Social Stress Promotes and γ-Aminobutyric Acid Inhibits Tumor Growth in Mouse Models of Non–Small Cell Lung Cancer

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Abstract
Psychologic distress is associated with increased lung cancer incidence and mortality. We have shown that non–small cell lung cancer (NSCLC) cells in vitro are stimulated by the cyclic AMP (cAMP)-dependent activation of cAMP-responsive element binding protein (CREB) and extracellular signal–regulated kinase (ERK) downstream of β-adrenergic receptors and that this pathway is inhibited by the neurotransmitter γ-aminobutyric acid (GABA). Because the stress neurotransmitters noradrenalin and adrenalin are β-adrenergic agonists, the current study has tested the hypothesis that social stress stimulates NSCLC growth in vivo and that GABA inhibits this effect. Social stress was induced in mice carrying xenografts from two NSCLC cell lines in the presence and absence of treatment with GABA. Xenograft sizes were measured after 30 days. Noradrenalin, adrenalin, cortisol, GABA, and cAMP were measured in blood and tumor tissues by immunoassays. Expression of nicotinic receptors in the xenografts was assessed by real-time PCR and Western blotting. Protein expression of phospho (p)-CREB, CREB, phospho (p)-ERK, ERK, and glutamate decarboxylase (GAD) 65 and 67 were determined by Western blotting. Xenograft sizes in stress-exposed mice were significantly increased. Nicotinic acetylcholine receptor (nAChR) subunits α3, α4, α5, and α7 in xenograft tissues showed posttranscriptional induction. Noradrenalin, adrenalin, and cortisol were elevated in serum and xenograft tissue whereas GABA was suppressed. Levels of cAMP, p-CREB, and p-ERK were increased whereas GAD65 and GAD67 were suppressed in tumor tissue. Treatment with GABA reversed the effects of stress. Our findings suggest that social stress stimulates NSCLC by increasing nAChR-mediated stress neurotransmitter signaling and that GABA is a promising novel agent for NSCLC intervention.


Introduction
Lung cancer is the leading cause of cancer death in developed countries, with non–small cell lung cancer (NSCLC) accounting for about 80% of cases (1, 2). NSCLC has a poor prognosis and most patients do not survive 2 years (3). New strategies for the effective prevention of this cancer are therefore urgently needed.

A low socioeconomic status has been associated with increased lung cancer risk and mortality (4). However, the reasons for the socioeconomic disparities in lung cancer risk and mortality are poorly understood. Furthermore, psychologic distress is a significant predictor of lung cancer mortality (5), and lung cancer patients with a high rate of psychologic distress at the time of diagnosis also have a history of preexisting psychologic stress (6). We have shown that β-adrenergic receptors (β-AR) stimulate the proliferation and migration of NSCLC cells in vitro via cyclic AMP (cAMP)-dependent signaling, resulting in the phosphorylation of the mitogen-activated protein kinases extracellular signal–regulated kinase 1/2 (ERK1/2) and the transcription factor cAMP-responsive element binding protein (CREB; refs. 7–11). Exposure of the cells to γ-aminobutyric acid (GABA), a neurotransmitter widely used as a nutritional supplement, blocked this pathway by inhibiting the activation of adenylyl cyclase, an effect mediated by the Gαi-coupled GABA-B receptor (11). Because the stress neurotransmitters noradrenalin and adrenalin are β-AR agonists, these findings suggested that the reported association of lung cancer incidence and mortality with chronic psychologic distress (4, 6) may be caused via β-adrenergic promotion of NSCLC in vitro, whereas GABA may inhibit this effect.

Most stressful stimuli are social in nature, and the majority of stressful stimuli associated with chronic psychologic...
distress and which also enhance the risk for stress-associated disorders are social in nature (12). Chronic social stress may also contribute to the reported excess lung cancer burden not associated with smoking in ethnic groups such as African Americans with significant populations of low socioeconomic status (2). A method for the experimental induction of social stress has been developed in male CD1 mice (12). Using this method, our current investigations have tested the hypothesis that chronic social stress stimulates the growth of NSCLC in mouse models via stress neurotransmitter-induced cAMP-dependent signaling, and that GABA inhibits these effects.

Methods

Animal experiment

The animal experiment was approved by the Institutional Animal Care and Use Committee. Male, 6-week-old athymic nude mice (Harlan Sprague Dawley Inc.) were housed in our laboratory animal facility under standard laboratory conditions with free access to food (autoclaved Purina rodent chow) and autoclaved water. The mice were randomly assigned 5 mice per cage to treatment groups (n = 20). Mice in 2 groups were exposed to social stress for 4 weeks according to published procedure (12) by changing the group composition of each cage twice per week. One group of these animals was then subcutaneously inoculated in the flank region with cells (3 × 10⁶ in 0.2 mL of PBS, viability > 95%) of the human lung AC cell line NCI-H322 (with activating point mutations in K-ras; European Collection of Cell Cultures; Health Protection Agency). The other group was subcutaneously injected in identical fashion with cells from the human lung AC cell line NCI-H441 (without ras mutations; American Type Culture Collection). Both cell lines were authenticated by species-specific PCR evaluation at Research Animal Diagnostic Laboratory (RADIL) in 2010 immediately prior to the start of the current experiments. Social stress was continued in both groups for another 30 days. Two additional groups of mice that were not exposed to social stress were inoculated with identical numbers of cancer cells from the NCI-H322 or NCI-H441 cell line, respectively. Four additional groups of mice (one group for each cell line with and without social stress) inoculated with NCI-H322 or NCI-H441 cells were treated by intraperitoneal injections of GABA (Sigma-Aldrich; 10 mg/kg 5 d/wk for 30 days). All animals were observed for 30 days after inoculation with cancer cells, and body weights were recorded weekly.

Two perpendicular diameters (length and width) of each xenograft were measured weekly, and tumor volumes were calculated as follows: (length/2) x (width/2). At the end of the 30-day observation period, the animals were euthanized by CO₂ inhalation. Blood samples were collected for the determination of adrenalin, noradrenalin, GABA, and cortisol in the serum and of cAMP in the cellular fraction of blood. The tumors were excised and snap frozen in liquid nitrogen for additional analyses.

Imмуnoassays for the detection of noradrenalin, adrenalin, cortisol, and GABA

Quantitative analyses of levels of noradrenalin, adrenalin, GABA, and the stress hormone cortisol were conducted by immunoassays of serum samples and in xenograft tissues. In addition, the levels of systemic cAMP were determined by immunoassays of samples from the cellular fraction of blood and in xenograft tissues. The assays were conducted according to the manufacturer’s instruction (adrenalin and noradrenalin: 2-CAT ELISA, GABA: GABA Elisa, Rocky Mountain Diagnostic, Inc.; cortisol: ELIA kit, Assay Designs; and cAMP: direct cyclic AMP enzyme immunoassay, Assay Designs). Absorbance was read with an ELISA reader at 450 nm for the neurotransmitters and cortisol and at 405 nm for cAMP.

Protein analysis of nicotinic acetylcholine receptors and their effectors by semiquantitative Western blotting

Protein samples were prepared with lysis buffer (T-PER tissue protein extraction reagent (Thermo Scientific), phenylmethylsulfonylfluoride, NaVO₄, dichlorodiphenyloxathione, Na deoxycholate, SDS, and 1 μg/mL of aprotinin, leupeptin, and pepstatin). After heat denaturation at 100°C for 5 minutes, equal amounts of protein were electrophoresed with 12% Novex SDS-PAGE gels (Invitrogen) and transferred onto nitrocellulose membranes and Western blotting carried out, using incubations overnight at 4°C with the following primary antibodies: total CREB (Upstate Biotechnology), phospho (p)-CREB, phospho (p)-ERK1/2, and ERK1/2 (Cell Signaling), nicotinic acetylcholine receptor (nAChR) subunits α3, α4, α5, α7, glutamate decarboxylase 65 (GAD65) and GAD67 (Millipore), and β-actin (Sigma-Aldrich). In addition to the characterization of these antibodies by the vendors, we have recently verified the specificity of the antibodies for nAChR subunits α7 and α4 by gene knockdown (13). Three independent Western blotting were conducted for each antibody for the semiquantitative assessment of protein expressions by densitometry, with NIH ImageJ software. Following background subtractions, mean densities of 4 rectangular areas of standard size per band were determined, and ratios of protein over actin (nAChR and GAD) or phosphorylated protein over unphosphorylated protein (p-CREB and p-ERK) were calculated.

Quantitative analysis of mRNA for nAChR subunits by real-time PCR

RNA isolation and quantitative analysis of mRNAs were done by real-time PCR as previously described by a Cepheid SmartCycler (14). The primers used for assessment of α5 subunit mRNA were forward 5’-aagggagaggccacctc-3’ and reverse 5’-ggcggcaggccactcatc-3’ (GenBank accession no. NM_000745), and the internal TaqMan probe was 6-FAM-ttaatcgtaggcaggtgtttaatgcca-BHQ1 (Bioscience Technologies). The real-time PCR conditions for α5 were 95°C for 120 seconds, followed by 45 cycles of 95°C, 15 seconds; 56°C, 10 seconds; and 72°C, 15 seconds. For the quantitative determination of mRNAs of nAChR subunits α3, α4,
and α7. Quantitect Primer assays (Qiagen) were used along with the Quantifast SYBR Green PCR Kit, following instructions by the vendor. 18S rRNA detection reagents (Eurogentec) were used for normalization of the data. Real-time PCR data were analyzed by the $2^{-\Delta\Delta C_{\text{t}}}$ method (15).

**Statistical analysis of data**

Statistical analysis was conducted with GraphPad InStat software. Statistical significance of differences between xenograft volumes from animals of all treatment groups ($n = 20$) were determined by unpaired, 2-tailed $t$ tests following verification of a Gaussian distribution of data by the method of Kolmogorov and Smirnov.

Statistical significance of differences between levels of noradrenalin ($n = 5$), adrenalin ($n = 5$), GABA ($n = 4$), cortisol ($n = 4$), and cAMP ($n = 5$) in blood and xenograft tissues was assessed by the nonparametric Mann–Whitney $U$ test.

Statistical significance of differences between 4 densitometric readings per protein bands from 3 independent Western blottings ($n = 12$) was assessed by the nonparametric Mann–Whitney $U$ test because these data did not quite pass the normality test by Kolmogorov and Smirnov.

Statistical evaluation of real-time PCR data ($n = 3$) was by unpaired 2-tailed $t$ test.

**Results**

**Effects of treatments on body weights**

The mice in all treatment groups weighed between 20 and 25 g throughout the experiment, with no statistically significant differences among individual treatment groups.

**Effects of social stress on systemic levels of neurotransmitters and cortisol**

The levels of noradrenalin in serum samples of stress-exposed mice with NCI-H322 xenografts were increased 2.93-fold increase ($P = 0.0079$, Mann–Whitney $U$ test; Table 1), whereas adrenalin was increased 2.73-fold ($P = 0.0079$, Mann–Whitney $U$ test). Similarly, the stress-exposed mice carrying xenografts from cell line NCI-H441 showed significantly increased serum levels of noradrenalin (3.1-fold, $P = 0.0286$, Mann–Whitney $U$ test) and adrenalin (2.6-fold, $P = 0.0286$, Mann–Whitney $U$ test). The serum levels of cortisol were increased 2.58-fold ($P = 0.0286$, Mann–Whitney $U$ test) in mice with NCI-H322 xenografts and 2.24-fold ($P = 0.0286$, Mann–Whitney $U$ test) in mice with NCI-H441 xenografts (Table 1). Systemic levels of GABA (Table 1) showed significant reductions in stress-exposed mice with xenografts from both cell lines (mice with NCI-H322 xenografts: 0.77-fold, $P = 0.0079$, Mann–Whitney $U$ test; mice with NCI-H441 xenografts: 0.58-fold, $P = 0.0286$, Mann–Whitney $U$ test).

**Effects of social stress on neurotransmitters and cortisol in xenograft tissues**

In vitro experiments have established that the proliferation of the NCI-H322 and NCI-H441 and of small airway epithelial cells is stimulated by β-adrenergic agonists via cAMP-dependent signaling (7–10) and that GABA inhibits this response. In addition, in vitro studies with small airway epithelial cells and cell line NCI-H322 have shown that the synthetic glucocorticoid dexamethasone increases intracellular cAMP via nongenomic mechanisms, resulting in a significant stimulation of cell proliferation (16). We therefore measured the levels of both stress neurotransmitters, cortisol and GABA, in xenograft tissues. As Table 1 shows, noradrenalin was increased 2.74-fold in NCI-H322 xenografts ($P = 0.0079$, Mann–Whitney $U$ test) and 2.52-fold in NCI-H441 xenografts ($P = 0.0286$, Mann–Whitney $U$ test). The levels of adrenalin were also significantly increased in xenografts from both cell lines (NCI-H322 xenografts: 2.08-fold, $P = 0.0079$, Mann–Whitney $U$ test; NCI-H441 xenografts: 2.14-fold, $P = 0.0286$, Mann–Whitney $U$ test). GABA was significantly decreased in xenograft tissues (Table 1) by both cell lines (NCI-H322 xenografts: 0.86-fold, $P = 0.0286$, Mann–Whitney $U$ test; NCI-H441 xenografts: 0.64-fold, $P = 0.0286$, Mann–Whitney $U$ test). Social stress increased the cortisol levels in xenografts from both cell lines (Table 1; NCI-H322: 2.3-fold, $P = 0.0286$, Mann–Whitney $U$ test; NCI-H441: 2.25-fold, $P = 0.0286$, Mann–Whitney $U$ test).

**Effects of social stress on xenograft sizes**

Chronic exposure to social stress was well tolerated by all mice as indicated by the absence of significant changes in body weight. Xenografts from the cell line that expresses activating point mutations in K-ras (NCI-H322) generally grew faster than NCI-H441 xenografts (without ras mutations), resulting in larger tumor sizes regardless of treatment group (Fig. 1). Thirty days after inoculation with tumor cells, xenografts from cell line NCI-H322 in mice exposed to social stress showed a 1.63-fold significant ($P = 0.0474$, unpaired 2-tailed $t$ test) increase in volume over that from unstressed animals (Fig. 1). The slower growing xenografts from NCI-H441 cells showed a 3.2-fold significant ($P = 0.0033$, unpaired 2-tailed $t$ test) stress-induced increase in volume (Fig. 1).

**Effects of social stress on nAChR subunits in xenograft tissues**

nAChRs containing the subunits α7, α5, and α3 stimulate the release of noradrenalin and adrenalin in neuronal tissues and in the adrenal glands (17, 18), whereas nAChRs containing the α4 subunits stimulate the release of GABA in neuronal tissues (19). In vitro studies have shown that the α7 nAChR regulates the synthesis and release of noradrenalin and adrenalin from small airway epithelial cells and NCI-H322 cells, whereas the α4β2 nAChR regulates the synthesis and release of GABA in these cells (13, 20). As expression of nAChR subunits α3, α4, α5, and α7 have been reported in airway epithelial cells (21), we assessed the expression levels of these nAChR subunits by semiquantitative Western blot analyses and real-time PCR in xenograft tissues of stress-exposed and unstressed mice. The protein expression of each of these investigated nAChR subunits was...
was significantly \( (P = 0.0001, \text{Mann–Whitney } U \text{ test}) \) in all cases) increased (up to 2.77-fold) by social stress in xenografts from both cell lines (Fig. 2). In contrast, mRNAs of the \( \alpha5 \) subunit were significantly \( (P = 0.0327, \text{unpaired } 2\text{-tailed } t \text{ test}) \) downregulated by social stress in NCI-H322 xenografts whereas remaining unchanged in NCI-H441 xenografts (Table 2). None of the other investigated \( \alpha \) subunits showed significant changes in mRNA levels in stress-exposed mice (Table 2).

### Effects of social stress on cAMP in blood cells and xenograft tissue

Binding of noradrenalin or adrenalin to the G-protein–coupled \( \beta1 \)- and \( \beta2 \)-ARs activates adenylyl cyclase, the rate-limiting step for the formation of intracellular cAMP (22). In addition, cortisol increases intracellular cAMP in small airway epithelial cells and NCI-H322 cells via nongenomic mechanisms (16). We therefore assessed by immunoassays whether the observed stress-induced increases in noradrenalin, adrenalin, and cortisol in fact lead to increased intracellular cAMP in xenograft tissues and in the cellular fraction of blood samples. As Fig. 3 shows, stress-exposed mice carrying xenografts from both cell lines showed significantly increased \( (P = 0.0079, \text{Mann–Whitney } U \text{ test}) \) systemic cAMP in blood cells (1.95-fold) in mice carrying NCI-H322 xenografts and 1.71-fold in mice carrying NCI-H441 xenografts. The stress-induced increases in intracellular cAMP in xenograft tissues (Fig. 3) were even higher (NCI-H322 xenografts: 3.22-fold, \( P = 0.0079, \text{Mann–Whitney } U \text{ test} \); NCI-H441 xenografts: 2.71, \( P = 0.0079, \text{Mann–Whitney } U \text{ test} \)).

### Effects of social stress on GAD65 and GAD67

In accordance with the observed significant decreases in systemic and xenograft GABA levels (Table 1), the protein expression of the GABA-synthesizing enzyme GAD65 (Fig. 2) was significantly decreased in xenografts from both cell lines (NCI-H322 xenografts: 0.55-fold, \( P = 0.0001, \text{Mann–Whitney } U \text{ test} \); NCI-H441 xenografts: 0.47-fold, \( P = 0.0001, \text{Mann–Whitney } U \text{ test} \)). Similarly, GAD67 protein expression in stress-exposed mice (Fig. 2) was significantly (\( P = 0.0001, \text{Mann–Whitney } U \text{ test} \)) reduced in xenografts from both cell lines (NCI-H322: 0.54-fold; NCI-H441: 0.51-fold).

### Effects of social stress on cAMP-mediated cellular signaling

In vitro studies with NCI-H322 cells and small airway epithelial cells have shown that exposure to \( \beta \)-adrenergic agonists stimulates the proliferation and migration of these cells via cAMP-dependent activation of protein kinase A (PKA), p-CREB, and PKA-dependent transactivation of the EGFR and its downstream effectors ERK1/2 (8–10). To assess a potential activation of this signaling cascade by social stress, we therefore determined the relative expression levels of p-CREB and p-ERK by semiquantitative Western blotting. As Fig. 4 shows, p-CREB was significantly (\( P = 0.0001, \text{Mann–Whitney } U \text{ test} \)) induced in xenografts from NCI-H322 xenografts (2.71, \( P = 0.0079, \text{Mann–Whitney } U \text{ test} \)).

### Table 1. Modulation of serum and xenograft levels of noradrenalin (\( n = 5 \)), adrenalin (\( n = 5 \)), cortisol (\( n = 4 \)), and GABA (\( n = 5 \)) in response to social stress

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Stress response</th>
<th>Mean</th>
<th>SD</th>
<th>( P^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H322</td>
<td>Serum noradrenalin</td>
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<td></td>
<td>Xenograft noradrenalin</td>
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<td>0.19</td>
<td>0.0079</td>
</tr>
<tr>
<td></td>
<td>Serum adrenalin</td>
<td>2.73</td>
<td>0.33</td>
<td>0.0079</td>
</tr>
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<td></td>
<td>Xenograft adrenalin</td>
<td>2.08</td>
<td>0.44</td>
<td>0.0079</td>
</tr>
<tr>
<td></td>
<td>Serum cortisol</td>
<td>2.58</td>
<td>0.16</td>
<td>0.0286</td>
</tr>
<tr>
<td></td>
<td>Xenograft cortisol</td>
<td>2.3</td>
<td>0.17</td>
<td>0.0286</td>
</tr>
<tr>
<td></td>
<td>Serum GABA</td>
<td>0.77</td>
<td>0.03</td>
<td>0.0079</td>
</tr>
<tr>
<td></td>
<td>Xenograft GABA</td>
<td>0.86</td>
<td>0.02</td>
<td>0.0286</td>
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<tr>
<td>NCI-H441</td>
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<tr>
<td></td>
<td>Xenograft noradrenalin</td>
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<td>0.0286</td>
</tr>
<tr>
<td></td>
<td>Serum adrenalin</td>
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<td>0.19</td>
<td>0.0286</td>
</tr>
<tr>
<td></td>
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<td>0.16</td>
<td>0.0286</td>
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<tr>
<td></td>
<td>Serum cortisol</td>
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<td>0.17</td>
<td>0.0286</td>
</tr>
<tr>
<td></td>
<td>Xenograft cortisol</td>
<td>2.25</td>
<td>0.10</td>
<td>0.0286</td>
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<tr>
<td></td>
<td>Serum GABA</td>
<td>0.58</td>
<td>0.03</td>
<td>0.0286</td>
</tr>
<tr>
<td></td>
<td>Xenograft GABA</td>
<td>0.64</td>
<td>0.06</td>
<td>0.0286</td>
</tr>
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</table>

NOTE: Data are mean values and SDs expressed as fold change in stress-exposed mice over mice not exposed to stress. \( P \) values were established by Mann–Whitney \( U \) tests.

\(^a\)The \( P \) values represented are that for NS and SS mice.
both cell lines (NCI-H322 xenografts: 3.38-fold; NCI-H441 xenografts: 2.48-fold). Similarly, p-ERK was significantly (\(P = 0.0001\), Mann–Whitney \(U\) test) induced by social stress in xenografts from both cell lines (NCI-H322 xenografts: 2.49-fold; NCI-H441 xenografts: 2.42-fold).

Cancer preventive effects of GABA treatment

GABA injections of mice carrying NCI-H322 or NCI-H441 xenografts significantly reduced the xenograft volumes in both stress-exposed (NCI-H322 xenografts, \(P = 0.0118\); NCI-H441 xenografts, \(P = 0.0001\)) and unstressed (NCI-H322 xenografts, \(P = 0.0001\); NCI-H441 xenografts, \(P = 0.0001\)) mice below the tumor sizes in animals not stimulated by stress (Fig. 1). Social stress significantly \((P = 0.0001)\) reduced this cancer preventive effect of GABA in NCI-H322 xenografts, whereas this difference was not significant in NCI441 xenografts (Fig. 1). In accordance with the documented function of GABA as a physiologic inhibitor of cAMP formation (23, 24), the systemic levels of cAMP in the cellular fraction of blood as well as in xenograft tissues were significantly \((P = 0.001)\) reduced in both social stress–exposed and unstressed animals treated with GABA (Fig. 3). In addition, the social stress–induced phosphorylation of CREB \((P = 0.0286)\) and ERK \((P = 0.0002)\) were significantly reduced by GABA treatment (Fig. 4).

Discussion

Our data show, for the first time, that social stress promotes whereas GABA prevents the growth of lung cancer in vivo. Social stress significantly promoted the growth of xenografts from both NSCLC cell lines, whereas GABA reversed this effect. In addition, GABA had strong inhibiting effects on base level growth of the unstimulated xenografts. These findings suggest that chronic social stress enhances the progression of NSCLC in human patients and identify GABA as a promising novel agent for the prevention and adjuvant therapy of NSCLC. The observed stress responses and their effects on xenograft growth are unlikely to be gender- or mouse strain–specific as female mice (25) have shown similar stress responses as males (12) to the social stress-inducing manipulations used by us, and studies in female severe combined immunodeficient (SCID) mice have revealed significant tumor promoting effects of psychologic stress associated with increased levels of stress neurotransmitters and \(\beta\)-adrenergic signaling in ovarian cancer xenografts in female SCID mice (26). Furthermore, the process of xenografting itself did not seem to cause stress responses in our study as...
serum cortisol levels (2.7–3.3 ng/mL) in our nonstress-exposed mice were similar to those reported in untreated mice without xenografts by another laboratory (12).

The observed systemic increases in cortisol, noradrenalin, and adrenalin in mice exposed to social stress are classic indicators of psychologic distress in humans (17) and confirm that the repeated regrouping of mice used in this study caused psychologic distress as established by another laboratory (12). Receptors for which these agents are the physiologic agonists (β-ARs for noradrenalin and adrenalin, glucocorticoid receptors for cortisol) are expressed in NSCLC cells and airway epithelial cells (8, 10, 16). In addition, it has been shown that airway epithelia and NSCLC harbor the complete machinery for the regulation and production of stress neurotransmitters (13, 20, 27).

The observed significant stress-induced increase in α7 nAChR protein is in accordance with similar findings in human airway epithelial cells or neuronal cells exposed to chronic nicotine or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK; refs. 13, 20, 28). In turn, binding of the increased noradrenalin and adrenalin to β-ARs in the xenograft tissues activated adenyl cyclase–dependent cAMP signaling, leading to activation of CREB and ERK. The simultaneous increase in systemic and xenograft cortisol levels may have, in addition, intensified the growth stimulating effects of this pathway as glucocorticoids activate cAMP-mediated signaling via nongenomic mechanisms in human airway epithelial cells and NSCLC cells (16).

The observed increases in protein expression of the α4β2 nAChR in conjunction with the reduction in GAD65, GAD67, and GABA in the xenografts of stress-exposed mice is suggestive of receptor upregulation in response to chronic agonist–induced desensitization that has been described in neuronal cells (28), in small airway epithelial cells and in NCI-H322 cells (13, 20). Our findings are also in accordance with reports that chronic stress reduced neuronal GAD65 and GABA, leading to functional impairment of the GABAergic network in mice (29, 30). As cAMP signaling downstream of β-ARs stimulates the growth and migration of NSCLC cells whereas GABA inhibits these effects (11), the stress-induced increases in noradrenalin, adrenalin, and cortisol and concomitant decrease in GABA provided an environment that significantly stimulated the growth of xenografts from both NSCLC cell lines. This interpretation is further supported by the observed significant inhibitory effects of GABA treatment on xenograft growth, cAMP levels, and the phosphorylation of cAMP-induced CREB and

### Table 2. Real-time PCR data of nAChR subunits α3, α4, α5, and α7 in NCI-H322 and NCI-H441 xenografts from mice exposed to SS and from NS

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mean</th>
<th>SD</th>
<th>P*</th>
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<tbody>
<tr>
<td>NCI-H322</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α3</td>
<td>1.28</td>
<td>0.03</td>
<td>0.7971</td>
</tr>
<tr>
<td>α4</td>
<td>0.91</td>
<td>0.34</td>
<td>0.5020</td>
</tr>
<tr>
<td>α5</td>
<td>0.49</td>
<td>0.19</td>
<td>0.0327</td>
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<tr>
<td>α7</td>
<td>1.41</td>
<td>1.11</td>
<td>0.6699</td>
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<tr>
<td>NCI-H441</td>
<td></td>
<td></td>
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<tr>
<td>α3</td>
<td>0.78</td>
<td>0.57</td>
<td>0.3410</td>
</tr>
<tr>
<td>α4</td>
<td>3.66</td>
<td>2.84</td>
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</tr>
<tr>
<td>α5</td>
<td>1.34</td>
<td>0.37</td>
<td>0.5110</td>
</tr>
<tr>
<td>α7</td>
<td>7.45</td>
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NOTE: Ct values corrected by 18S control values are expressed as fold change of SS over NS. RNA for the α5 subunit in stress-exposed NCI-H322 xenografts was down-regulated (0.49). Data are mean values and SDs of triplicate samples. The P values were established by unpaired, 2-tailed t tests.

*The P values represented are that for NS and SS mice.

**P values of 0.05 were considered significant.

### Figure 3. Results of immunoassays for the determination of cAMP in the cellular fraction of blood (A) and in xenograft tissues (B) from mice carrying xenografts from NCI-H322 and NCI-H441 cells and exposed to chronic SS and from NS and GABA-treated animals with (SS + G) and without (NS + G) stress. Columns in the graphs represent mean values and SDs (n = 5) expressed as fold increase or decrease of values from stress-exposed or GABA-treated mice over those from untreated unstressed mice.
the functional significance of social stress-induced protein increase of nAChR subunits α3 and α5 is less clear. Receptors containing these subunits can assemble in a variety of configurations either together or with subunits α4 or α6 and contribute to the regulation of stress neurotransmitters in the adrenal gland (17, 18, 35). However, their function in different types of lung cells and lung cancers is poorly understood. It has been shown that an nAChR with the composition α3β5β2 contributes to wound repair processes in the airway epithelium by stimulating the migration of cells (36). In addition, genome-wide association studies have identified single-nucleotide polymorphisms in African Americans associated with lung cancer risk, particularly NSCLC, in genes CHRNA3 and CHRNA5 for the α3 and α5 nAChR subunits (37).

Our data suggest that chronic social stress is a risk factor for NSCLC that facilitates the development and promotes the growth of this cancer by modulating the expression and function of nAChRs and the neurotransmitters regulated by these receptors. In addition, our findings imply that psychologic stress may significantly reduce the efficacy of cancer preventive and therapeutic agents. Reversal of stress-induced tumor promotion by GABA suggests that GABA may improve therapeutic and preventive outcomes of this malignancy. GABA has been used as a safe nutritional supplement for many years because of its calming and relaxing effects and numerous fruits, vegetables, and grains are rich in GABA. Cancer intervention with this agent can therefore be achieved by a nutritional approach. A recent publication has reported a significant stress-induced increase in the metastasis of orthotopic breast cancer xenografts (38). On the other hand, women receiving β-blocker therapy showed a significant reduction in breast cancer metastasis, recurrence, and mortality (39). Moreover, noradrenalin-induced stimulation of cancers in other organs, including stomach, colon, prostate, and ovary have been reported (40–43). GABA may therefore also be suitable for prevention and adjuvant therapy of these malignancies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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