Wildtype epidermal growth factor receptor (Egfr) is not required for daily locomotor or masking behavior in mice

Reade B Roberts1, Carol L Thompson2, Daekee Lee1, Richard W Mankinen1, Aziz Sancar2 and David W Threadgill*1

Address: 1Department of Genetics, CB 7264, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA and 2Department of Biochemistry, CB 7260, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

Email: Reade B Roberts - reader@hcgs.unh.edu; Carol L Thompson - CarolTh@alleninstitute.org; Daekee Lee - daekee@med.unc.edu; Richard W Mankinen - richard_mankinen@med.unc.edu; Aziz Sancar - aziz_sancar@med.unc.edu; David W Threadgill* - dwt@med.unc.edu

* Corresponding author

Abstract

Background: Recent studies have implicated the epidermal growth factor receptor (EGFR) within the subparaventricular zone as being a major mediator of locomotor and masking behaviors in mice. The results were based on small cohorts of mice homozygous for the hypomorphic Egfrwa2 allele on a mixed, genetically uncontrolled background, and on intraventricular infusion of exogenous EGFR ligands. Subsequently, a larger study using the same genetically mixed background failed to replicate the original findings. Since both previous approaches were susceptible to experimental artifacts related to an uncontrolled genetic background, we analyzed the locomotor behaviors in Egfrwa2 mutant mice on genetically defined, congenic backgrounds.

Methods: Mice carrying the Egfrwa2 hypomorphic allele were bred to congenicity by backcrossing greater than ten generations onto C57BL/6J and 129S1/SvImJ genetic backgrounds. Homozygous Egfrwa2 mutant and wildtype littermates were evaluated for defects in locomotor and masking behaviors.

Results: Mice homozygous for Egfrwa2 showed normal daily locomotor activity and masking indistinguishable from wildtype littermates at two light intensities (200–300 lux and 400–500 lux).

Conclusion: Our results demonstrate that reduced EGFR activity alone is insufficient to perturb locomotor and masking behaviors in mice. Our results also suggest that other uncontrolled genetic or environmental parameters confounded previous experiments linking EGFR activity to daily locomotor activity and provide a cautionary tale for genetically uncontrolled studies.

Background

The epidermal growth factor receptor (EGFR) pathway plays key roles in the development and maintenance of many tissue and organ systems [1]. Recent reports have suggested that the EGFR pathway mediates two aspects of behavior, diurnal locomotor activity and suppression of locomotion in response to light (masking). Levels of the EGFR ligand transforming growth factor alpha (TGFA) fluctuate with a circadian rhythm within the suprachiasmatic nucleus (SCN) [2,3], which is located within the hypothalamus and is considered the primary anatomical circadian clock, and are associated with circadian time-
dependent changes in gene expression [4]; similarly, EGFR ligands are expressed within cells of the retina, which modulates masking behavior [3]. Both of these structures appear to input into the subparaventricular zone (SPZ), a hypothalamic region that is required for circadian rhythms [5] and that expresses high levels of EGFR [3]. This anatomical network has been experimentally manipulated, with infusion of TGFA into the hamster hypothalamus reversibly suppressing locomotor activity [3,6]. However, exogenous administration of receptor ligands can lead to non-physiological responses, indicating what a protein can do, not necessarily its normal biological function [7].

Perhaps the most compelling evidence implicating the EGFR pathway in locomotor activity and masking was found in the behavior of mice homozygous for the Egfrwa2 allele, which produces a hypomorphic receptor with reduced kinase activity [8,9]. The Egfrwa2 allele is a valuable genetic reagent for dissecting biological functions of EGFR since homozygosity for Egfr null alleles results in lethality [1,10], while Egfrwa2 homozygous animals can survive to adulthood. Both abnormally high diurnal activity and strong masking defects were reported in four of the six animals tested [3,6]. Surprisingly, the vast majority of the previous study reporting Egfrwa2-associated abnormalities [3] described [3]. Unlike what happened in the previous study, the majority of the abnormally high daytime activity seen in the two affected mice was not sporadic, but rather occurred in a discrete time period anticipating the dark cycle and thus resulting in a consistent phase shift (Fig. 1A). Similar to the previous study, the negative masking in mice was delayed, with normal suppression of activity in the first hour of the three-hour light pulse (Fig. 1B). Thus, the current results on controlled genetic back-
grounds show substantially less penetrance and expressivity than originally reported (18% versus 80% penetrance) [3].

The discrepancy between previous results using the B6EiC3H mixed genetic background and our current results with congenic mice is particularly surprising since most abnormal phenotypes increase in severity with inbreeding, this being particularly striking for phenotypes associated with Egfr [1, 12]. However, use of the B6EiC3H-a/A-Egfrwa2 Wnt3a\textsuperscript{vt} stock to study effects of the Egfrwa2 allele is immediately problematic since Egfrwa2 homozygotes are also homozygous for the linked Wnt3a\textsuperscript{vt} mutation, a hypomorphic allele of Wnt3a known for producing the vestigial tail phenotype [13]. Since Wnt3a-deficient mice exhibit defects in the hippocampus and central nervous system [14], the Wnt3a\textsuperscript{vt} allele is possibly responsible for the activity defects. Additionally, the non-inbred B6EiC3H background, maintained through a cross-outcross mating scheme, harboring the Egfrwa2 and Wnt3a\textsuperscript{vt} alleles in cis segregates known and unknown mutations from the C57BL/6Ei and C3H/HeSnJ strains [15]. Consequently, defects like those previously reported for locomotor activity cannot be attributed to Egfr because an appropriate control does not exist for the mixed B6EiC3H background.

Wildtype inbred mice from the C57BL/6Ei and C3H/HeSnJ strains have different masking thresholds and vary in diurnal locomotor activity in a manner that is likely multigenic [16, 17]. One strong candidate for such a modifier mutation is retinal degeneration (Pdebrd1), which is carried by the C3H/HeSnJ strain but not C57BL/6EiJ, and causes progressive and selective degeneration of photoreceptor cells [18, 19]; the Pdebrd1 mutation alone has a highly significant effect on masking behavior [16]. Melatonin production is also vastly different between the two strains contributing to the B6EiC3H mixed background, with C3H mice exhibiting high, rhythmic melatonin levels, and C57BL/6 mice exhibiting low to undetectable melatonin levels caused by mutation of at least one gene (N-acetyltransferase 2) related to melatonin production [20, 21]. The previous Egfrwa2 homozygous cohort that had a high penetrance of activity defects may have fortuitously contained a high frequency of these or other genetic modifiers given the small number of individuals tested.

Previous studies utilizing direct infusion of ligand to hyperactivate EGFR in the brain have shown that abnormally high EGFR signaling is capable of perturbing light-associated locomotor activity control [3, 6]. However, such an approach can produce non-physiological responses that are artifactual or neomorphic in nature rather than being representative of normal biology [7]. Thus, the suggestion that locomotor activity is strongly dependent on normal levels of EGFR signaling is not supported, especially since extensive testing of B6EiC3H-a/A\textsuperscript{Egfrwa2 Wnt3a\textsuperscript{vt}} mice across a wide range of lighting conditions failed to detect any differences in masking response [11]. Using appropriately controlled genetic conditions, our results conclusively demonstrate that EGFR activity is not required to produce and is not a major mediator of abnormal activity phenotypes, and that other environmental, genetic, or stochastic effects are required.

Table 1: Activity measurements at 200–300 lux

| Mouse number | Strain            | Genotype | Total activity | Daytime activity | Masking |
|--------------|-------------------|----------|----------------|------------------|---------|
| 1            | C57BL/6J          | +        | 30154          | 1.82             | 99.33   |
| 2            | C57BL/6J          | +        | 37951          | 0.28             | 99.45   |
| 3            | C57BL/6J          | +        | 44485          | 0.86             | 97.35   |
| 4            | C57BL/6J Wnt3a\textsuperscript{vt} |        | 23509          | 1.43             | 99.72   |
| 5            | B6.129 F1         | +        | 51014          | 1.44             | 95.82   |
| 6            | B6.129 F1         | +        | 39133          | 0.74             | 96.10   |
| 7            | C57BL/6J Egfrwa2/wa2 |       | 27630          | 30.22            | 66.25   |
| 8            | C57BL/6J Egfrwa2/wa2 |    | 21821          | 1.30             | 95.82   |
| 9            | C57BL/6J Egfrwa2/wa2 |      | 40884          | 0.08             | 99.97   |
| 10           | C57BL/6J Egfrwa2/wa2 |      | 33975          | 1.16             | 97.80   |
| 11           | C57BL/6J Egfrwa2/wa2 |      | 31997          | 0.55             | 97.97   |
| 12           | C57BL/6J Egfrwa2/wa2 |      | 38165          | 1.95             | 80.49   |
| 13           | C57BL/6J Egfrwa2/wa2 |      | 35818          | 0.98             | 99.37   |
| 14           | C57BL/6J Egfrwa2/wa2 |      | 37774          | 0.39             | 99.27   |
| 15           | C57BL/6J Egfrwa2/wa2 |      | 21888          | 1.92             | 97.39   |
| 16           | B6.129 F1         | Egfrwa2/wa2 | 43930         | 17.55            | 26.63   |
| 17           | B6.129 F1         | Egfrwa2/wa2 | 46505         | 1.41             | 97.50   |

\[\text{Average number of wheel revolutions per twenty-four hour period.}\]

\[\text{Percentage of total wheel revolutions during light cycle.}\]

\[\text{Percent suppression of activity during three-hour light pulse given during the dark cycle, relative to activity in the same dark cycle time period on the previous day.}\]
Figure 1

Locomotor activity in Egfr\textsuperscript{wa2/wa2} mice during 12 h:12 h light:dark cycles and three-hour light pulses given during the dark cycle. Horizontal white and black bars represent light and dark exposure, respectively. Vertical axis indicates wheel-running activity. (A) The majority of Egfr\textsuperscript{wa2/wa2} mice tested were indistinguishable from wildtype controls in behavior, though two Egfr\textsuperscript{wa2/wa2} mice did exhibit a phase shift resulting in abnormally high daytime activity, as well as abnormally high activity during a three hour light pulse. (B) Abnormal wheel running activity during three-hour light pulses followed a characteristic one-hour of activity suppression in three Egfr\textsuperscript{wa2/wa2} mice (red), while wildtype (dark blue) and the majority of Egfr\textsuperscript{wa2/wa2} mice (light blue) demonstrated suppression of activity throughout the pulse. Grey areas, lights off; white area, light pulse.
to reveal abnormal phenotypes. A recent report revealed significant intrastrain and intraindividual fluctuations of EGFR ligand levels in the SCN of inbred mice [2]. Thus we cannot eliminate the possibility that homozygosity for $Egf^{wa2}$ may cause individuals to be sensitized to phenotypically express abnormal locomotor activities. For example, $Egf^{wa2}$ homozygotes have variable eye defects [8] that could combine with other factors to contribute to light-associated activity abnormalities. In fact one of the $Egf^{wa2}$/wa2 individuals in our study supports the presence of intraindividual variability, exhibiting an abnormal masking response in one trial followed by a near-normal response in a subsequent trial (data not shown).

Conclusion

The mice used to originally implicate EGFR as a major mediator of locomotor activity carried numerous other mutations and allelic variants that could contribute to the observed results. Since the animals were not properly controlled for genetic background, numerous other interpretations (such as unequal genetic background distribution in control and test mice) most likely contributed to the erroneous results. Our data using genetically uniform congenic lines indicate that EGFR is not a required mediator of the locomotor or masking behaviors, though it may modulate the activity of other pathways involved in control of locomotor activity. Further investigation is required to properly elucidate the molecular pathways and factors mediating locomotor activity.

Competing interests

The author(s) declare that they have no competing interests.

Authors’ contributions

RBW participated in all experiments, in the analysis and discussion of the results, and in the writing of the manuscript. CLT participated in all experiments, in the analysis and discussion of the results, and in the writing of the manuscript. DL participated in all experiments and in the analysis and discussion of the results. RWM participated in all experiments and in the analysis and discussion of the results. DWT participated in the conceptualization of the experiments, in the analysis and discussion of the results, and in the writing of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by grants from the National Institutes of Health (CA092479 and HD039896) to DWT and (GM031082) to AS.

References

1. Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichiti U, Yee D, LaMania C, Mourton T, Herrup K, Harris RC, Barnard JA, Yuspa SH, Coffey RJ, Magnuson T: Targeted disruption of mouse EGFR receptor: effect of genetic background on mutant phenotype. Science 1995, 269:230-234.
2. Van der Zee EA, Roman V, Ten Brinke O, Meerlo P: TGFα and AYF in the mouse suprachiasmatic nucleus: anatomical relationships and daily profiles. Brain Res 2005, 1054(2):159-166.
3. Kramer A, Yang FC, Snodgrass P, Li X, Scammell TE, Davis FC, Weitz CJ: Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling. Science 2001, 294(5551):2511-2515.
4. Zak DE, Hao H, Yadegaripelli R, Miller GM, Ogunnaikke BA, Schwaber J: Systems analysis of circadian time-dependent neuronal epidermal growth factor receptor signaling. Genome Biol 2006, 7:R48.1-15.
5. Saper CB, Scammell TE, Lu J: Hypothalamic regulation of sleep and circadian rhythms. Nature 2005, 437:1257-1263.
6. Snodgrass-Belt P, Gilbert JL, Davis FC: Central administration of transforming growth factor-alpha and neuroregulin-1 suppress active behaviors and cause weight loss in hamsters. Brain Research 2005, 1038:171-182.
7. Wakefield LM, Yang Y, Dukhanina O: Transforming growth factor-beta and breast cancer: lessons learned from genetically altered mouse models. Redox Biol Cancer Research 2000, 2:100-106.
8. Luetteke NC, Phillips HK, Qiu T, Copeland NG, Earp HS, Jenkins NA, Lee DC: The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. Genes and Development 1994, 8:399-413.
9. Fowler KJ, Walker F, Axelson W, Hibbs ML, Nice EC, Bohmer RM, Mann GB, Thumbwood C, Maglilio R, Danks JA, Cheyty R, Burgess AW, Dunn AR: A mutation in the epidermal growth factor receptor in waved-2 mice has a profound effect on the receptor biochemistry that results in impaired lactation. Proceedings of the National Academy of Sciences USA 1995, 92:1465-1469.
10. Sibilia M, Wagner EF: Strain-dependent epithelial defects in mice lacking the EGF receptor. Science 1995, 269:234-238.
11. Mrosovsky N, Redlin U, Roberts RB, Threadgill DW: Masking in waved-2 mice: EGF receptor control of locomotion questioned. Chronobiol Int 2005, 22:963-974.
12. Strunk KE, Amann V, Threadgill DW: Phenotypic variation resulting from a deficiency of epidermal growth factor receptor in mice is caused by extensive genetic heterogeneity that can be genetically and molecularly partitioned. Genetics 2004, 167:1821-1832.
13. Greco TL, Takada S, Newhouse MM, McMahon JA, McMahon AP, Camper SA: Analysis of the vestigial tail mutation demonstrates that Wnt-3a gene dosage regulates mouse axial development. Genes and Development 1996, 10:313-324.
14. Lee SM, Tole S, Grove E, McMahon AP: A local Wnt-3a signal is required for development of the mammalian hippocampus. Development 2000, 127:457-467.
15. Staff: Genetic background effects: can your mice see? In JAX Notes Bar Harbor, Jackson Laboratory; 2002.
16. Mrosovsky N, Foster RG, Salmon PA: Thresholds for masking responses to light in three strains of retinally degenerate mice. J Comp Physiol [A] 1999, 184(4):423-428.
17. Tankersley CG, Irizarry R, Flanders S, Rabold R: Circadian rhythm variation in activity, body temperature, and heart rate between C3H/HeJ and C57BL/6j inbred strains. J Appl Physiol 2002, 92(2):870-877.
18. White JA, Burgess BJ, Hall RD, Nadol JB: Pattern of degeneration of the spiral ganglion cell and its processes in the C57BL/6j mouse. Hear Res 2001, 161(1-2):175-196.
19. Wang S, Villegas-Perez MP, Vidal-Sanz M, Lund RD: Progressive optic axon dystrophy and vacular changes in rd mice. Invest Ophthalmo Vis Sci 2000, 41(2):537-545.
20. Ebihara S, Marks T, Hudson DJ, Menaker M: Genetic control of melanin synthesis in the pineal gland of the mouse. Science 1986, 231(4737):491-493.
21. Vivien-Roels B, Malan A, Rettori MC, Delgrange P, Jeanniot JP, Pevet P: Daily variations in pineal melanotonin concentrations in inbred and outbred mice. J Biol Rhythms 1998, 13(5):403-409.