Genetic contributions to generalized arousal of brain and behavior

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We have identified a generalized arousal component in the behavior of mice. Analyzed by mathematical/statistical approaches across experiments, investigators, and mouse populations, it accounts for about 1/3 of the variance in arousal-related measures. Knockout of the gene coding for the classical estrogen receptor (ER-α), a ligand-activated transcription factor, greatly reduced arousal responses. In contrast, disrupting the gene for a likely gene duplication product, ER-β, did not have these effects. A combination of mathematical and genetic approaches to arousal in an experimentally tractable mammal opens up analysis of a CNS function of considerable theoretical and practical significance.

estrogen | motivation | mice | genomics | estrogen receptor

Hormonal, neural, and genetic mechanisms for simple sex behaviors in rats and mice have been worked out in some detail. Underlying all of these is sexual arousal. Concepts of arousal are essential for helping to explain broad classes of behavior, but they also have been murky and ill-defined. In humans, "arousal" is intuitively obvious, but what about in experimental animals?

To justify mechanistic studies of arousal, we propose that neurophysiological and behavioral responsivity to external stimuli constitute elementary requirements for animal life. But whether there is a generalized arousal function has been hotly debated. Electrophysiological evidence from recordings across the cerebral cortex after manipulations of the brainstem said "yes" (1–3). However, some cognitive neuroscientists argued (4) that the concept of arousal has become hopelessly subdivided. A clear theoretical resolution can be found, for the first time, in an equation‖

$$A = \mathrm{F}(Kg{\cdot}Ag + Ks_1{\cdot}As_1 + Ks_2{\cdot}As_2 + Ks_3{\cdot}As_3 + \cdots + Ks_n{\cdot}As_n), \quad [1]$$

which combines both generalized (Ag) and various specific forms of arousal [$As_{(1\ \mathrm{to}\ n)}$] such as sex, hunger, fear, etc. (see also Fig. 1). Here, we show that principal components analysis (5) of published experimental data (6–9) shows quantitatively that generalized arousal influences the behavior of mice. New data from mice with the classical estrogen receptor (ER)-α or ER-β genes disrupted here illustrate how the gene for a particular nuclear receptor contributes to arousal.

Methods

Mice. The original five populations of ovariectomized female mice, the raw data from which have been reanalyzed by principal components analysis, were described in detail in refs. 6–9. Additional female mice were those in which the gene coding for ER-α had been functionally disrupted ($n = 8$). These mice were originally obtained from the colony at the National Institute on Environmental Health Sciences (NIEHS, Research Triangle Park, NC) and had been backcrossed to a C57 background for at least eight generations. All their experimental procedures were run in parallel with WT littermate controls ($n = 9$). Likewise, ER-β knockout females ($n = 10$) were initially obtained from NIEHS, maintained and bred in our colony, and experimented in parallel with WT ($n = 10$) littermate controls. All females were ovariectomized at least 1 wk before beginning experiments and were housed individually. These mice were assayed for responsiveness to stimuli and for voluntary motor activity, as follows.

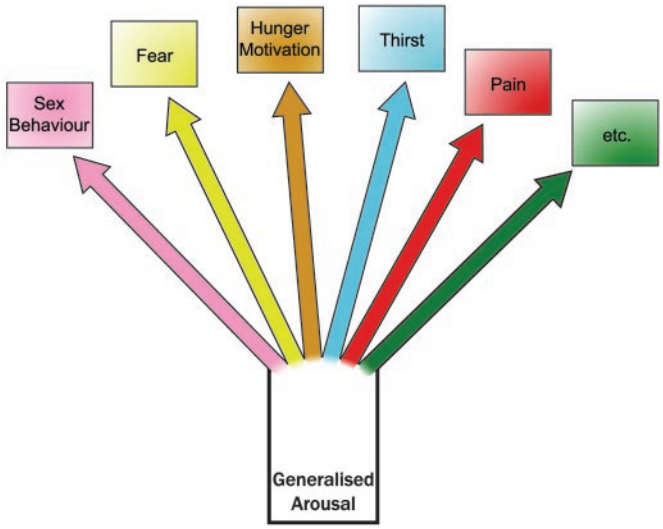

Fig. 1. Simplified schematic of the concepts embodied in Eq. **1**. Generalized arousal of the brain coupled with each specific form of arousal fosters that specific type of biologically motivated behavior. Note that the concept allows for interactions among arousal states; e.g., alterations in sexual arousal could influence response to pain.

Assays. For a concrete, experimental approach to the arousal problem, we set forth a clear operational definition of generalized arousal. The operational definition was as follows: a more aroused animal is more responsive to a wide variety of external stimuli spanning sensory modalities and is more motorically active. This definition yields easily gathered quantitative, physical measures of activity.

Abbreviations: ER, estrogen receptor; PCA, principal components analysis; ERKO, ER knockout.

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‖A = arousal, as a function (F) of generalized arousal (Ag) and specific forms of arousal (As). The + sign is not meant to imply simple linearity, but rather to indicate that A is an increasing function of the variables Ag and $As_{(1\ \mathrm{to}\ n)}$, sometimes additive, sometimes multiplicative, and therefore potentially complex. Whereas the constants [Kg and $Ks_{(1\ \mathrm{to}\ n)}$] reflect traits of the individual, arousal components (Ag, As) are determined by the immediate environment.

Table 1. Characterization of the one-factor solution as a measure of generalized arousal

	One-factor solution (representing generalized arousal)	Control values	Significant?
Correlation to first conventional factor of n factors*	0.677 ± 0.07	Theoretical, if identical: 1.00	$P < 0.001$
% data (variance) accounted for†	37.5 ± 2.55	Random numbers: 13.4 ± 0.3	$P < 0.001$
% data (variance) accounted for‡	43.8	Scrambled data: 15.3 ± 0.26	$P < 0.001$

Complete datasets can be found at www.rockefeller.edu/labheads/pfaff/pfaff-lab.html.

*This correlational analysis shows that the one-factor solution is not identical to the first factor of an *n*-factor solution. If it were, the average correlation from the data sets would be 1.0. Calculations: Is the one-factor solution simply identical to the dominant, first-factor of a principal components analysis in which several factors have eigenvalues >1? In each of five databases referred to in the text, we calculated the correlation between the contributions of variables in the one-factor solution, compared with the first of *n* factors. Then, we averaged that correlation value across the databases and compared the average with the theoretical value of 1.0 (expected if the two types of analyses were identical). The one-factor solution's correlation was significantly less than 1.0 (*t* test, $P < 0.001$).

†Using all of the datasets, the percentage variance accounted for by the one-factor solution was significantly greater than the same calculation applied to random numbers. Calculations: The percentages of variance accounted for by a one-factor solution were calculated across the databases referred to in the text. Their mean, 37.5%, was significantly greater than the percentage obtained using a table of random numbers (*t* test, two-tailed, $P < 0.001$). For more details on the random numbers tables, see Pfaff laboratory web site, as above.

‡Using one of the datasets, from ref. 9, we scrambled the data while keeping marginal values the same and compared that control with the actual one-factor result from that dataset. There was a significant difference between the actual data and the control. Calculations: For one of the databases (from ref. 9), the percentage variance accounted for by a one-factor solution (43.8%) was compared with a table of the identical data in which marginal means were held constant but the order was scrambled. The difference was significant at $P < 0.001$ (*t* test, two-tailed).

The assay for responsiveness to external stimuli was conducted early in the light phase of a 12:12 light:dark daily cycle. We aimed to reduce variability in the results by assaying mice when they are minimally active, during the first 4 h of lights-on. Nevertheless, sleep is not a homogeneous state. Therefore, we matched times of assay, pair by pair, for WT and knockout mice. To begin each series of stimuli, it was required that the animal be sleeping and motionless for 2 min. Then, the vestibular stimulus (a 90° rotation of the entire cage around the vertical axis) was applied, and the responses measured. After the animal again became motionless for 2 min, a tactile stimulus (hair deflection only) was applied. The tactile stimulus that was administered consisted of a brief air puff strong enough to deflect the hair on the back of the test mouse. The air puff was administered via a small desk fan 10 cm above the back of the animal, applied for 5 s. Again, after recovery to the motionless state, an olfactory stimulus was introduced. Many different odors were tested during preliminary trials with other mice. The maximal response came from straw-

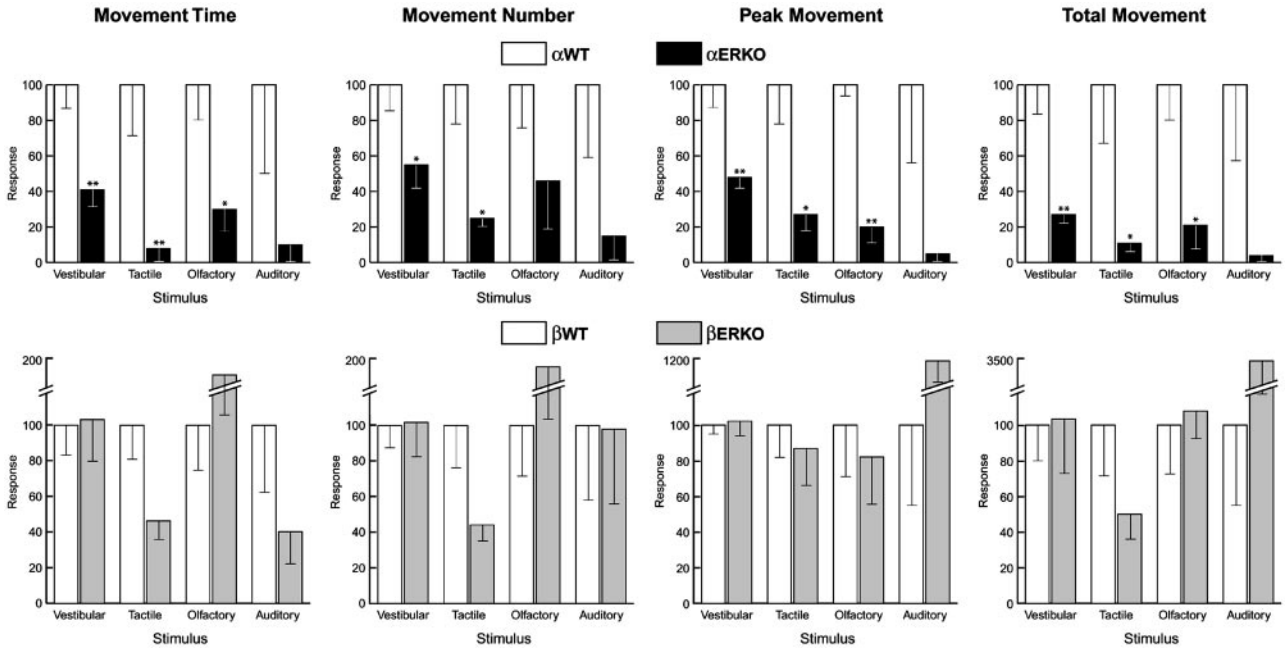

Fig. 2. α-ERKO female mice were less aroused by vestibular, tactile, olfactory, or auditory stimuli than their littermate WT controls (see *Statistics*). *, $P < 0.05$; **, $P < 0.01$. This result was not true of β-ERKO females. Complete databases can be found at www.rockefeller.edu/labheads/pfaff/pfaff-lab.html. Note that none of the comparisons of the β-ERKO with β-WT females were statistically significant. Therefore, there was a marked difference between the results with α-ERKO and β-ERKO mice. In fact, a nonsignificant trend for the β-ERKO females in the opposite direction (see www.rockefeller.edu/labheads/pfaff/pfaff-lab.html) can be seen in the full datasets.

berry flavored powder mixed with water. A saturated solution was made and used to soak a cotton swab, which was lowered through the cage to a position 2 cm above the animal's back. It remained there for 5 s and then was removed. Then, after full recovery to a motionless state, an auditory stimulus was introduced: a continuous burst of loud white noise for 5 s from a battery-operated buzzer that was positioned 15 cm above the home cage. There was a trend for decreasing responsivity from the first stimulus to the last.

Response measures recorded were movement number, peak movement (per minute), total distance traveled, and total movement duration. By using VERSAMAX equipment and software (Accuscan, Columbus, OH), these measures were calculated for each stimulus for each mouse.

After assays of responses to external stimuli, mice were tested in running wheels (Mini-Mitter, Sunriver, OR) attached to their home cages. It is recognized that running wheel locomotor activity is a quantifiable but highly specialized form of locomotion and is assayed here in the safe environment of the home cage rather than on a flat surface in a novel environment. It is intended as a precise measure of voluntary activity in the absence of fear. After at least 72 h of adaptation to the running wheels, data were collected for 14 days, and total revolutions per day were plotted.

Principal Components Analysis (PCA). The mathematical approach PCA was chosen to avoid falling prey to a false dichotomy that "generalized arousal comprises 100% (vs. 0%)" of arousal mechanisms (5). PCA mathematically separates and analyzes the variations of behavioral responses during experiments employing many mice and many arousal-related assays (6–9). Data were analyzed by using SPSS software to perform PCA. We obtained factor loadings and percentage variance accounted for by requesting a one-factor solution to reflect generalized arousal. In addition, we obtained factor loadings on the same databases for those components having initial eigenvalues >1, and obtained the percentage variance accounted for of the first component of rotation sums of squared loadings for eigenvalues >1 solutions by using the varimax rotation. The original databases are those that gave rise to refs. 6–9, and can be accessed at the Pfaff laboratory web site, www.rockefeller.edu/labheads/pfaff/pfaff-lab.html. In summary, this quantitative approach allows the structure of arousal to be revealed by the responses of the mice themselves, rather than by the experimenter's preconceptions. Calculating from a matrix of cross-mouse correlations, it can identify several factors that contribute to arousal responses. Importantly, the one-factor solution mathematically identifies the most global component, in our case designating generalized arousal, and reveals how significantly it influences the animals' behaviors (10).

Statistics. From each experiment, response measures were normalized so that the mean WT values equal 100%. For the experiments with genetically modified mice, a small number of outlying values (by convention, farther than 2 SD from the mean) were not included in calculations of the means. For statistical analysis of these experiments, ANOVAs were used, followed by Bonferroni-corrected *t* tests.

Results

In all experiments reanalyzed by PCA, generalized arousal accounted for a significant amount of the data (about one-third), with the lowest contribution at 29.7% and the highest at 45%. Surprisingly, this range held true despite (*i*) different populations of mice, (*ii*) different investigators, (*iii*) different experimental manipulations and details of response measures, and (*iv*) different configurations of individual, particular factor analysis solutions involving four to six factors for each experiment. The one-factor solution was robust, shown three ways: (*i*) It was never identical to the first factor of a particular multifactor analysis. Indeed it was, as might be expected, correlated, but that correlation was always significantly <1.0 ($P < 0.001$). (*ii*) It accounted for significantly more data than in a random-number control. (*iii*) It accounted for significantly more data than in a control in which marginal averages were held constant but the individual data entries were scrambled randomly (Table 1). All of these arguments and the biological data below indicate that the mathematical structure of arousal includes a primitive, undifferentiated form that accounts for about one-third of the data in female mice.

To begin exploring genetic influences, we used gene knockouts for the estrogen receptors ER-α and ER-β, two very similar transcription factors, probably gene duplication products (11, 12). Estrogens, sex behavior, and sexual arousal are useful as a "bridge" to the discovery of fundamental arousal mechanisms. Because estrogens strongly drive female sex behaviors, could the genes coding for their nuclear receptors be involved in a more global brain function? In transient transfection assays, both ER-α and ER-β respond strongly to natural ovarian estrogens such as estradiol-17β by mediating hormone-induced transcription. Can functional inactivation ("knockout") of their respective genes influence arousal responses?

We studied female mice, individually housed, sleeping in their home cages as they do during the *light phase* of the daily light cycle. Thus, imprecision due to ongoing activities and biases due

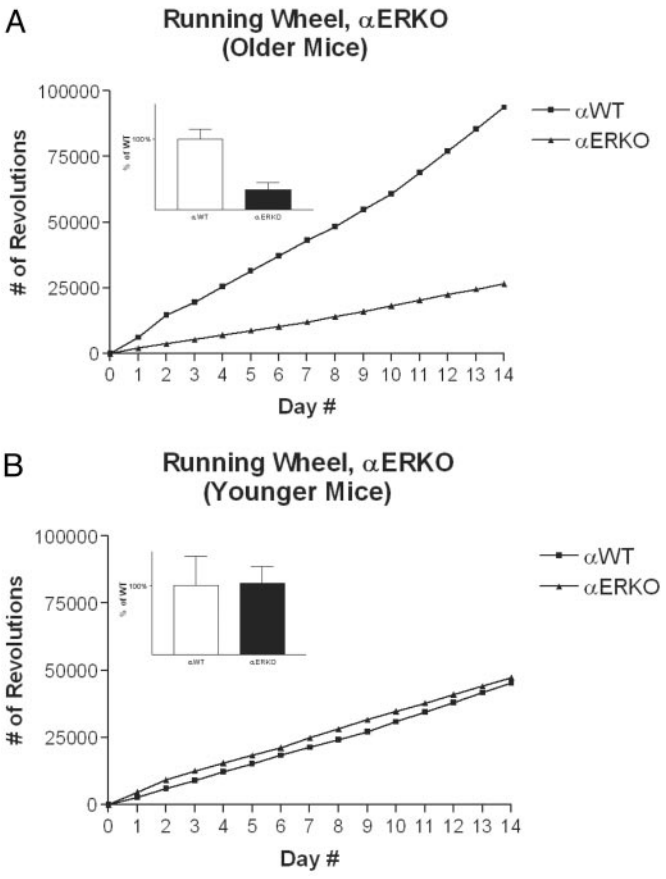

Fig. 3. Comparisons of locomotor activity among genotypes. (*A*) Older α-ERKO females (35 wk) were significantly less motorically active on running wheels in their home cages, compared with their WT littermate controls ($P < 0.01$). (*B*) This result was not true of younger animals (14 wk). Further comparisons of β-ERKO females with their littermate WT controls yielded no significant differences (data not shown).

to fear and anxiety were removed from the experimental protocol. For all sensory modalities tested, α-ER knockout (α-ERKO) female mice were less responsive to sensory stimuli than their WT female littermate controls (Fig. 2). Surprisingly, disruption of the gene for the closely related ER-β, a likely gene duplication product, did not have the same effect. Generally, ER-β has a reciprocal relation to ER-α, often opposing ER-α actions. In our data, in fact, with respect to auditory stimuli, there was a trend for the ER-β effect to be the opposite: heightened responsiveness. Such data in mice may, in turn, underlie ER-β's influences on a more complex state, anxiety (6, 13).

Likewise, in terms of locomotor activity in running wheels, highest during the dark phase of the daily light cycle, α-ERKO females were less active. Interestingly, this phenotype depended on age; older α-ERKO females were subject to the genetic effect, whereas the younger α-ERKO females were not (Fig. 3). Again, the differences between β-ERKO females and their WT littermate controls were not significant (data not shown). The graphs for running wheel activity of β-ERKO mice and their controls were virtually identical. In sum, α-ERKO females were less responsive to external stimuli and less motorically active.

Discussion

Thus, behavioral results from mice indicate the existence of a generalized arousal function. The mathematical/statistical analysis of behavioral data above resonates with (*i*) neuroanatomical delineations of massive systems ascending in the mammalian brainstem and affecting crucial basal forebrain neuronal groups (14–17); (*ii*) electrophysiological demonstrations of multimodally responsive neurons that could serve to alert the animal (or person) to virtually any incoming stimulus (18, 19); and (*iii*) ongoing discoveries of genes obviously contributing to arousal-related functions (20–22). Here, it is intriguing that measures of arousal are affected differentially by disruption of genes for two very similar hormone-activated transcription factors, likely gene duplication products. The fact that β-ERKO females' results were so different from those of alpha-ERKOs poses a challenge to understand the protein chemistry of these nuclear receptors, which act as transcription factors in neurons.

Elementary arousal is of broad general significance. Its explanation can contribute to the mechanistic understanding of a large number of behavioral states. Moreover, approaching its mechanisms in the systematic manner exemplified by the papers cited above heralds a shift in emphasis from the exclusive concentration of specific stimulus-response combinations to the explanation of CNS states that govern entire classes of behaviors. Besides the status of arousal as a "holy grail" in neurobiology, its deficits can contribute to disorders of cognition (e.g., attention deficit hyperactivity disorder, Alzheimer's disease, autism), and its erosion can account for some of the mental difficulties during aging. That is, arousal pathways "feed" circuits for higher cognitive functions that are being understood at cortical levels (23, 24). Its thorough understanding will allow us to enhance vigilance during the day (as in military applications) and sleep at night (25). In medical terms, its mechanistic analysis will lead to a more precise anesthesiology. Our growing ability to measure and manipulate arousal closes a stage in neurobiology during which it seemed, exclusively, that only the "specificity of CNS functions" could be grasped and appreciated. Overall, this field of work (e.g., refs. 14–22) opens an era in which broader, more enduring influences on cognition and emotion can be understood in mechanistic detail.

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