

Pathological aggression in 'fierce' mice corrected by human nuclear receptor 2E1 (*NR2E1*)

^{1,2}Brett S. Abrahams, ²Melvin C.H. Kwok, ²Eric Trinh,
²Saeed Budaghzadeh, ²Sazzad M. Hossain², ^{2,3}Elizabeth M. Simpson

¹Graduate Program in Neuroscience, ²Centre for Molecular Medicine & Therapeutics,
British Columbia Research Institute for Children's and Women's Health, and Department
of Medical Genetics, University of British Columbia, 980 West 28th Avenue, Vancouver,
British Columbia. V5Z 4H4, Canada

³Correspondence should be addressed to EMS (simpson@cmmt.ubc.ca)

Abstract

Violence is a primary cause of mortality. 'Fierce' mice deleted for nuclear receptor 2E1 (*Nr2e1*; previously *Mtll*, *Tailless*, *Tll*, *Tlx*) show brain-eye abnormalities and pathological aggression. Whether human *NR2E1* is similarly involved in the modulation of development and behavior, however, is unknown. To test whether human *NR2E1* can modulate behavior, we generated mice carrying human *NR2E1* under the control of its endogenous regulatory elements and bred them to fierce. Structural brain defects were eliminated and eye abnormalities ameliorated in fierce mutants carrying human *NR2E1*. Excitingly, behavior in these 'rescue' mice was indistinguishable from controls. These data suggest that mechanisms underlying the behavioral abnormalities in fierce are conserved in humans. Moreover, variation at *NR2E1* may contribute to human behavioral disorders. Use of this paradigm will permit the direct evaluation of human genes hypothesized to play a causal role in behavior or psychiatric disease, but for which evidence is lacking or equivocal.

The study of mental illnesses is exceedingly difficult. Despite strong evidence for genetic modulation of disease risk¹, positional cloning has shown only limited success in the identification of psychiatric disease genes. Genetic studies in human are hampered by incomplete penetrance and extensive phenotypic variability, resulting in part from polygenic inheritance² and gene-environment interactions^{3,4}. Moreover, genetic heterogeneity between individuals undermines both population-based strategies and the replication of results between groups. Mouse genetics can address these concerns, ensuring near identical environments and genetic make-up between subjects. However, because of the differences between mouse and human, determining which observations from mouse generalize to humans remains challenging. From this perspective, we reasoned that establishing a paradigm in which one could functionally evaluate the ability of a human gene to shape behavior and simultaneously retain the advantages associated with a model system would be powerful. Towards this end, we generated transgenic mice expressing the human form of orphan nuclear receptor 2E1 (*NR2E1*; also *Tailless*, *Tll*, and *Tlx* previously) and evaluated the ability of this human gene to modulate the behavior of mice, which in its absence would have demonstrated pathological violence.

The use of *NR2E1* in the brain of embryonic and adult mice is multifaceted and the subject of active investigation⁵⁻⁷. However, an emerging hypothesis suggests that the primary function of this nuclear receptor is to suppress the differentiation of neuronal stem cells⁷⁻⁹. Consistent with this hypothesis, *Nr2e1* is transcribed in the embryonic and adult mouse within regions important for neurogenesis^{7,10}. Moreover, although mice deleted for *Nr2e1* show a complex phenotype¹¹⁻¹³, the developmental hypoplasia observed within the forebrain, eye, and olfactory bulbs, may be secondary to a reduced

proliferative capacity. Thus, at the cellular level, *NR2E1* may normally act to suppress differentiation, thereby enabling an expansion of the neuronal stem cell pool ultimately available for neurogenesis.

Despite the exciting implications of these results for mouse *Nr2e1*, it is unclear the extent to which they may apply to humans. First, it is well established that subtle interspecies differences within individual genes can give rise to striking phenotypic variation¹⁴. Second, *Nr2e1* transcript distribution in mouse is both spatially complex and temporally dynamic. It is first seen at approximately embryonic day (E)8 in a few adjacent neuroepithelial cells at the anterior limit of the prosencephalon, but by E8.5 the transcript has spread to the presumptive diencephalon and is also present in the newly formed optic and olfactory evaginations¹⁰. Transcription peaks at E13.5 in ventricular and subventricular zones, and although almost undetectable in the perinatal brain¹⁰, is seen later in the adult brain at high levels in the dentate gyri and subventricular zones and at lower levels scattered throughout the cortex⁷. Because data from human is limited¹⁵, it is unclear whether the regional expression of the molecule across time is conserved between species. Third, *NR2E1* acts in more than one cell type⁷, and at least one of its functions appears to be cell-type specific¹⁶. Fourth, functional equivalence between mouse and human *NR2E1* would require both that upstream and downstream signaling pathways and the molecular interactions within them be conserved between species. Fifth, the question of whether human *NR2E1*, like its mouse homolog¹¹⁻¹³, might modulate behavior has not been addressed. In fact, it remains unclear the extent to which the mechanisms mediating behavior are conserved between mouse and human in general. Together these data

underscore the complexity of *Nr2e1* function and highlight the necessity of a behaving *in vivo* system in which to study the human gene.

Our results show that the presence of human *NR2E1* under its endogenous regulatory elements is sufficient to eliminate structural brain defects, ameliorate eye abnormalities, and restore normal behavior to fierce mice. The correction of fierce brain-behavior abnormalities by human *NR2E1*, supports conserved underlying mechanisms for behavior modulation and suggests that variation at *NR2E1* may contribute to human behavioral disorders. Moreover, use of this same approach with other genes may prove useful in the genetic dissection of endophenotypes that contribute to psychiatric disease as well as the systematic evaluation of candidate genes for which involvement in disease is controversial.

Methods

Transgenesis. Pronuclear injection of a 141-kb PAC clone spanning human *NR2E1* (pacEMS1, Clone ID dJ429G5, GenBank ALO78596.7) was performed as previously described¹⁷ to produce eight transgenic founders from which five independent strains were successfully established. Two strains were used here (C57BL/6J.Cg-Tg(*NR2E1*pacEMS1B)10Ems and C57BL/6J.Cg-Tg(*NR2E1*pacEMS1D)11Ems) with mice at N6 (98.4% C57BL/6J) to N10 (99.9% C57BL/6J) prior to use. These strains will be represented by C57BL/6J (+/+, Tg(Hum)/+) or Tg(Hum).

Breeding. Two strains bearing the fierce mutation were used; C57BL/6J.Cg-*Nr2e1*^{frc} that will be represented by C57BL/6J (fierce/+, +/+), and 129S1/SvImJ.Cg-*Nr2e1*^{frc} that will be represented by 129S1/SvImJ (fierce/+, +/+). Both strains had been backcrossed more

than ten times (to C57BL/6J and 129S1/SvImJ, respectively) and thus were greater than 99.9% inbred prior to use. Homozygotes for the *Nr2e1^{frc}* allele will be represented by fierce.

Experimental mice were obtained by a two generation mating scheme. C57BL/6J (fierce/+, +/+) mice were crossed to C57BL/6J (+/+, Tg(Hum)/+) mice to obtain C57BL/6J (+/+, Tg(Hum)/+) females. These females were then crossed to 129S1/SvImJ (fierce/+, +/+) males to produce experimental animals. Thus, experimental mice were first generation C57BL/6J x 129S1/SvImJ (B6129F1) offspring. Three separate PCR assays were used to genotype each individual. oEMS650 (5'-GGCGGAGGGAGCTTAAATAG-3') and oEMS1368 (5'-GATTCATCCTATTCCACAAAGTCA-3') span the fierce deletion region and give a product only in its absence. oEMS1859 (5'-CTGGGCCCTGCAGATACTC-3') and oEMS1860 (5'-GGTGGCATGATGGGTAAGTCA-3') detect mouse but not human *NR2E1*. oEMS800 (5'-CCCAGCAGCTGCGGTTTTGC-3') and oEMS801 (5'-GCAGCGCTCCAGGCAGGAC-3') detect human but not mouse *NR2E1*. Reactions were put through 30 cycles of the PCR as described previously¹⁸ but with the addition of 10% DMSO for oEMS1859/60 and 800/801.

Histology. Whole brains were dissected following intracardial perfusion of Avertin-anaesthetized mice with formalin. Sectioned brains were prepared from a separate set of animals, sacrificed by cervical dislocation. Brains were rapidly removed, embedded in OCT (VWR, Delta, BC) and frozen on dry ice. Cryosections were prepared at 15µM then stained with Cresyl Violet and Luxol Fast Blue.

Funduscopy. Direct funduscopy was performed between 18 and 24 weeks with a Kowa Genesis Small Animal Fundus Camera (Pacific Medical, Delta, BC) in conjunction with a Volk 90D lens (Topcon Canada, Calgary, AB) as described¹⁹. Eyes were dilated with 25% atropine 30 min prior to examination, at which time mice were lightly sedated with Avertin.

Behavior Testing. Mice were housed under a reverse light-dark cycle and tested in the dark²⁰. To control for the possible confound of environmental effects²¹ and gene-environment interactions²² on behavior, all experimental mice were individually housed from weaning at 18 days until sacrifice in adulthood for histological analyses. Struggle was measured in males and females at 13 weeks for 3 min using a PHM-300TSS Mouse Tail Suspension System (Med Associates, St. Albans, VT). The apparatus was calibrated to offset animal weight before testing, and system settings for struggle and gain were 15 and 4, respectively. Percent time struggle was calculated by expressing the number of msec during which force exceeded the struggle threshold (set to 15) as a proportion of test time. Force exerted struggling was calculated by summing the force applied to the system across the testing period.

Spontaneous activity was measured in mice of both sexes at 12 weeks using a 3 min open-field paradigm. The apparatus we employed (Med Associates, St. Albans, VT), was digiscan photocell-equipped to permit automated data capture. Ambulatory counts equaled the number of beam breaks recorded during the test period. Ambulatory time represented the amount of time (in sec) spent in motion during the test period. Number of ambulatory episodes was calculated by summing the number of individual episodes

(defined as a series of sequential ambulatory counts separated in time by no more than 500 msec).

A 4-10 min resident-intruder paradigm¹³ was used to assess aggression in 16- to 18-week-old experimental males. Wild-type mice matched to experimental animals for genetic background, sex and weight served as intruders. Video analysis software (Observer Video Pro, Noldus, The Netherlands) was used to score frequency and duration of tail rattling (rapid quivering or thrashing of the tail), wrestling (close contact that escalates to rolls and tumbling), attack (biting of the opponent mouse), sniffing (sniffing the head or snout of the partner), and anogenital investigation (exploration of the intruder's hindquarters). Tail rattling, wrestling, and attack behaviors were combined to give an index of aggression. Aggression per minute and percent time aggression were calculated by expressing the number of aggressive behaviors and time engaged in aggressive behaviors over minutes of testing and as a proportion of total test time, respectively. Sniffing and anogenital investigation were combined to give an index of social behavior. Percent time social was calculated by expressing the amount of time engaged in social behaviors as a proportion of total test time.

Statistical Analyses. Data were analyzed using JMP 4.0 (SAS, Cary, NC), with one-way ANOVAs for genotype (and the Tukey-Kramer as needed) or the Wilcoxon/Kruskal-Wallis test. Significant Wilcoxon/Kruskal-Wallis results were attributed to a single group when $p > 0.05$, following its removal from the original data set. Nominal data (radial asymmetry and mottling) was analyzed using the Pearson Chi square test.

Results

To test the functionality of human *NR2E1* we established transgenic mice carrying the human gene under the control of its endogenous regulatory elements. We then crossed transcription-positive strains (data not shown) to fierce mice shown previously to carry a deletion that spans *Nr2e1* but leaves neighboring genes intact and transcriptionally active²³. Previously identified abnormalities that make up core features of *Nr2e1* mutants were then used to make blinded comparisons between mice from each of the following four groups: wild-type (+/+, +/+), fierce (fierce/fierce, +/+), transgenic (+/+, Tg(Hum)/+), and 'rescue' (fierce/fierce, Tg(Hum)/+).

Multiple brain abnormalities in fierce but not transgenic or rescue mice (Fig. 1) were present at the gross and microscopic level. Compared to wild-type controls, fierce brains showed gross hypoplasia of the olfactory bulbs (arrow) and anterior cortex leaving midbrain colliculi exposed (between dashed lines) (Fig. 1a). Quantitative analysis of surface areas for the olfactory bulbs (Fig. 1b) and cortex (Fig. 1c) confirmed significant reductions in both regions (49.1 and 38.9%, respectively) for fierce relative to each of the other genotypes. In contrast, brains of wild-type, transgenic, and rescue mice were phenotypically indistinguishable.

We observed characteristic abnormalities in sectioned brains from every fierce mouse examined but no abnormalities in any transgenic or rescue animals (Fig. 2). Sections through the fierce forebrain showed an overall reduction in size relative to wild-type controls (Fig. 2a). An unusually shaped cingulum (Cg), poorly defined piriform cortex (Pir), and smaller anterior commissure (Ac) were also evident in fierce. At higher magnification, abnormal cortical lamination (based on cell size and density) was noted

(Fig. 2b), consistent with the previous observation²⁴ that *Nr2e1* is required for proper formation of layers II and III. A reduction in striatal (Str) volume was also observed in fierce (Fig. 2c) as a result of misspecification of the ventral lateral ganglionic eminence from which it develops⁶. Fierce mice showed an enlargement of corticostriatal fibers that pass through the striatum (arrows), with clustering towards its medial border. As well, the external capsule of the corpus callosum (Ec) was thin in fierce relative to wild-type (Fig. 2c).

Abnormalities observed in the fierce eye by funduscopy were each corrected or ameliorated in transgenic and rescue animals (Fig. 3a). Asymmetry of the radial vasculature, mottling of the retinal pigment epithelium (arrow), and a reduction in retinal vessel number were characteristic features of the fierce eye. All fierce mice showed abnormal radial symmetry, and thus radial symmetry was significantly reduced relative to wild-type, transgenic, and rescue mice (Fig. 3b). Similarly, only fierce showed mottling of the retinal pigment epithelium (Fig. 3c). Vessel number was lowest in fierce and significantly reduced relative to rescue mice, which showed the next lowest vessel number (Fig. 3d; see †). However, for vessel number only, rescue animals were reduced significantly relative to wild-type and transgenic mice (Fig. 3d, see ‡). Nevertheless, because rescue eyes were largely normal both by qualitative and quantitative analyses, we conclude that the presence of human *NR2E1* had a corrective influence on the structural development of the fierce eye. The slight reduction in vessel number may reflect the observation by others that development of the mouse eye is exquisitely sensitive to changes in gene dosage²⁵⁻²⁷.

Multiple behavior tests identified fierce mice as distinct from the three other groups, whereas no differences were found between wild-type, transgenic, and rescue animals. We employed a tail-suspension test to quantify the hard-to-handle phenotype previously observed in fierce mice^{11,13}. In fierce mice, percent time struggling and force exerted struggling were significantly increased relative to mice of other genotypes (Fig. 4a and b). Similarly, fierce showed significant increases in spontaneous activity relative to the other groups as measured by ambulatory counts (Fig. 4c), ambulatory time (Fig. 4d), and ambulatory episodes (Fig. 4e). Additional measures obtained during this test also showed significant differences between fierce and all other genotypes (increased ambulatory distance and decreased resting time; data not shown). Fierce mice showed significantly heightened aggression in the resident-intruder test relative to the other genotypes (Fig. 4f and g). The proportion of time fierce mice engaged in social behavior was reduced relative to mice from each of the other genotypes (Fig. 4h). Most strikingly, unlike fierce males who attack, wound, and kill female mates¹³, male rescue animals were not aggressive towards female partners and mated successfully. Together, these results indicate that the presence of human *NR2E1* under its endogenous regulatory elements renders the behavior of fierce mice indistinguishable from that of wild-type controls.

Discussion

Our results demonstrate that human *NR2E1* substitutes appropriately for its mouse homolog *in vivo*, correcting the structural brain and behavioral abnormalities present in fierce. Given the transcriptional complexity of *NR2E1* and its role in both development¹¹⁻¹³ and adulthood⁷, we were surprised at obtaining complete correction of brain abnormalities. Furthermore, given that it was unclear the extent to which

normalization of structural abnormalities would predict behavioral performance we were further surprised by the complete correction of behavioral abnormalities. Together these observations indicate that *NR2E1* protein and regulatory sequences are comparable between mouse and human and that conservation is sufficient to maintain the function of both up- and downstream signaling pathways. That the human transgene was able to correct the behavioral abnormalities observed in *fierce*, supports a conserved underlying mechanism between these species. Our results also suggest that allelic variation at *NR2E1* may be associated with abnormal behavior in humans.

Particularly germane is that genome scans for each of Schizophrenia (SCD, OMIM 603175)²⁸⁻³⁰ and Bipolar Disorder (BPD)³¹⁻³⁴, have repeatedly obtained significant linkage to the interval to which *NR2E1* maps (human 6q21-22). That *NR2E1*-interacting genes^{5,7,12,16} are themselves implicated in mental illness³⁵⁻⁴⁵ also supports the hypothesis that *NR2E1* itself may be involved in psychiatric disease. The role of *NR2E1* in neuronal proliferation is also of interest given that regional cell loss is observed in SCD and BPD⁴⁶ and that drugs used to treat these disorders result in increased neurogenesis⁴⁷⁻⁴⁹. Together with our results documenting the ability of human *NR2E1* to correct abnormal behavior in *fierce*, these results call for a more careful consideration of *NR2E1* and *NR2E1*-signalling in the etiology of mental illness. Although our data supports the conservation of mechanisms underlying *fierce* behavior abnormalities between mouse and human, the clinical presentation of a common cellular defect may be phenotypically distinct between them. One possibility that would account for several converging lines of evidence supporting *NR2E1* in SCD and BPD, is that a common polymorphism in the human gene has an effect on a shared endophenotype and thereby

modulates risk to both disorders. Use of the humanized strains we have generated may be useful in understanding the specific role of NR2E1 in risk for psychiatric illness and the manner by which this risk interacts with changes in the environment.

More generally, we have established an experimental paradigm in which to functionally evaluate the role of human genes in behavior. These data represent the first example of a human gene correcting mouse behavior. Whereas cell-based endpoints are suitable for some kinds of disease, others, particularly psychiatric disorders, require *in vivo* assay systems. Moreover, as the number of coding, and most importantly, non-coding variants implicated in disease increases, so will the need for systems in which to assess their function. Experiments similar to those we have described will be useful to clarify the role of other genes and alleles in human behavior and psychiatric disease.

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References

1. Tandon, K. & McGuffin, P. The genetic basis for psychiatric illness in man. *Eur J Neurosci* **16**, 403-7. (2002).
2. Owen, M. J., Williams, N. M. & O'Donovan, M. C. The molecular genetics of schizophrenia: new findings promise new insights. *Mol Psychiatry* **9**, 14-27 (2004).
3. Caspi, A. *et al.* Role of genotype in the cycle of violence in maltreated children. *Science* **297**, 851-4 (2002).
4. Caspi, A. *et al.* Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* **301**, 386-9. (2003).
5. Stenman, J., Yu, R. T., Evans, R. M. & Campbell, K. Tlx and Pax6 co-operate genetically to establish the pallio-subpallial boundary in the embryonic mouse telencephalon. *Development* **130**, 1113-22. (2003).
6. Stenman, J. M., Wang, B. & Campbell, K. Tlx controls proliferation and patterning of lateral telencephalic progenitor domains. *J Neurosci* **23**, 10568-76. (2003).
7. Shi, Y. *et al.* Expression and function of orphan nuclear receptor TLX in adult neural stem cells. *Nature* **427**, 78-83. (2004).
8. Younossi-Hartenstein, A. *et al.* Control of early neurogenesis of the Drosophila brain by the head gap genes *tll*, *otd*, *ems*, and *btd*. *Dev Biol* **182**, 270-83 (1997).
9. Nguyen, V. *et al.* Morphogenesis of the optic tectum in the medaka (*Oryzias latipes*): a morphological and molecular study, with special emphasis on cell proliferation. *J Comp Neurol* **413**, 385-404. (1999).
10. Monaghan, A. P., Grau, E., Bock, D. & Schütz, G. The mouse homolog of the orphan nuclear receptor *tailless* is expressed in the developing forebrain. *Development* **121**, 839-53 (1995).
11. Monaghan, A. P. *et al.* Defective limbic system in mice lacking the *tailless* gene. *Nature* **390**, 515-7. (1997).
12. Yu, R. T. *et al.* The orphan nuclear receptor Tlx regulates Pax2 and is essential for vision. *Proceedings of the National Academy of Sciences USA* **97**, 2621-2625 (2000).
13. Young, K. A. *et al.* Fierce: a new mouse deletion of *Nr2e1*; violent behaviour and ocular abnormalities are background-dependent. *Behavioural Brain Research* **132**, 145-158 (2002).
14. Enard, W. *et al.* Molecular evolution of FOXP2, a gene involved in speech and language. *Nature* **418**, 869-72 (2002).
15. Jackson, A., Panayiotidis, P. & Foroni, L. The human homologue of the Drosophila *tailless* gene (TLX): Characterization and mapping to a region of common deletion in human lymphoid leukemia on chromosome 6q21. *Genomics* **50**, 34-43 (1998).
16. Kobayashi, M., Yu, R. T., Yasuda, K. & Umesono, K. Cell-type-specific regulation of the retinoic acid receptor mediated by the orphan nuclear receptor TLX. *Mol Cell Biol* **20**, 8731-9 (2000).

17. Abrahams, B. S. *et al.* Metaphase FISHing of transgenic mice recommended: FISH and SKY define BAC-mediated balanced translocation. *Genesis* **36**, 134-41. (2003).
18. Banks, K. G. *et al.* Retroposon compensatory mechanism hypothesis not supported: Zfa knockout mice are fertile. *Genomics* **82**, 254-60. (2003).
19. Hawes, N. L. *et al.* Mouse fundus photography and angiography: a catalogue of normal and mutant phenotypes. *Molecular Vision* **5**, 22-29. (1999).
20. Hossain, S. M., Wong, B. K. Y. & Simpson, E. M. The Dark Phase Improves Genetic Discrimination for Some High Throughput Mouse Behavioral Phenotyping. *Genes Brain and Behavior* **3**, 167-177 (2004).
21. Van Loo, P. L., Mol, J. A., Koolhaas, J. M., Van Zutphen, B. F. & Baumans, V. Modulation of aggression in male mice: influence of group size and cage size. *Physiol Behav* **72**, 675-83 (2001).
22. Ouagazzal, A. M., Moreau, J. L., Pauly-Evers, M. & Jenck, F. Impact of environmental housing conditions on the emotional responses of mice deficient for nociceptin/orphanin FQ peptide precursor gene. *Behav Brain Res* **144**, 111-7 (2003).
23. Kumar, R. A. *et al.* Unexpected embryonic stem (ES) cell mutations represent a concern in gene targeting: Lessons from "fierce" mice. *Genesis* **38**, 51-7. (2004).
24. Land, P. W. & Monaghan, A. P. Expression of the transcription factor, *tailless*, is required for formation of superficial cortical layers. *Cereb Cortex* **13**, 921-31. (2003).
25. Schedl, A. *et al.* Influence of PAX6 gene dosage on development: overexpression causes severe eye abnormalities. *Cell* **86**, 71-82. (1996).
26. Brown, N. L. *et al.* Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis. *Development* **125**, 4821-33. (1998).
27. Chang, B. *et al.* Haploinsufficient Bmp4 ocular phenotypes include anterior segment dysgenesis with elevated intraocular pressure. *BMC Genet* **2**, 18. Epub 2001 Nov 6. (2001).
28. Cao, Q. *et al.* Suggestive evidence for a schizophrenia susceptibility locus on chromosome 6q and a confirmation in an independent series of pedigrees. *Genomics* **43**, 1-8. (1997).
29. Martinez, M. *et al.* Follow-up study on a susceptibility locus for schizophrenia on chromosome 6q. *American Journal of Medical Genetics* **88**, 337-43 (1999).
30. Levinson, D. F. *et al.* Multicenter Linkage Study of Schizophrenia Candidate Regions on Chromosomes 5q, 6q, 10p, and 13q: Schizophrenia Linkage Collaborative Group III. *Am J Hum Genet* **67** (2000).
31. Dick, D. M. *et al.* Genomewide linkage analyses of bipolar disorder: a new sample of 250 pedigrees from the National Institute of Mental Health Genetics Initiative. *Am J Hum Genet* **73**, 107-14. Epub 2003 May 27. (2003).
32. Pato, C. N. *et al.* Genome-wide scan in Portuguese Island families implicates multiple loci in bipolar disorder: Fine mapping adds support on chromosomes 6 and 11. *Am J Med Genet* **127B**, 30-4. (2004).
33. Middleton, F. A. *et al.* Genomewide linkage analysis of bipolar disorder by use of a high-density single-nucleotide-polymorphism (SNP) genotyping assay: a

- comparison with microsatellite marker assays and finding of significant linkage to chromosome 6q22. *Am J Hum Genet* **74**, 886-97. Epub 2004 Apr 1. (2004).
34. Schulze, T. G. *et al.* Loci on chromosomes 6q and 6p interact to increase susceptibility to bipolar affective disorder in the national institute of mental health genetics initiative pedigrees. *Biol Psychiatry* **56**, 18-23 (2004).
35. Johnston-Wilson, N. L. *et al.* Disease-specific alterations in frontal cortex brain proteins in schizophrenia, bipolar disorder, and major depressive disorder. The Stanley Neuropathology Consortium. *Mol Psychiatry* **5**, 142-9. (2000).
36. Davis, S. *et al.* Glial fibrillary acidic protein in late life major depressive disorder: an immunocytochemical study. *J Neurol Neurosurg Psychiatry* **73**, 556-60. (2002).
37. Chen, Y. H., Tsai, M. T., Shaw, C. K. & Chen, C. H. Mutation analysis of the human NR4A2 gene, an essential gene for midbrain dopaminergic neurogenesis, in schizophrenic patients. *Am J Med Genet* **105**, 753-7 (2001).
38. Buervenich, S. *et al.* NURR1 mutations in cases of schizophrenia and manic-depressive disorder. *Am J Med Genet* **96**, 808-13. (2000).
39. Iwayama-Shigeno, Y. *et al.* Distribution of haplotypes derived from three common variants of the NR4A2 gene in Japanese patients with schizophrenia. *Am J Med Genet* **118B**, 20-4 (2003).
40. Heyman, I. *et al.* Psychiatric disorder and cognitive function in a family with an inherited novel mutation of the developmental control gene PAX6. *Psychiatr Genet* **9**, 85-90 (1999).
41. Stober, G. *et al.* Functional PAX-6 gene-linked polymorphic region: potential association with paranoid schizophrenia. *Biol Psychiatry* **45**, 1585-91 (1999).
42. Goodman, A. B. Three independent lines of evidence suggest retinoids as causal to schizophrenia. *Proc Natl Acad Sci U S A* **95**, 7240-4. (1998).
43. Krezel, W. *et al.* Impaired locomotion and dopamine signaling in retinoid receptor mutant mice. *Science* **279**, 863-7 (1998).
44. Grabe, H. J., Ahrens, N., Rose, H. J., Kessler, C. & Freyberger, H. J. Neurotrophic factor S100 beta in major depression. *Neuropsychobiology* **44**, 88-90 (2001).
45. Rothermundt, M., Ponath, G. & Arolt, V. S100B in schizophrenic psychosis. *Int Rev Neurobiol* **59**, 445-70 (2004).
46. Benes, F. M., Kwok, E. W., Vincent, S. L. & Todtenkopf, M. S. A reduction of nonpyramidal cells in sector CA2 of schizophrenics and manic depressives. *Biol Psychiatry* **44**, 88-97. (1998).
47. Chen, G., Rajkowska, G., Du, F., Seraji-Bozorgzad, N. & Manji, H. K. Enhancement of hippocampal neurogenesis by lithium. *J Neurochem* **75**, 1729-34. (2000).
48. Dawirs, R. R., Hildebrandt, K. & Teuchert-Noodt, G. Adult treatment with haloperidol increases dentate granule cell proliferation in the gerbil hippocampus. *J Neural Transm* **105**, 317-27. (1998).
49. Wakade, C. G., Mahadik, S. P., Waller, J. L. & Chiu, F. C. Atypical neuroleptics stimulate neurogenesis in adult rat brain. *J Neurosci Res* **69**, 72-9. (2002).
50. *The Mouse Brain in Stereotaxic Coordinates* (eds. Franklin, K. B. J. & Paxinos, G.) (Academic Press, Inc., London, 1997).

Figure Captions.

Fig. 1: Human *NR2E1* corrects structural abnormalities seen in the fierce olfactory bulbs and forebrain. (a) Reduced olfactory bulb size (arrow) and incomplete extension of the cortex (dashed lines) was observed only in fierce. In contrast, wild-type (Wt), transgenic (Tg(Hum)), and rescue brains were indistinguishable. The surface areas of the (b) olfactory bulbs ($H_{3,34}=17.5854$, $p=0.0005$, *; $n=11, 6, 8, 9$ for Wt, fierce, Tg(Hum), and rescue, respectively) and (c) cortex ($H_{3,38}=17.4616$, $p=0.0005$, *; $n=11, 8, 9, 10$ for Wt, fierce, Tg(Hum), and rescue, respectively) were significantly different between groups as a result of fierce alone.

Fig. 2: Human *NR2E1* corrects abnormalities observed in forebrain sections. (a) Fierce coronal sections were small and showed abnormalities of the cingulum (Cg), piriform cortex (Pir), and anterior commissure (Ac). (b) The fierce cortex was shortened, consistent with hypoplasia across layers II and III. (c) The striatum (Str) and external capsule (Ec) were underdeveloped in fierce, whereas corticostriatal fibers (arrows) were enlarged and clustered medially. In contrast, transgenic (Tg(Hum)) and rescue sections were indistinguishable from wild type (Wt). Sections (Bregma +0.26mm⁵⁰) were stained with cresyl violet and luxol fast blue ($n=4, 5, 2, 5$ for Wt, fierce, Tg(Hum), and rescue, respectively).

Fig. 3: Human *NR2E1* ameliorates multiple aspects of the fierce eye. (a) Fierce fundus photos show radial asymmetry, mottling (arrow), and reduced vessel number. (b) Asymmetry was unique to fierce ($H_{3,46}=9.644$, $p<0.0001$, *; $n=12, 8, 12, 14$ for wild-type (Wt), fierce, transgenic (Tg(Hum)), and rescue, respectively). (c) Only fierce showed mottled pigment epithelium ($H_{3,32}=6.3$, $p=0.0978$; $n=12, 6, 7, 7$ for Wt, fierce, Tg(Hum), and rescue, respectively). (d) Fierce vessel number was significantly reduced relative to rescue ($H_{1,21}=12.3144$, $p<0.0004$, †; $n=7, 14$ for fierce, and rescue, respectively). Rescue vessel number was significantly reduced relative to Wt and Tg(Hum) ($H_{2,34}=21.4298$, $p<0.0001$, ‡; $n=11, 12, 14$ for Wt, Tg(Hum), and rescue, respectively).

Fig. 4: Human *NR2E1* corrects fierce behavior. In a tail suspension test, fierce was significantly different from other groups for (a) percent time struggling ($H_{3,47}=12.7877$, $p=0.0051$, *; $n=16, 6, 15, 10$ for wild-type (Wt), fierce, transgenic (Tg(Hum)), rescue) and (b) force exerted struggling ($H_{3,47}=12.9260$, $p=0.0048$, *). In an open field test (c) number of ambulatory counts ($H_{3,57}=23.2696$, $p<0.0001$, *; $n=17, 12, 17, 11$ for Wt, fierce, Tg(Hum), and rescue, respectively), (d) total ambulatory time ($H_{3,57}=25.4062$, $p<0.0001$, *), and (e) number of ambulatory episodes ($H_{3,57}=14.2440$, $p<0.0001$, *) were all significantly elevated in fierce. Aggression in a resident-intruder paradigm was also elevated; (f) instances of aggression per minute ($H_{3,49}=9.3130$, $p<0.0254$, *; $n=14, 7, 12, 16$ for Wt, fierce, Tg(Hum), and rescue, respectively), and (g) the percentage of time engaged in aggressive behaviors ($H_{3,49}=11.6736$, $p<0.0086$, *) were increased significantly in fierce relative to other genotypes. As well, (h) the percentage of time engaged in social behaviors was reduced in fierce relative to all other genotypes

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($H_{3,49}=9.8362$, $p<0.0200$, *). No significant differences were observed between the other three groups in any of the measures assessed in any of the three paradigms. AU = Arbitrary Units.

