetitious, everyday stressors of laboratory life (19), the isolated rats and their mammary tissues were likely exposed throughout adulthood to prolonged pulses of higher levels of corticosterone with relatively low corticosterone levels between stressful events; the low basal levels could also feedback to increase steady-state glucocorticoid receptor (GR) expression allowing a highly robust intermittent response to acute stressor-induced glucocorticoids (31). This pattern of hypocortisolemia and prolonged elevated corticosterone in response to an intermittent acute stressor may contribute to tumor initiation and growth, since GR activation has been associated with anti-apoptotic signaling in epithelial cells, including malignant human breast epithelial cells and premalignant mammary epithelial cells such as MCF10A-Myc cells (11).

Prior to the current study,

only

in young adulthood

in late middle age

F4

Here, we also demonstrate that in social isolation, mammary gland GR was more commonly found in the nucleus. While in lymphocytes, activation and translocation of the ligand-bound receptor to the nucleus is associated with cell death (32), in cultured malignant human breast epithelial cells, glucocorticoid-mediated GR activation prevents programmed cell death (apoptosis). Furthermore, in a mouse model of xenografted human breast cancer, glucocorticoid treatment and nuclear translocation of tumor GR is associated with resistance to chemotherapy-induced apoptosis and ultimately larger tumor growth (11, 12). Inhibition of apoptosis may also be driven by GR translocation to the mitochondria (33), consistent with the observed tendency toward greater variance in the relative subcellular distribution of GR between the cytoplasm and the nucleus. Since the GR has different mechanisms of activating signaling pathways in the nucleus versus non-nuclear locations, there are several possible mechanisms that might alter tumor growth in the mammary glands of isolated animals exposed to increased glucocorticoid action. Furthermore, the adrenergic components of the stress response on angiogenesis could further increase tumor growth (34).

Recently, a study of C3 (1)/SV40 large T-antigen (Tag) transgenic mice predisposed to developing invasive mammary gland carcinomas (35), revealed that social isolation in this species is also associated with increased glucocorticoid reactivity to an acute stressor as well as an increased tumor growth rate. In the SV40 T-antigen model, chronic social isolation from weaning was associated with significantly increased expression of genes encoding key metabolic pathway enzymes in the mammary gland before invasive cancer formation. Specifically, prolonged social isolation was associated with up-regulation of genes encoding key lipid synthesis and glycolytic pathway enzymes in the mammary tissues of 15-week-old mice, well before microscopic evidence of differences in invasive carcinoma between the two housing groups. In addition, gene expression differences reflecting inflammatory pathways were uncovered, supporting the hypothesis that differences in stress-induced inflammation (18) may also contribute to the susceptibility of genetically predisposed individuals to malignancy under conditions of chronic social isolation. **inflammatory dysregulation**

The majority (60%) of naturally occurring mammary tumors examined here expressed estrogen receptors (ER) and progesterone receptors (PR); the role of estrogens and progestins in mammary carcinogenesis and growth is well established (25). In this rat model, however, social isolation was associated with senescent ovaries at the age of tumorigenesis and growth; few isolated rats had hormonally active follicles or corpora lutea, (23), confirmed here by the condition of their vaginal epithelia. Therefore, ovarian function at best plays a permissive role, and likely does not account for the larger and more malignant tumors in isolates.

The most frequent neoplasia in these naturally occurring rat mammary tumors was ductal epithelial: premalignant intraductal hyperplasia (IDH), ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC). The spectrum of these diagnoses is also common in human breast cancer, suggesting that the Sprague–Dawley rat is an excellent model for human mammary cancer development and progression. However, equally important is the wide variety of spontaneously arising non-neoplastic mammary tumors, “benign” disease in which stromal tissue proliferated until it occupied as much as 54% of the female rat’s body weight (M = 6.7%) and became a significant metabolic and physical burden. This mammary gland disease diversity provides the opportunity to study the psychosocial mechanisms that might affect whether hyperplasias ultimately follow a malignant versus benign trajectory.

In summary, our model for investigating environmentally initiated psychosocial effects of mammary cancer initiation and growth provides a complementary approach to the current emphasis on inherited and spontaneous genetic alterations leading to changes in gene expression. First, it is a model of

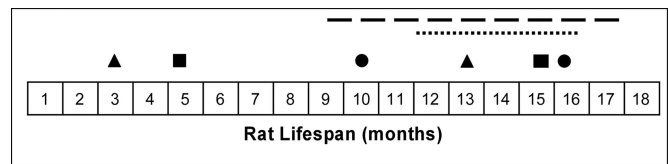


Fig. 5. Experimental protocol of biobehavioral assays: ▲ Corticosterone stress responses; ■ Exploration stress responses; ● vigilance testing, ovarian cyclicity, and --- tumor palpation.

bottom line of figure box is cut off

spontaneous tumors, which recapitulate most malignant and benign tumors in humans more accurately than the more expedient models using transgenes or carcinogens to stimulate rapid cancer growth or xenografts in which human cancer cells are injected into immunodeficient mice. Because this is a life span animal model of disease that examines dynamic interactions between the organism and its environment over a relatively long period, it illustrates not only the interaction between early stress responsiveness and later tumor pathology, but also tradeoffs between short-term benefits early in the life span, such as enhanced fertility of early ovarian maturity, yet increased disease burden at its end. Our data also point to the important and the somewhat unexpected role of the stress hormones in cancer biology. Specifically, in this animal model, adrenal dysregulation, not lengthening of estrogen or progesterone exposure, was associated with increased mammary tumor development and progression. (23)

Interestingly, loneliness and stress vulnerability have been associated previously with gene expression changes in isolated ovarian cancer and immune cells in humans (36–38). This animal model is also another powerful tool for identifying the links in a network between specific aspects of social context, psychological states, neuroendocrine mechanisms, and tissue gene expression changes that culminate in benign hyperplasia or mammary cancer. Translating these findings into successful targets for intervention to reduce cancer risk will require transdisciplinary research that considers the diversity of human circumstance and the complexity of cancer biology (39).

Materials and Methods

Female Sprague–Dawley rats ($n = 40$) were bred at Charles River Laboratories, Inc., weaned into same sex groups at 21 days of age, and shipped to our laboratory at 28 days of age (Fig. 5; pubertal data and methods are in ref. 23). In short, we randomly assigned 20 rats to socially isolated housing in a rack of single cages ($26 \times 23 \times 22$ cm) and 20 rats to four groups of five, each group in a large cage ($46 \times 61 \times 36$ cm). Cages had wire mesh floors over shared bedding pans and animals could smell and hear each other, but interact socially only in the group cages. All resided in the same colony room of an American Association for the Accreditation of Laboratory Animal Care accredited facility; the Institutional Animal Care and Use Committee of the University of Chicago approved all protocols. Detailed information for this and all methods are in *SI Text*.

Mammary Tumor Burden. In a series of bi-monthly health checks (Fig. 5), trained technicians palpated all mammary glands of middle-aged animals and noted the location and size of all nodules, using standard technique (23, 40). Estimates of tumor weight by palpation predicted the dissected tumor weight. Total tumor burden, the sum of all tumors within an animal, was determined at 15.0 ± 0.1 months of age, chosen for cross-sectional measurements to optimize the tradeoff between a longer opportunity to express the tumor phenotype and increasing mortality. At 15 months, 95% were still alive, 100% of animals that eventually developed a mammary tumor had already done so, and 97.8% of those who were tumor-free remained so.

Tumor Diagnosis. Necropsy occurred later (18.8 ± 0.5 months of age; Fig. 5). Surgical pathologists specializing in breast cancer pathology diagnosed a randomly selected tumor subset from representative histological sections,

MEDICAL SCIENCES
 PSYCHOLOGICAL AND COGNITIVE SCIENCES
 F5
 STXT

γ / ^

classifying them (40) and categorizing them as malignant [2; including premalignant intraductal hyperplasia (41)], benign (1), or no tumor (0).

Hormone Receptor Status. Tissue microarrays (TMA) contained two different 1 mm cores per tumor. Primary antibodies were: anti-estrogen receptor α (C1355, 1:800, Millipore) anti-progesterone receptor (Ab 13, 1:200, Lab Vision Corporation) and anti-glucocorticoid receptor (3D5, 1:400, Abcam Inc.). After incubation with HRP-labeled polymer, reactions were completed with the Envision detection system using 3–3' diaminobenzidine as the chromogen (DakoCytomation; staining intensity was excellent for GR, ER, and PR (all median and modal values = 3 on a 3-point scale).

In this study of GR in rat mammary tissue, we scored the cytoplasm and nucleus of both epithelial and the stromal cells [% cells with positively GR stain, coded as: (0) negative stain, (1) 5% (range 1 to 10% of cells), (2) 30% (range 11–50%), (3) 65% (range 51–80%), and (4) 90% (81%–100%)]. Following standard clinical method for assessing ER and PR status of mammary tumors and cancers, we recorded their presence or absence in the nuclei of epithelial and stromal cells.

Corticosterone Response to Stressors. At the beginning of behavioral night (lights-on), rats were stress-tested in an adjacent room. At 2 months of age, they spent 30 min in an unfamiliar cage scented with predator (fox) urine (Fig. 5) and at 13 months of age, 30 min in an unfamiliar restraint tube (Harvard Apparatus). Tail-blood samples were taken within 2 min to measure the adrenal stress hormone corticosterone at: prestress baseline, after 30 min of imposed stress and during recovery, 1 and 2 h after returning to their home cage. Serum concentrations of corticosterone were assayed by RIA (ICN Biomedicals), with slight modifications to increase sensitivity (intra-assay coefficient of variance = 9.4%; inter-assay variance = 8.1)

Ovarian Senescence. Vaginal cytology were analyzed daily (12–16 months of age; Fig. 5), quantifying estrogenization levels, cycle length, and reproductive state (22).

Behavioral Response to Stressors. Exploration in an unfamiliar environment is a classic stressor for rodents (42). We modified the technique to avoid arousal confounds by gently placing each animal in the home corner, important protection for this thigmotactic species, seated in a heavy ceramic bowl serving as a home base from which to remain vigilant or explore. A detailed ethological analysis was used to measure: exploration stress, anxiety, and boldness. Similar exploration responses to opening the home cage were quantified by the latency to emerge and touch the cage rim.

Statistical Analysis. Statistical analyses were conducted with Statview (SAS Institute; NS = not significant ($P > 0.05$, two-tailed tests), all means \pm SEM.). Log-transformed tumor weights, arcsine transformed arcsine [sqrt (p)] percentages and z-scores met parametric distribution requirements.

ACKNOWLEDGMENTS. We thank M. Tsakalis, K. Eisenmann, M. Kerr, and J. Yee for behavioral, endocrine, and tumor data collection, and especially J. Hoffman and H. You, for necropsies, laboratory and data management, insightful critiques, persistence, and stamina. We are indebted to John Borla for Biopsychological Sciences Building operations crucial for this longitudinal research. Research support from P50 ES012382 National Institute of Environmental Health Sciences/National Cancer Institute, Idea Award BC 061754 U.S. Army MRRMC under W81XWH-07-1-0296, T32 HD009007 at University of Chicago, R25 MH071584, the State of Connecticut Department of Mental Health and Addiction Services, and T32 MH19961 Department of Psychiatry Yale University.

1. Reiche EM, Nunes SO, Morimoto HK (2004) Stress, depression, the immune system, and cancer. *Lancet Oncol* 5:617–625.
2. Lillberg K, et al. (2003) Stressful life events and risk of breast cancer in 10,808 women: A cohort study. *Am J Epidemiol* 157:415–423.
3. Fox CM, Harper AP, Hyner GC, Lyle RM (1994) Loneliness, emotional repression, marital quality, and major life events in women who develop breast cancer. *J Community Health* 19:467–482.
4. Sephton SE, Sapolsky RM, Kraemer HC, Spiegel D (2000) Diurnal cortisol rhythm as a predictor of breast cancer survival. *J Natl Cancer Inst* 92:994–1000.
5. Antoni MH, et al. (2006) The influence of bio-behavioral factors on tumor biology: Pathways and mechanisms. *Nat Rev Cancer* 6:240–248.
6. Grimm MS, Emerman JT, Weinberg J (1996) Effects of social housing condition and behavior on growth of the Shionogi mouse mammary carcinoma. *Physiol Behav* 59:633–642.
7. Kerr L, et al. (1997) Effects of social housing condition on the response of the Shionogi mouse mammary carcinoma (SC115) to chemotherapy. *Cancer Res* 57:1124–1128.
8. Joosten HF, et al. (2004) Genotoxicity of hormonal steroids. *Toxicol Lett* 151:113–134.
9. Singh H, et al. (1994) In vitro and in vivo genotoxicity evaluation of hormonal drugs. II. Dexamethasone. *Mutat Res* 308:89–97.
10. Yee JR, et al. (2009) Individual variation in stress-induced corticosterone dynamics and spontaneous mammary tumors. *Carcinogenesis*.
11. Moran TJ, Gray S, Mikosz CA, Conzen SD (2000) The glucocorticoid receptor mediates a survival signal in human mammary epithelial cells. *Cancer Res* 60:867–872.
12. Pang D, et al. (2006) Dexamethasone decreases xenograft response to paclitaxel through inhibition of tumor cell apoptosis. *Cancer Biol Ther* 5:933–940.
13. Fauquier D, Gulland F, Haulena M, Spraker T (2003) Biliary adenocarcinoma in a stranded northern elephant seal (*Mirovunga angustirostris*). *J Wildl Dis* 39:723–726.
14. Davis R, Stevenson GT, Busch KA (1956) Tumor incidence in normal Sprague–Dawley female rats. *Cancer Res* 16:194–197.
15. McClintock MK, et al. (2005) Mammary cancer and social interactions: Identifying multiple environments that regulate gene expression throughout the life span. *J Gerontol B Psychol Sci Soc Sci* 60:32–41.
16. Barnett SA (1963) *The Rat* (University of Chicago Press, Chicago).
17. Calhoun JB (1961) Determinants of social organization exemplified in a single population of domesticated rats. *Trans N Y Acad Sci* 23:437–442.
18. Hermes GL, Rosenthal L, Montag A, McClintock MK (2006) Social isolation and the inflammatory response: Sex differences in the enduring effects of a prior stressor. *Am J Physiol Regul Integr Comp Physiol* 290:R273–282.
19. Sharp JL, Zammit TG, Azar TA, Lawson DM (2003) Stress-like responses to common procedures in individually and group-housed female rats. *Contemp Top Lab Anim Sci* 42:9–18.
20. Cavigelli SA, Yee JR, McClintock MK (2006) Infant temperament predicts life span in female rats that develop spontaneous tumors. *Horm Behav* 50:454–462.
21. Yee JR, Cavigelli SA, Delgado B, McClintock MK (2008) Reciprocal affiliation among adolescent rats during a mild group stressor predicts mammary tumors and life span. *Psychosom Med* 70:1050–1059.
22. LeFevre J, McClintock MK (1991) Isolation accelerates reproductive senescence and alters its predictors in female rats. *Horm Behav* 25:258–272.
23. Hermes GL, McClintock MK (2008) Isolation and the timing of mammary gland development, gonadarche, and ovarian senescence: Implications for mammary tumor burden. *Dev Psychobiol* 50:353–360.
24. Antonova L, Mueller CR (2008) Hydrocortisone down-regulates the tumor suppressor gene *BRCA1* in mammary cells: A possible molecular link between stress and breast cancer. *Genes Chromosomes Cancer* 47:341–352.
25. Shyamala G, et al. (2002) Cellular expression of estrogen and progesterone receptors in mammary glands: Regulation by hormones, development and aging. *J Steroid Biochem Mol Biol* 80:137–148.
26. Dix MJ, et al. (2003) Energy restriction and the risk of spontaneous mammary tumors in mice: A meta-analysis. *Int J Cancer* 106:766–770.
27. Keenan K, Soper KA, Smith PF, Ballam GC, Clark RL (1995) Diet, overfeeding, and moderate dietary restriction in control Sprague–Dawley rats: I. Effects on spontaneous neoplasms. *Toxicol Pathol* 23:269–286.
28. Bremner D, Vermetten E, Kelley ME (2007) Cortisol, dehydroepiandrosterone, and estradiol measured over 24 hours in women with childhood sexual abuse-related posttraumatic stress disorder. *J Nerv Ment Dis* 195:919–927.
29. Chrousos GP (2009) Stress and disorders of the stress system. *Nat Rev Endocrinol* 5:374–381.
30. Pyter LM, et al. (2009) Peripheral tumors induce depressive-like behaviors and cytokine production and alter hypothalamic-pituitary-adrenal axis regulation. *Proc Natl Acad Sci USA* 106:9069–9074.
31. Yehuda R, et al. (1991) Lymphocyte glucocorticoid receptor number in posttraumatic stress disorder. *Am J Psychiatry* 148:499–504.
32. DeVries A, Craft, TK, Glasper, ER, Neigh, GN, Alexander, JK (2007) 2006 Curt P. Richter award winner: Social influences on stress responses and health. *Psychoneuroendocrinology* 32:587–603.
33. Chen JQ, Brown TR, Yager JD (2008) Mechanisms of hormone carcinogenesis: Evolution of views, role of mitochondria. *Innovative Endocrinology of Cancer, Advances in Experimental Medicine and Biology Volume 630*, eds Bernstein LM, Santen RJ (Bio-science and Springer Science Business Media, Boston, MA), Vol 630, pp 1–18.
34. Thaker PH, et al. (2006) Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. *Nat Med* 12:939–944.
35. Williams JB, et al. (2009) Chronic social isolation is associated with altered gene expression and increased tumor growth in transgenic mice. *Cancer Prev Res*.
36. Lutgendorf SK, et al. (2009) Depression, social support, and beta-adrenergic transcription control in human ovarian cancer. *Brain Behav Immun* 23:176–183.
37. Cole SW, et al. (2007) Social regulation of gene expression in human leukocytes. *Genome Biol* 8:R189.
38. Miller GE, et al. (2008) A functional genomic fingerprint of chronic stress in humans: Blunted glucocorticoid and increased NF- κ B signaling. *Biol Psychiatry* 64:266–272.
39. McClintock MK, et al. (2009) Overcoming health disparities: The power of a transdisciplinary approach to environmental regulation of gene expression. (*Royal Society of Canada*), In press.
40. Russo J, Russo IH (2000) Atlas and histologic classification of tumors of the rat mammary gland. *J Mammary Gland Biol Neoplasia* 5:187–200.
41. Tran DD, Lawson JS (2002) Microcysts and breast cancer: A study of biological markers in archival biopsy material. *Breast Cancer Res Treat* 75:213–220.
42. Roth KA, Katz RJ (1979) Stress, behavioral arousal, and open field activity—A reexamination of emotionality in the rat. *Neurosci Biobehav Res* 3:247–263.

AQ: D

replace
-ment
text in
blue
popup

8

2010

AQ: E

in *Genes and Social Environment Changing Boundaries: Issues and Challenges*, eds Maheu L, Macdonald RA (McGill-Queen's University Press, Montreal), In press.

Supporting Information

Hermes et al. 10.1073/pnas.0910753106

STXT

SI Text

Animals. The colony room and the adjacent testing room were maintained at $22 \pm 1^\circ\text{C}$, $40 \pm 5\%$ relative humidity, and a 14/10-h light/dark cycle (lights on at 1000 h CST). Water and food (Rodent Diet 8604) were available ad libitum. Harlan Teklad Rodent Diet 8604 has metabolizable energy in the low normal range for a standard laboratory diet (3.1 kcal/g), grain-based (corn primary, soybean meal secondary), and low normal fat content (4.4 g/100 g diet; standard range 4.0 to 6.0 g/100 g).

Protocol Time Line. Hormonal and behavioral responses to stressors were assessed repeatedly from young adulthood through late middle age (see Fig. 5). Corticosterone stress responses were assessed at 3 and 13 months of age. Behavioral responses during an exploration stressor were assessed at 5 and 15 months of age. Behavioral responses to the every-day husbandry procedure of opening the home cage were assessed among isolates at 10 and 15 months of age. Ovarian function was quantified during late middle age (12 to 16 months of age) and anatomy at necropsy. All 12 mammary glands were palpated for mammary tumors, beginning at 10 months of age and ending with necropsy at 18.8 ± 0.5 months of age when tumors were excised, diagnosed, and characterized by levels of glucocorticoid, estrogen, and progesterone receptors.

Mammary Tumor Burden. Animals were palpated for mammary tumors beginning at 9 months of age. Tumors were first palpable at 1-mm diameter, a sensitivity facilitated by location of thin flat mammary sheets between skin and the underlying muscle and bone. The diameter of palpable mammary tumors was coded in situ, measured by caliper, and converted to tumor weights based on volume of a prolate ellipsoid with the short axes equal tumor weight grams = $\{\text{length (cm)} \times [\text{width (cm)}]^2\} / 2$ (4). In a cross-sectional data collection at 15 months of age, all animals were diagnosed as being tumor-free or having developed at least one tumor. The number of palpable tumors was recorded along with the total tumor burden. The age for the cross-sectional study was set at 15 months to balance maximizing the opportunity tumor development against the loss of animals to the cross-sectional study because of early tumor-related deaths.

Tumor Tissue Collection And Processing. Animals were necropsied beginning at 16 months of age, if their tumors interfered with their well being (e.g., became infected or the animal cachectic, determined in consultation with a veterinarian) continuing to 22 months of age when the study ended. These criteria afforded the opportunity to validate 15 months of age as appropriate for determining tumor condition.

At 15 months of age, animals diagnosed as tumor-free or having only micromasses (0.11 ± 0.02 g; total tumor burden ≤ 1.00 g), remained tumor free and lived longer than those with tumors, (7 additional months, Log rank $\chi^2 = 24.6$, $P \leq 0.0001$). New tumors developed rarely (in 0% of rats without any palpable tumors at 15 months of age; only 2.2% of tumors found at necropsy had developed after 15 months of age). Thus, in Sprague–Dawley rats, mammary tumors are a disease of middle age; 15 months is an age by which genetic risk for different mammary pathology has been manifest (presence, benign or malignant). African-American women have a similar age-dependent risk, highest during middle age, in contrast to European-American women whose probability of cancer increases with age.

Once an animal developed a palpable diagnosable tumors, she typically lived only an additional 2 months before the disease interfered with her well being (2.0 ± 0.3 months). The average age of necropsy was 18.8 ± 0.5 months of age; no difference in age of necropsy in the two social conditions: isolates 18.9 ± 0.6 months, group-housed = 19.3 ± 0.5 months, Mantel-Cox $\chi^2 = 0.06$, $P = 0.80$).

The ovoid tumors were typically encapsulated and easily excised from each of the mammary quadrants (left and right pectoral, left and right inguinal). The total tumor burden of animals with diagnosable tumors (68% of 39 rats) was greater than those with only micromasses ($17.8 \pm 6.9\%$ vs. $2.6 \pm 0.4\%$ body weight), which were equally likely to be isolated or group-housed animals (37% vs. 34%; Fisher's Exact $P = 0.99$).

Tumor tissue was fixed in 10% formalin and embedded in paraffin blocks. Sections measuring $4 \mu\text{m}$ were stained with hematoxylin and eosin (H&E), read for diagnosis by at least two surgical pathologists (Pathology Department of The University of Chicago Hospitals).

Two representative sites were chosen for 1-mm cores of tissue to be mounted in a tissue microarray, constructed using the automated Beecher tissue arrayer ATA-27 (Beecher Instrument, Inc.). TMA sections were $4 \mu\text{m}$. After deparaffinization in xylene, slides were rehydrated through consecutive graded alcohols to distilled water. After blocking endogenous peroxidase activity, sections were heated for antigen retrieval with citrate buffer. The sections were then incubated with the primary antibodies for 1 h at room temperature (described in the main article).

The microarray technology permits >40 experimental cores per slide, so that tissues from both experimental conditions are processed identically on the same slide, and all slides can be processed simultaneously in a single batch, reducing error introduced by variation between batches of a large number of slides.

Animals with only micromasses (0.11 g) were excluded from analyses of tumor types ($n = 13$) because (i) most micromasses were not diagnosed and (ii) total micromass burden did not predict diagnosis (subset analysis of rats with diagnosed micromasses: 62% malignant diagnosis with micromass burden ≥ 1 gm, 38% malignant given burden <1 g; Fisher's Exact $P = 0.32$).

Corticosterone Stress Response. During the nadir of the diurnal rhythm in corticosterone (the rat equivalent of cortisol) 2-month-old rats were placed for 30 min in an unfamiliar cage scented with predator (fox) urine and the corticosterone concentration attained at the end of this stressor measured hormonal stress response. Corticosterone sampled 30 and 90 min after returning to the home cage assessed stressor recovery, defined as the drop between samples taken 30 and 90 min after stress (see Fig. 2).

When the rats became 13 months old, we modified this protocol. Other studies in our laboratory indicated that baseline levels could differ between social conditions in middle age (2), even though they did not in young animals. Moreover, prolonged recovery from stress is a hallmark of aging of the hypothalamic-pituitary-adrenal axis (3). Therefore, we added a baseline sample immediately before the imposed stressor, and measured hormonal recovery with two samples taken 60 and 120 min post-stressor.

The rise and recovery of the stress response were expressed as a change from baseline concentrations (4) (See Fig. 2). For

equal

$\frac{l \wedge}{\frac{w \wedge}{2}}$

Tumor

(weight)

parametric statistical analysis, values were z scored, standardized to the time of sample collection. Recovery z-scores at 60 and 120 min after stress were correlated within individuals ($r = 0.60$, $P \leq 0.0003$), and therefore averaged into a single recovery z score corresponding to recovery 90 min after stress, as measured at 3 months of age.

Exploration Stress, Anxiety, and Boldness. Exploration arena. The exploration arena measured 110 cm wide \times 55.2 cm high \times 109.1 cm long, consisting of four sides joined to form a square. Three sides were dark sealed wood; the fourth clear Plexiglas enabling videography from an oblique angle. The arena rested on the floor; covered with a layer of wood chip cage bedding changed after each trial. A ceramic bowl in a corner served as the home base into which the animal was gently placed at the beginning of 4-min exploration trial. An overhead video camera, sensitive in dim red light, allowed recording of the rats' nocturnal activity during its "behavioral day." Behavioral sequences were analyzed using Ethovision (Noldus).

Ethogram. When rats are placed in their home bowl set in the corner of an open field, they explore with a stereotyped pattern, and exhibit species-typic behaviors indicating anxiety and vigilance as well as confidence and curiosity. Some rats take a long time to leave their home bowl, and then move slowly along one wall (from one to five rat body lengths), returning home before going out again along the same or adjacent wall. Other rats will then round the next corner, return, and explore around the corner on the opposite wall. Less than one-third of rats will eventually move around all four sides, with sorties away from the wall cutting corners or completely traversing the open field. We created a composite score to quantify the animals' responses to the open field, measuring the balance of exploration and vigilance, a behavioral stress response.

Exploration behaviors were quantified with four measures: latency to move from home base, distance traveled (measured by the number of grid lines crossed, new areas entered, and thigmotaxis (time in contact with the wall)). A confirmatory oblique factor analysis of the z-scores substantiated averaging the z-scores into a single score: latency to leave home bowl (-0.94), new areas entered ($+0.93$), distance covered ($+0.93$), and time in contact with the walls ($+0.71$); Eigen value = 3.1, Bartlett's Chi Square = 114.6, proportion of variance = 0.78, $P \leq 0.0001$).

Anxiety. The frequencies of anxiety behaviors were summed and expressed as a z score, standardized to the mean for each age of testing: freezing, piloerection, urination, or defecation outside the toilet area, stereotyped grooming).

Boldness was quantified similarly by summing bold behaviors and expressing as a z score (standardized to the mean for each age of testing): rearing on hind legs, with or without a supporting paw, standing, lack of hesitant gait, progression through the four stages of exploration.

Exploration stress. Average to low levels of exploration behaviors were validated as an anxiety measure and high exploration levels were validated as a boldness measure. Animals who explored with average to below average levels ($\leq +0.25$ z score) also exhibited a variety of anxiety and vigilance behaviors, increasing linearly with less exploration (piloerection, freezing, defecation/urination in the field, and stereotypical grooming; $r = -0.51$, $P \leq 0.0001$).

Females with above average exploration scores exhibited bold behaviors (rearing on their hind legs, standing supported by a vertical surface, speed ($0, \leq 1$ ft/s, > 1 ft/s), and progress through the stages of species-typic exploration ($r = 0.64$, $P \leq 0.0001$). Nonetheless, among females with below average exploration, exploration did not have a linear relationship with boldness behaviors.

Therefore, the inverse of the exploration factor score was used as an indicator of Exploration Stress, associated within individual rats at mid to high ranges with anxiety and vigilance, and in the low range boldness and curiosity.

Vigilance During Every-Day Stressors. Standard husbandry procedures are routine rodent stressors (5). When the home cage is opened daily to check food and health, or to obtain a vaginal cytology sample, some rats cower in the back, while others quickly come to the front and even rising up to inspect the investigator. These individual differences in vigilance and behavioral stress reactivity to repeated every-day stressors were quantified by leaving the cage open for up to 5 min and recording the latency for the rat to emerge from the back and latency to stand at the front with a paw on the rim.

Reproductive Senescence. Dynamic ovarian function. Reproductive senescence in the rat is indicated by the onset of irregular cycles and, in some animals, entry into a state of tonic unopposed estrogen (constant estrus), a dynamic temporal pattern, which must be measured noninvasively with vaginal cytology following a well-established protocol. From daily vaginal lavages (mid-dark dark-phase), the changing proportion of cornified epithelial cells, nucleated epithelial cells, and leukocytes indicates estrous cycle phase (6, 7). The number of periovulatory surges of estrogen (i.e., during proestrus) included those that began a complete cycle (marked by two successive proestrous days) as well the proestrous day beginning a cycle that was not completed by the end of the observation period. Estrogenization level was quantified by percent of 14 days with only nucleated or cornified vaginal epithelial cells, a well-established bioassay for estrogen level (8).

Ovarian anatomy. In middle-aged animals, we determined which animals had reached the irreversible state of estropause, that is, ovarian senescence. There are two patterns of ovarian senescence in this species—one of irregular cycles, followed by constant estrus, and a return to irregular cycles. Other females simply maintain irregular cycles during middle age. Therefore, months of continuous vaginal cytology records are needed to characterize which aging pattern the animal takes and whether irregular cycles represent irreversible ovarian senescence. Here, we needed a single measure close in time to the assessment of mammary tumor burden that could unequivocally determine the state of ovarian senescence—the anatomy of the ovary. Moreover, it was known that social isolation accelerated reproductive senescence measured dynamically with daily vaginal smears (9); here, we sought to verify this by quantifying ovarian function directly.

For animals necropsied at 16 months of age, ovaries were weighed, formalin fixed and paraffin embedded and serial sections ($10 \mu\text{m}$) were stained with hematoxylin and eosin. The presence of tertiary follicles, ova and recent or involuting corpora lutea confirmed ovulation and steroidally active ovarian tissue. Secondary and atretic follicles were also identified (10).

1. Simpson-Herren L, Lloyd HH (1970) Kinetic parameters and growth curves for experimental tumor systems. *Cancer Chemother Rep* 54:143–174.
2. Hermes GL, Rosenthal L, Montag A, McClintock MK (2006) Social isolation and the inflammatory response: Sex differences in the enduring effects of a prior stressor. *Am J Physiol Regul Integr Comp Physiol* 290:R273–282.
3. Sapolsky RM (1996) Stress, glucocorticoids, and damage to the nervous system: The current state of confusion. *Stress* 1:1–19.

4. Vahl TP, et al. (2005) Comparative analysis of ACTH and corticosterone sampling methods in rats. *Am J Physiol Endocrinol Metab* 289:E823–828.
5. Cavigelli SA, et al. (2005) Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms. *J Endocrinol* 184:153–163.
6. Gans SE, McClintock MK (1993) Individual differences among female rats in the timing of the preovulatory LH surge are predicted by lordosis reflex intensity. *Horm Behav* 27:403–417.

7. LeFevre J, McClintock MK (1988) Reproductive senescence in female rats: A longitudinal study of individual differences in estrous cycles and behavior. *Biol Reprod* 38:780–789.
8. Nequin LG, Alvarez J, Schwartz NB (1979) Measurement of serum steroid and gonadotropin levels and uterine and ovarian variables throughout 4 day and 5 day estrous cycles in the rat. *Biol Reprod* 20:659–670.
9. LeFevre J, McClintock MK (1991) Isolation accelerates reproductive senescence and alters its predictors in female rats. *Horm Behav* 25:258–272.
10. Hermes GL, McClintock MK (2008) Isolation and the timing of mammary gland development, gonadarche, and ovarian senescence: Implications for mammary tumor burden. *Dev Psychobiol* 50:353–360.

PNAS proof
Embargoed



Fig. S4: Statistical analyses. Exploration Stress: Social Condition $F(1, 36) = 52.1, P \leq 0.0001$; Age $F(1, 36) = 1.8, NS$; Interaction $F(1, 36) = 4.8, P \leq 0.03$. Isolated animals did not improve with age as did those in groups. Anxiety: Social Condition $F(1, 34) = 13.80, P \leq 0.001$; Age $F(1, 34) = 0.1, NS$; Interaction $F(1, 34) = 0.0, NS$. Boldness: Social Condition $F(1, 34) = 57.5, P \leq 0.0001$; Age $F(1, 34) = 0.0, NS$; Interaction $F(1, 34) = 0.2, NS$.

Sf1

This legend has been moved to the main article, and inserted at the end of Figure 4 Figure legend.

PNAS proof
Embargoed

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES

1

done A—Au: Please contact Betty Cherniak at PNASProofReturn@cadmus.com or 410-691-6452 if you have questions about the editorial changes, this list of queries, or the figures in your article. Please include your manuscript number in all e-mail correspondence (the number will begin with 09 and be followed by five additional numbers, e.g., 09-01234). Furthermore, if returning proofs electronically, please e-mail them to the above address. Please (i) review the author affiliation and footnote symbols carefully, (ii) check the order of the author names, and (iii) check the spelling of all author names and affiliations. Please indicate that the author and affiliation lines are correct by writing OK in margin next to the author line. Please note that this is your opportunity to correct errors in your article prior to publication. Corrections requested after online publication will be considered and processed as errata.

OK B—Au: PNAS does not allow statements of novelty or priority; therefore, descriptors such as 'new,' 'novel,' and 'the first' have been removed.

bottom edges cut off, as noted in text C—Au: If your article contains figures that were submitted in an unsupported or problematic format (e.g., any format other than .eps or .tif), please check the quality of the figures closely. Please note that if figures need to be replaced, the standard charges listed on the reprint form may be incurred.

published reference substituted D—Au: Please provide updated publication information for reference 10. Please note that PNAS does not allow citation of unpublished information, and thus, if this manuscript is not yet 'In press' this reference must be removed and the reference renumbered accordingly.

in text E—Au: Please provide updated publication information for main text reference 39 if available.

OK F—Au: Please review the information in the author contribution footnote carefully. Please make sure that the information is correct and that the correct author initials are listed. Note that the order of author initials matches the order of the author line per journal style. You may add contributions to the list in the footnote; however, funding should not be an author's only contribution to the work.

OK G—Au: Each corresponding author is required to return a completed Reprint and Publication Charges form, whether or not reprints are ordered. Please complete the form, including payment information for applicable publication charges (purchase order number or credit card information), and return the form with your proof corrections. Failure to return a completed

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES

2

form may result in publication delays.

H—Au: Reminder: You have chosen not to pay an additional \$1200 (or \$850 if your institution has
OK a site license) for the PNAS Open Access option.

I—Au: A legend is provided for Fig. S4 but this figure is not cited anywhere in the main text or SI,
and no Figs. S1—S3 are mentioned. Please mark this legend for deletion if appropriate, or
renumber the figure and provide a citation of the figure in the main text (a PNAS requirement
for all elements of the SI).

This legend has been moved to the main
article, and inserted at the end of Figure 4
Figure legend.
