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The Transgenic TGF-alpha or EGFR1 Overexpression Mouse Model for Symptom
Complex Research

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14. ABSTRACT: Running wheel studies were conducted on two strains of transgenic mice to test the hypothesis that ligands of the EGFR inhibit hypothalamic modulatory centers regulating 24 hour rest/activity and other circadian functions. Running wheel activity (RWA) was used first to assess the structure (PERIOD) of the rest and activity phases in 12 hour lights on and off (LD) and in constant darkness (DD) conditions. Period was found to be ~ 24 hours in LD and DD conditions for both the transgenic TGF- α and EGFR overexpression mice and similar to the control animals. From this we concluded that the supra chiasmatic nucleus (SCN, master clock) in not disrupted in these animals. The second set of measurements focused on the fractional time active for the mice over 24 hours (ALPHA). These data showed that TGF- α overexpression animals, and not the EGFR animals, had significantly lower ALPHA. These data are consistent with a model of the EGFR ligands can produce disruption of neural signaling downstream from the SCN. We have interpreted this as a cause for fatigue associated with the overexpression of TGF- α as could be found in cancer patients with a tumor that produces this ligand. Next, TGF- α mice were treated with an EGFR tyrosine kinase inhibitor (AG1478), given by the subcutaneous route, to detect an effect in ALPHA. We could find no evidence for such an effect in this model system. Nevertheless, symptoms in cancer patients might be related to the production of neurally active cytokines and growth receptor ligands of the epidermal growth factor family. This suggests that clinical studies should examine for not only changes in tumor growth per se, but for improvement in quality of life and specific symptoms of fatigue in patients receiving anti-EGFR agents.			
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Introduction:

Symptom clusters in cancer patients include fatigue, appetite loss, and sleep disruption. Fatigue is the most common symptom and the cause is likely related to multiple factors. One mechanism for fatigue that has been recently elucidated is the loss of rhythmic circadian activity in animals that receive an intracerebral-ventricular infusion of ligands of the epidermal growth factor receptor, EGFR [1,2,3]. This receptor has been shown to control several rhythmic behaviors in the laboratory rodent. We hypothesize that animals that over express the ligand of the EGFR would show measurable signs of decreased activity.

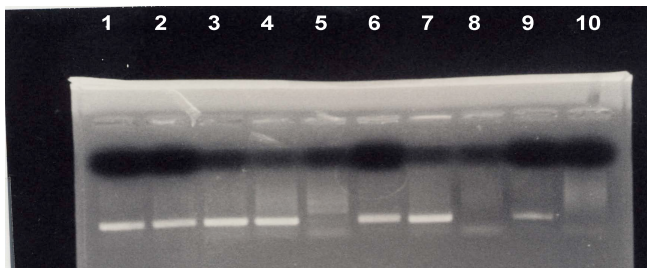
Task 1- Assessment of 24 hour rest activity patterns in wild type and transgenic over expression TGF- α mice:

Our laboratory studies commenced with circadian running wheel behavior of transgenic mice over expressing TGF- α . This work began with setting up a breeding colony of transgenic mice in our core vivarium facilities. The mice started producing offspring after the age of 6 weeks. Because of the propensity for these mice to develop tumors after the age of 6 months active breeding pairs beyond this age were not kept. After starting the breeding colony and having success we noticed that mice aged approximately 4-5 months become less fertile; breeding pairs after the age of 4 months were then retired from continued breeding.

Once the offspring are weaned they are ear tagged and assessed for genotype. The genotype procedure consists of snipping a small piece of the distal tail under auspices of an approved ACUC animal protocol. The tailpiece is digested and assessed for the TGF- α gene expression using a PCR technique. In the first figure below are shown examples of transgenic TGF- α over expression mice where the bright band in the electrophoresis gel demonstrates the presence of the gene in each animal. Once each animal has been genotyped it is now ready to be used in the running wheel experiments.

Figure 1.

Transgenic TGF- α overexpression model:



PCR Analysis:

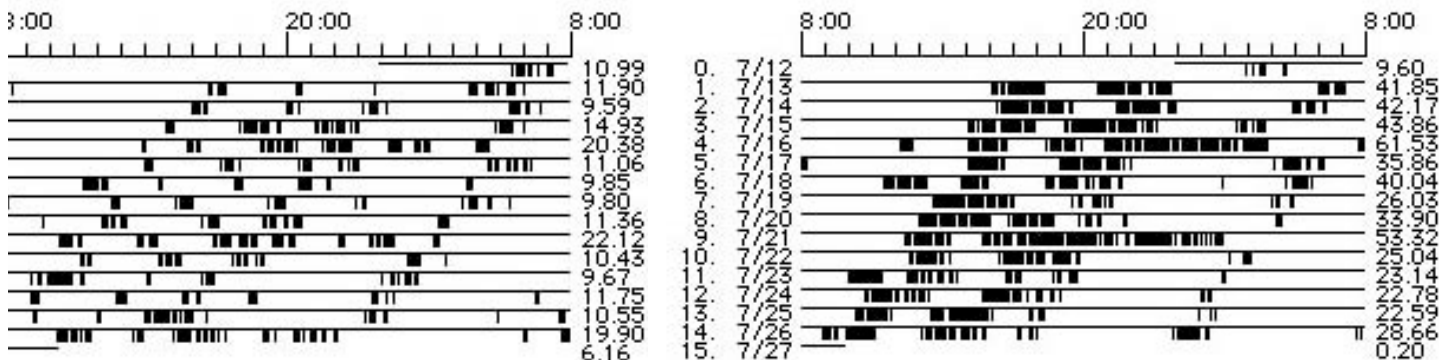
Lanes 1-4, 6-7, and 9 all show positive results indicating TGF-alpha expression.

Lanes 5, 8, and 10 show a negative result and can be used as control mice.

Running wheel experiments are performed in a specialized light and temperature controlled environment and where the daily activity is recorded on a specialized computer log. One delay in initiating our project was the arrival of a new electronics system, which required several months to debug. Once we discovered that there was an

incompatibility between the motherboard and the PC board in the first computer, we did not experienced any further problems. This, however, did result in loss of time in being able to obtain usable data.

Figure 2. Shown below are actigrams of wild type or a transgenic TGF- α mouse in a constant darkness. The left panel shows the TGF- over expression animal and the control is on the right.



Condition. The panel above shows a display of free running behavior with a diagonal structure based on the onset of running wheel activity, which can be compared, to the control C57 black mice shown in the adjacent panel. Both actigrams are obtained on mice in constant dark conditions. These figures show less running wheel activity in transgenic compared to the wild type. These data are displayed with shareware software running on a Macintosh computer. Subsequent data show running wheel raw data collected and generated with an updated software system from Respirationics, Inc.

The next two figures (3 and 4) are the statistical analysis with sufficient numbers of mice per group. LD stands for 12 hours lights on and 12 hours darkness; the DD stands for constant darkness. These results show that the running wheel behavior for the experimental TGF- α over expression mice compared to the controls shows no statistical difference between the period of the running wheel behavior in either LL or LD conditions.

Figure 3

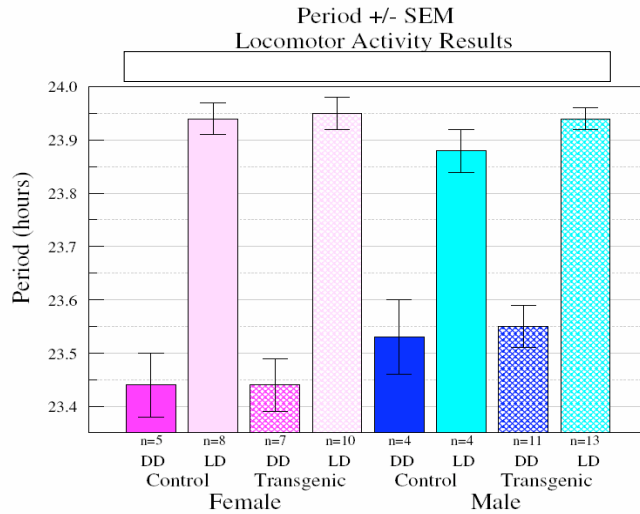
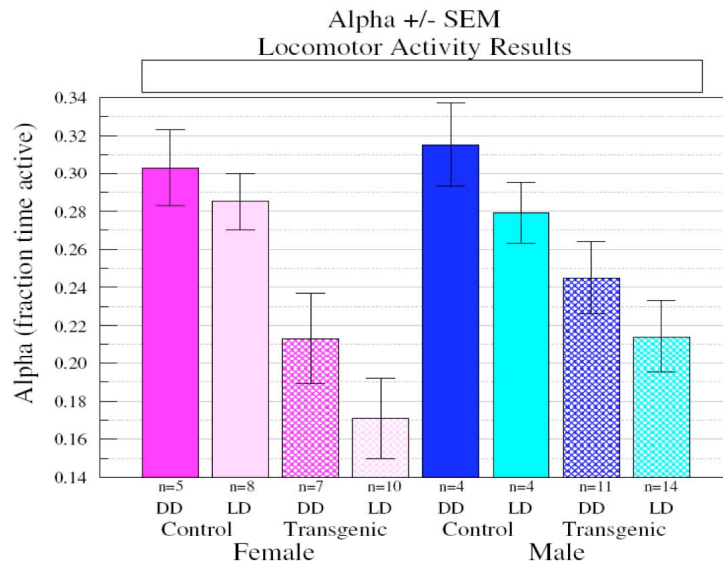


Figure 3 shows there is no difference in PERIOD (the 24 hour rest activity phases) in either LD or DD conditions for the transgenic over expression TGF- α animals compared to the controls

Figure 4



Conclusion: Controls exhibit more relative active time than do transgenics.

Alpha is a measure of the relative duration active (a value of zero meaning no activity, a value of unity corresponding to no rest).

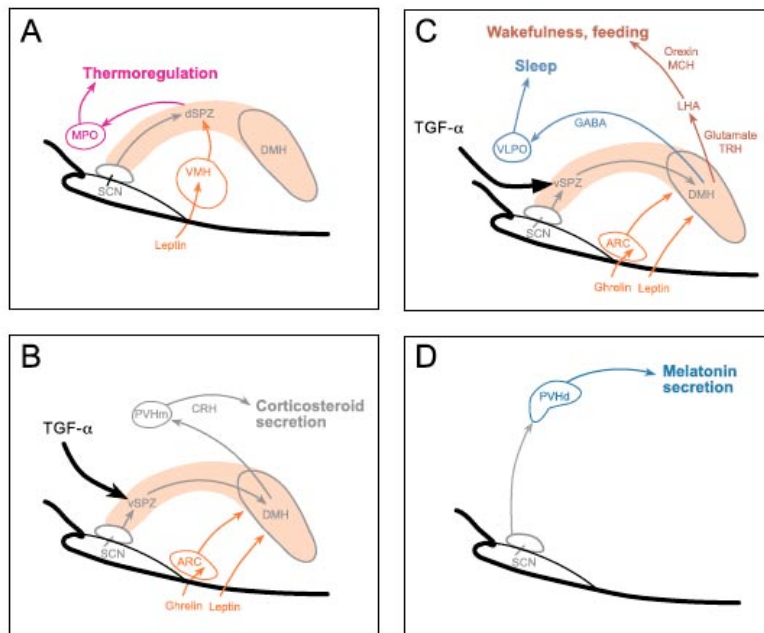
p-value 2-sided < 0.0001 (Welch's t-test used because variances not equivalent; p(SD) = 0.0011)

Figure 4. Shows significantly lower ALPHA (fraction of time active per 24 hours) for the transgenic TGF- α animals compared to the control.

The data shown in figures 3 and 4 are consistent with our hypothesis shown in the Figure 5 where both rat and hamster hypothalamic nuclei ablation data suggests that the modulation of the circadian running wheel behavior occurs downstream from the level of the central clock in the SCN [4,5]. See the figures below indicating where the interruption by TGF- α might be acting.

Figure 5.

HYPOTHALAMIC MODULATION OF CIRCADIAN BEHAVIOR



The model shown here is consistent with modulation of the clock signal downstream from the SCN at the vSPZ nucleus in the hypothalamic paraventricular region. This suppression of activity is consistent with our hypothesis that TGF- α causes disruption of the signal from the SCN to downstream hypothalamic nuclei.

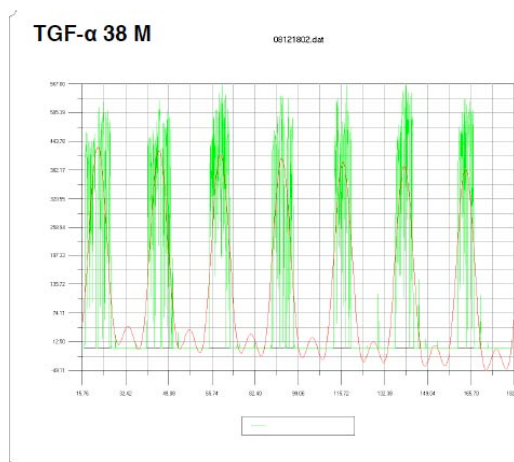
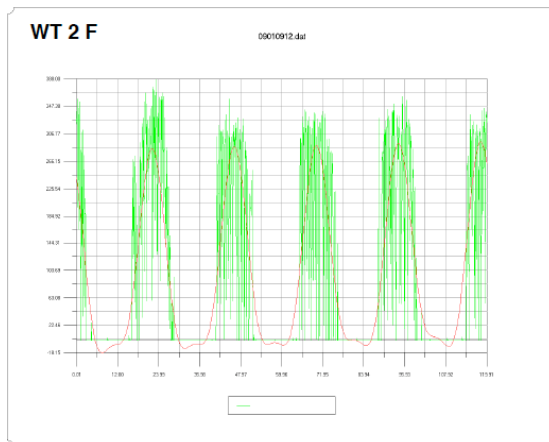
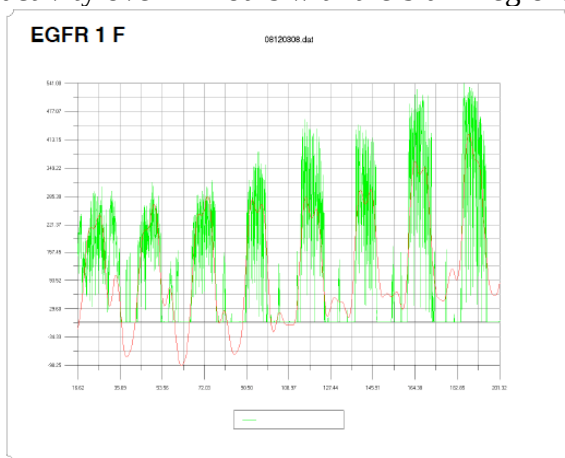
THIS COMPLETES THE FIRST PART OF TASK 1.

Task 1b. **THE SECOND PART OF TASK 1:** to compare the activity levels (ALPHA) of EGFR transgenic animals to wild type:

A. Genotyping of the EGFR overexpression animals was performed in a similar fashion as shown above with the TGF- α animals (Data not shown).

B. Genotyped EGFR animals were run in LL conditions and the activity of the EGFR over expression animals was compared to the wild type and TGF- Shown in Figures 6 below are actigrams (these look different than those shown above because of a different computer program's display)

Figure 6. These are representative actigrams showing the amount of running wheel activity in green each 24 hours. Shown are actigrams from EGFR, TGF- α , and wild type animals. They all show oscillations in the running wheel activity over 24 hours with the blank regions representing the rest phase (light on when the animals rest).



Analysis of the data from the EGFR animals shows:

EGFR animals exhibited greater locomotor activity than did both TGF-alpha animals and wild type. The ALPHA for EGFR animals was 0.397 (n= 9 series of animal rest/activity runs) compared to an ALPHA of 0.330 for the wild type animals (n= 13 series of animal rest/activity runs). The latter figure is consistent with the control values found in figure 4.

Comment: the data described in Task 1 illustrate that only the TGF- α over expression animals behaved according to the hypothesis. Ligands of the EGF receptor may be nerually active and produce a suppression of hypothalamic signaling manifested by a lower proportion of time active (ALPHA).

Task 2. Reversal of the inhibitory effects of TGF- α on activity in the transgenic overpression animal. Can the administration of a tyrosine kinase inhibitor result in a lower ALPHA?

Using the running wheel model to measure ALPHA, we next moved to examine the effects of injection of an EGFR tyrosine kinase inhibitor and measure changes in activity. We used a commercial tyrosine kinase inhibitor, AG1478 that is a lipophilic tyrosine kinase inhibitor of the EGFR family. In vivo studies with this agent show that it penetrates the blood brain barrier and can be found in measureable concentrations in the rodent brain [6].

The results of four separate experiments using sub cutaneous injection of AG1478 into control and transgenic mice are shown below:

AlphaRho

Analytical Results -- Averages ($\pm$ SEM) -- Censored Data Series

Parameter	Pre-TGF- α (N=23 series)	During-TGF- α (N=4 series)	Post-TGF- α (N=5 series)	EGFR (N=9 series)	WT (N=13 series)
$\overline{ALPHA}$	0.332 $\pm$ 0.014	0.327 $\pm$ 0.038	0.304 $\pm$ 0.028	0.397 $\pm$ 0.016	0.330 $\pm$ 0.025
$\langle ALPHA \rangle$	0.331 $\pm$ 0.011	0.319 $\pm$ 0.036	0.308 $\pm$ 0.030	0.391 $\pm$ 0.018	0.315 $\pm$ 0.022

Analytical Parameter Significance Probabilities

Parameter	ANOVA probability	Significant pairs
$\overline{ALPHA}$	0.12	-----
$\langle ALPHA \rangle$	0.06	-----

AlphaRho Parameter Definitions:

$\overline{ALPHA}$ = arithmetic mean fraction-active (24-hour reference period)

$\langle ALPHA \rangle$ = error-weighted mean fraction-active (24-hour reference period)

Comment: The AlphaRho analysis shows there is no effect of the AG1478 injections on the activity of these animals. In fact, there appears to be a slight loss of activity with the administration of this inhibitor (opposite to what was expected) based on a higher level of ALPHA for the EGFR animals.

Task 3. Correlations of tissue and serum samples of TGF- α , EGFR, and pro inflammatory cytokine with running wheel parameters assessed above.

This task was not fully addressed because we ran out of resources to run the assays planned. One ELISA on the serum collected from TGF- α mice indicated a measureable level of ~ 40 picograms/ml of TGF- α in the serum. Any measureable level of TGF- α is considered abnormal since in a mature animal there should be no measureable levels in the serum.

Key research accomplishments:

1. In spite of having difficulties with the transgenic mouse breeding colony and production of sufficient numbers of transgenic mice and with electronic difficulties in the initial setup of the computerized running wheel system, we obtained statistically significant data with the TGF- α animals that supports the hypothesis regarding the effect of TGF- α on running wheel activity. These data show that the TGF- α transgenic animal has a suppression of overall activity (measured here as ALPHA) which is consistent with a loss of signal transmission at the hypothalamic nucleus downstream from the SCN where the presence and excess TGF- α is believed to produce decrease active behavior.
2. The circadian time structure of RWA in the transgenic TGF- α animal is preserved. This finding is consistent with the lack of a direct effect of TGF- α on the clock time keeping mechanism itself.
3. The EGFR animals do not show altered levels of running activity like the TGF - α animals. In fact, these animals exhibit greater locomotor activity than either the TGF- α or wild type animals and a higher ALPHA.

Reportable outcomes:

1. These data add to the behavioral studies that have been conducted on the transgenic TGF- α rodent. Prior studies focused on aggression and depression in reference to the breast cancer carcinogenesis model. Our studies have been conducted in contrast from a mechanistic based hypothesis regarding fatigue and circadian rest/activity as related to the signaling by the ligands of the EGFR. Our data can be considered as a beginning for support of the hypothesis that this family of growth factors contributes to the alterations in behavior that accompany cancer. They suggest that there is an additional dimension to the presence of circulating levels of ligands of the EGFR family that can potentially affect outcomes in two distinct ways. One is by the direct cellular proliferation effects caused by the signaling pathways of the tumor on cancer growth, and secondly on the effects that the cancer has on the host through signaling in the brain that coordinates psychoneuroimmune status

Conclusion:

The data we have obtained support our hypothesis that the peripheral production of TGF- α in the transgenic over expression model can be associated with loss of activity. We interpret this finding as form of “fatigue” in this animal model. These findings are predicted by the model based on neuroanatomic studies and functional activity of ligands of the EGFR in the circadian signaling pathway. The relevance to cancer treatment is the potential use of tyrosine kinase inhibitors in the targeted management of symptoms in patients. These data are consistent with our observation of a normalization of 24 hour rest/activity rhythms in cancer patients given systemic Iressa [7]. In summary, these data suggest there may be a new way to treat fatigue in cancer patients by using a tyrosine kinase inhibitor of the EGFR pathway.

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