Social stress promotes and γ-aminobutyric acid inhibits tumor growth in mouse models of non small cell lung cancer.

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Abstract.

Psychological 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 cAMP-dependent activation of CREB and ERK downstream of beta-adrenergic receptors and that this pathway is inhibited by the neurotransmitter $\gamma$-aminobutyric acid (GABA). Because the stress neurotransmitters noradrenalin and adrenaline are beta-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, adrenaline, 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 p-CREB, CREB, 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 $\alpha_3, \alpha_4, \alpha_5, \alpha_7$ in xenograft tissues showed posttranscriptional induction. Noradrenalin, adrenaline and cortisol were elevated in serum and xenograft tissue while GABA was suppressed. Levels of cAMP, p-CREB and p-ERK were increased while GAD 65 and GAD 67 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 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 socio-economic status has been associated with increased lung cancer risk and mortality (4). However, the reasons for the socio-economic disparities in lung cancer risk and mortality are poorly understood. Furthermore, psychological distress is a significant predictor of lung cancer mortality (5) and lung cancer patients with a high rate of psychological distress at the time of diagnosis also have a history of pre-existing psychological stress (6). We have shown that beta-adrenergic receptors (β-ARs) stimulate the proliferation and migration of NSCLC cells in vitro via cAMP-dependent signaling, resulting in the phosphorylation of the mitogen-activated kinases Erk1/2 and the transcription factor CREB (7-11). Exposure of the cells to γ-aminobutric 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 beta-adrenergic receptor agonists, these findings suggested that the reported association of lung cancer incidence and mortality with chronic psychological distress (4, 6) may be caused via beta-adrenergic promotion of NSCLC in vivo while GABA may inhibit this effect.

Most stressful stimuli are social in nature and the majority of stressful stimuli associated with chronic psychological 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 socio-economic 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., Indianapolis, IN, USA) 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 five mice per cage to treatment groups (n=20). Mice in two 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 (3X10^6 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, Porton Down, Salisbury, Wiltshire, UK). 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, Manassas, VA, USA). Both cell lines were authenticated by species-specific PCR evaluation at Research Animal Diagnostic Laboratory (RADIL, Columbia, MO, USA) 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 one without social stress) inoculated with NCI-H322 or NCI-H441 cells were treated by intraperitoneal injections of GABA (Sigma, St Louis, MO; 10 mg/Kg 5 days/week for 30 days). All animals were observed for 30 days after inoculation with cancer cells and bodyweights were recorded weekly.

Two perpendicular diameters (length and width) of each xenograft were measured weekly and tumor volumes were calculated (length/2) X (width²). 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.

*Immunoassays 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., Colorado Spring, CO, USA; cortisol: EIA kit: Assay Designs, Ann Arbor, MI, USA cAMP: direct cyclic AMP enzyme immunoassay, Assay Designs, Ann Arbor, MI, USA). Absorbance was read with an ELISA reader at 450 nm for the neurotransmitters and cortisol and at 405 nm for cAMP.
**Protein analysis of nAChRs and their effectors by semi-quantitative Western Blotting:**

Protein samples were prepared using lysis buffer (T-PER tissue protein extraction reagent (Thermo Scientific, Rockford, IL), PMSF, Na$_3$ VO$_4$, DDT, Na deoxycholate, SDS, and 1µg/ml of aprotinin, leupeptin, and pepstatin). After heat denaturation at 100°C for five minutes, equal amounts of protein were electrophoresed using 12% Novex SDS-polyacrylamide gels (Invitrogen) and transferred onto nitrocellulose membranes and Western blots performed, using incubations overnight at 4°C with the following primary antibodies: total CREB (Upstate Biotechnology, Lake Placid, NY, USA), p-CREB, p-ERK1/2 and ERK1/2 (Cell Signaling, Danvers, MA, USA), nAChR subunits $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_7$, glutamate decarboxylase 65 (GAD 65) and GAD 67 (Millipore, Billerica, MA, USA) and $\beta$-actin (Sigma-Aldrich). In addition to the characterizations of these antibodies by the vendors, we have recently verified the specificity of the antibodies for nAChR subunits $\alpha_7$ and $\alpha_4$ by gene knockdown (13). Three independent Western blots were conducted for each antibody for the semi-quantitative assessment of protein expressions by densitometry, using NIH ImageJ software. Following background subtraction, mean densities of four rectangular areas of standard size per band were determined and ratios of protein over actin (nAChRs and GADs) or phosphorylated protein over unphosphorylated protein (p-CREB, 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 using a Cepheid smart cycler (14). The primers used for assessment of $\alpha_5$ subunit mRNA were forward 5´- aaggccggagcctaca -3´ and reverse 5´- gctggcaggcaatctaatc -3´ (GenBank accession no. NM_000745), and the internal TaqMan probe was 6-FAM-
ttaatcggaggtgtattatagcc -BHQ1 (Biosearch Technologies, Novato, CA, USA). The real-time PCR conditions for \( \alpha_5 \) were 95°C for 120 s, followed by 45 cycles of 95°C, 15 s; 56°C, 10 s; and 72°C, 15 s. For the quantitative determination of mRNAs of nAChR subunits \( \alpha_3 \), \( \alpha_4 \), and \( \alpha_7 \), QuantiTect Primer assays (Qiagen, Valencia, CA) were used along with the QuantiFast SYBR Green PCR kit, following instructions by the vendor. 18S rRNA detection reagents (Eurogentec, San Diego, CA, USA) were used for normalization of the data. Real-time PCR data were analyzed using the \( 2^{-\Delta\Delta C_T} \) method (15).

**Statistical Analysis of Data.**

Statistical analysis was performed using Graphpad Instat software. Statistical significance of differences between xenograft volumes from animals of all treatment groups (n = 20) were determined by unpaired, two-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 non-parametric Mann-Whitney test.

Statistical significance of differences between four densitometric readings per protein bands from three independent Western blots (n = 12) was assessed by the non parametric Mann-Whitney 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 two-tailed t-test.
Results.

Effects of treatments on bodyweights: The mice in all treatment groups weighed between 20 and 25 grams 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 test; Table 1) while adrenalin was increased 2.73-fold (p = 0.0079, Mann-Whitney 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 test) and adrenalin (2.6-fold, p = 0.0286, Mann-Whitney test). The serum levels of cortisol were increased 2.58-fold (p = 0.0286, Mann-Whitney test) in mice with NCI-H322 xenografts and 2.24-fold (p = 0.0286, Mann-Whitney 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 test; mice with NCI-H441 xenografts: 0.58-fold, p = 0.0286, Mann-Whitney 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 beta-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 non-genomic 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 test) and 2.52-fold in NCI-H441 xenografts (p = 0.0286, Mann-Whitney 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 test; NCI-H441 xenografts: 2.14-fold, p = 0.0286, Mann-Whitney test). GABA was significantly decreased in xenograft tissues (Table 1) from both cell lines (NCI-H322 xenografts: 0.86-fold, p = 0.0286, Mann-Whitney test; NCI-H441 xenografts: 0.64-fold, p = 0.0286, Mann-Whitney 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 test; NCI-H441: 2.25-fold (p = 0.0286, Mann-Whitney 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 bodyweight. 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 (Figure 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 two-tailed t-test) increase in volume over that from unstressed animals (Figure 1). The slower growing xenografts from NCI-H441 cells showed a 3.2-fold significant (p = 0.0033, unpaired two-tailed t-test) stress-induced increase in volume (Figure 1).
Effects of social stress on nAChR subunits in xenograft tissues: Nicotinic acetylcholine receptors containing the subunits α7, α5, and α3 stimulate the release of noradrenalin and adrenalin in neuronal tissues and in the adrenal glands (17, 18) while nAChRs containing the α4 subunits stimulate the release of GABA in neuronal tissues (19). In vitro studies have shown that the α7nAChR regulates the synthesis and release of noradrenalin and adrenalin from small airway epithelial cells and NCI-H322 cells while the α4β2nAChR 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 semi quantitative 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 significantly (p = 0.0001 by Mann-Whitney test in all cases) increased (up to 2.77-fold) by social stress in xenografts from both cell lines (Figure 2). By contrast mRNAs of the α5 subunit was significantly (p = 0.0327 by unpaired two-tailed t-test) down regulated by social stress in NCI-H322 xenografts while 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 β1 and β2-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 non-genomic mechanisms (16). We therefore assessed by immunoassays if 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 Figure 3 shows, stress-exposed mice carrying
xenografts from both cell lines demonstrated significantly increased (p = 0.0079, Mann-Whitney 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 (Figure 3) were even higher (NCI-H322 xenografts: 3.22-fold, p = 0.0079, Mann-Whitney test; NCI-H441 xenografts: 2.71, p = 0.0079, Mann-Whitney test).

Effects of social stress on GAD65 and GAD67: In accord with the observed significant decreases in systemic and xenograft GABA levels (Table 1), the protein expression of the GABA synthesizing enzyme GAD 65 (Fig. 2) was significantly decreased in xenografts from both cell lines (NCI-H322 xenografts: 0.55-fold, p = 0.0001, Mann-Whitney test; NCI-H441 xenografts: 0.47-fold, p = 0.0001, Mann-Whitney test). Similarly, GAD67 protein expression in stress-exposed mice (Fig. 2) was significantly (p = 0.0001, Mann-Whitney 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 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 phosphorylated CREB and phosphorylated ERK by semi quantitative Western blotting. As Figure 4 shows, p-CREB was significantly (p = 0.0001, Mann-Whitney test) induced in xenografts from 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 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 (Figure 1). Social stress significantly (p = 0.0001) reduced this cancer preventive effect of GABA in NCI-H322 xenografts while this difference was not significant in NCI441 xenografts (Fig. 1). In accord with the documented function of GABA as a physiological 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 while GABA prevents the growth of lung cancer in vivo. Social stress significantly promoted the growth of xenografts from both NSCLC cell lines while 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 SCID mice have revealed significant tumor promoting effects of psychological 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 appear to cause stress responses in our study as serum cortisol levels (2.7-3.3 ng/ml) in our non stress 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 psychological distress in humans (17) and confirm that the repeated re-grouping of mice used in this study caused psychological distress as established by another laboratory (12). Receptors for which these agents are the physiological 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 a7nAChR protein is in accord with similar findings in human airway epithelial cells or neuronal cells exposed to chronic nicotine or NNK (13, 20, 28). In turn, binding of the increased noradrenalin and adrenalin to β-ARs in the xenograft tissues activated adenylly cyclase-dependent cAMP signaling, leading to activation of CREB and ERK. The simultaneous increase in systemic and xenograft cortisol levels may have additionally intensified the growth stimulating effects of this pathway as glucocorticoids activate
cAMP-mediated signaling via non-genomic mechanisms in human airway epithelial cells and NSCL cells (16).

The observed increases in protein expression of the α4β2nAChR in conjunction with the reduction in GAD65, GAD67 and GABA in the xenografts of stress-exposed mice is suggestive of receptor up regulation 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 accord 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 while 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 phosporylation of cAMP-induced CREB and ERK in stress-exposed and unstressed mice. It has also been shown that GABA inhibits nicotine-induced growth of pancreatic cancer xenografts that express a beta-adrenergic regulatory pathway (31) as well as the noradrenalin-induced migration of colon cancer (32) and breast cancer (33) in vitro. GABA is the main inhibitory neurotransmitter in the brain where it counteracts numerous stimulating responses to Gαs-coupled neurotransmitter receptors by binding to the Gαi-coupled GABA-B receptor, thus blocking the activation of adenylyl cyclase (34). In analogy to this function in the brain, GABA inhibited beta-adrenergic receptor-induced cAMP-dependent growth and migration of NSCLC cells and this effect was abrogated by gene knockdown of the GABA-B receptor (11).
The functional significance of social stress-induced protein increase of nAChR subunits \(\alpha_3\) and \(\alpha_5\) is less clear. Receptors containing these subunits can assemble in a variety of configurations either together or with subunits \(\alpha_4\) or \(\alpha_6\) 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 \(\alpha_3\alpha_5\beta_2\) 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 \(\alpha_3\) and \(\alpha_5\) 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 psychological 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 beta-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.

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References.


Figure Legends.

Figure 1. Effects of social stress on the size of xenografts from human NSCLC cell lines NCI-H322 and NCI-H441 and the effects of GABA treatment on NCI-H322 and NCI-H441 xenografts in unstressed (NS+G) stress exposed (SS+G) mice. The photographs in A exemplify the tumor sizes in the 4 treatment groups. The graph (B) illustrates tumor volumes calculated from two perpendicular diameters of each xenograft. Data in the graph are mean values and standard deviations of xenograft volumes from 20 mice per group expressed as fold change of xenograft volumes from the stress-exposed and GABA treated groups over that from untreated mice not exposed to stress.
Figure 2. Representative examples of Western blots (A) for the assessment of protein expression of the nAChR subunits α3, α4, α5 and α7 and of the two isozymes of the GABA synthesizing enzyme GAD in xenografts from NSCLC cell lines NCI-H322 and NCI-H441 of mice exposed to social stress (SS) and unstressed mice (NS). Columns in the graph (B) represent mean values and standard deviations of four densitometric readings per protein band adjusted for actin from three independent Western blots after background subtraction (n = 12).

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 social stress (SS) and from unstressed mice (NS) and GABA treated animals with (SS+G) and without (NS+G) stress. Columns in the graphs represent mean values and standard deviations (n = 5) expressed as fold increase or decrease of values from stress-exposed or GABA treated mice over those from untreated unstressed mice.

Figure 4. Western blots (A) exemplifying the protein expression of p-ERK and p-CREB in xenograft tissues from NCI-H 322 and NCI-H441 cells in untreated mice without stress (NS), social stress exposed mice (SS) and from GABA treated mice without (NS+G) and with (SS+G) stress. Columns in the graph (B) are mean values and standard errors of densitometry ratios of phosphorylated protein/total protein calculated from 4 densitometric readings per band of 3 independent Westerns blots (n = 12) and are expressed as fold increase/decrease of values over untreated animals without stress (NS).
Figure 1

Xenograft Volumes (fold increase)

A

NCI-H322

NS  SS

NS+G SS+G

NCI-H441

NS  SS

NS+G SS+G

10 mm
Figure 2

Mean Density (fold change)

A

H322

H441

α3

α4

α5

α7

GAD65

GAD67

Actin

B

NS | SS
α3 | α4 | α5 | α7 | GAD65 | GAD67

H322

H441
Figure 3

(A) Cellular Fraction of Blood

(B) Xenograft Tissue

Legend:
- NS
- SS
- NS+G
- SS+G
Figure 4
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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. Data are mean values and standard deviations expressed as fold change in stress exposed mice over mice not exposed to stress. P values were established by Mann-Whitney tests.
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 social stress (SS) and from unstressed animals (NS). 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 standard deviations of triplicate samples. The p-values were established by unpaired, two-tailed t-tests.

| Gene   | Fold Change | p-Value |  |
|--------|-------------|---------| |
|        | Mean  | SD    | NS  | SS |
| NCI-H322: |       |       |     |    |
| α3     | 1.28  | 0.03  | 0.7971 | |
| α4     | 0.91  | 0.34  | 0.5020 | |
| α5     | 0.49  | 0.19  | 0.0327* | |
| α7     | 1.41  | 1.11  | 0.6699 | |
| NCI-H441: |       |       |     |    |
| α3     | 0.78  | 0.57  | 0.3410 | |
| α4     | 3.66  | 2.84  | 0.5980 | |
| α5     | 1.34  | 0.37  | 0.5110 | |
| α7     | 7.45  | 6.23  | 0.9324 | |
Social stress promotes and γ-aminobutyric acid inhibits tumor growth in mouse models of non small cell lung cancer


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