ABSTRACT

The pathophysiology of epilepsy is intimately linked with imbalances of excitation and inhibition (E/I) in the brain. Although the loss of GABAergic inhibition is strongly implicated as a mechanism by which E/I imbalances arise in human epilepsy disorders, the molecular mechanisms that govern synaptic inhibition, and thereby maintain E/I balance, have been largely obscure. Recently, we discovered a novel inhibitory synaptic protein, Rogdi, whose gene is strongly linked to a human epilepsy disorder, Kohlschütter-Tönz syndrome. Preliminary data indicate that Rogdi may function either at the pre- or postsynapse where I hypothesize that it may regulate the synaptic vesicle cycle or protein trafficking. I propose to test this hypothesis and demonstrate Rogdi's functional role at inhibitory synapses. Importantly, I will also determine if the loss of Rogdi is causal for seizures in mice. The successful completion of the proposed research will generate a new preclinical mouse model for studying Kohlschütter-Tönz syndrome and can be expected to generate an understanding of Rogdi's molecular function and its contribution towards the etiology of a human epilepsy disorder.

PROJECT NARRATIVE

Epilepsy, the condition of spontaneous recurrent seizures, is a devastating neurological disorder. Rare genetic mutations provide unique opportunities to understand the molecular mechanisms that drive susceptibility to seizures and epilepsy. This proposed work will study a human epilepsy candidate gene and can be expected to generate a deeper understanding of the molecular mechanisms underlying epilepsy.

RELEVANCE STATEMENT

Epilepsy, the condition of spontaneous recurrent seizures, is a devastating neurological disorder. Rare genetic mutations provide unique opportunities to understand the molecular mechanisms that drive susceptibility to seizures and epilepsy. This proposed work will study a human epilepsy candidate gene and can be expected to generate a deeper understanding of the molecular mechanisms underlying epilepsy.

BIBLIOGRAPHY

1. Aswath N, Ramakrishnan SN, Teresa N, Ramanathan A. A novel ROGDI gene mutation is associated with Kohlschutter-Tonz syndrome. Oral Surg Oral Med Oral Pathol Oral Radiol. 2018 Jan;125(1):e8–e11. PMID: 29153277

2. Tucci A, Kara E, Schossig A, Wolf NI, Plagnol V, Fawcett K, Paisán-Ruiz C, Moore M, Hernandez D, Musumeci S, Tennison M, Hennekam R, Palmeri S, Malandrini A, Raskin S, Donnai D, Hennig C, Tzschach A, Hordijk R, Bast T, Wimmer K, Lo C-N, Shorvon S, Mefford H, Eichler EE, Hall R, Hayes I, Hardy J, Singleton A, Zschocke J, Houlden H. Kohlschütter-Tönz syndrome: mutations in ROGDI and evidence of genetic heterogeneity. Hum Mutat. 2013 Feb;34(2):296–300. PMCID: PMC3902979

3. Huckert M, Mecili H, Laugel-Haushalter V, Stoetzel C, Muller J, Flori E, Laugel V, Manière M-C, Dollfus H, Bloch-Zupan A. A Novel Mutation in the ROGDI Gene in a Patient with Kohlschütter-Tönz Syndrome. Mol Syndromol. 2014 Dec;5(6):293–298. PMCID: PMC4281576

4. Schossig A, Wolf NI, Kapferer I, Kohlschütter A, Zschocke J. Epileptic encephalopathy and amelogenesis imperfecta: Kohlschütter-Tönz syndrome. Eur J Med Genet. 2012 May;55(5):319–322. PMID: 22522085

5. Mory A, Dagan E, Illi B, Duquesnoy P, Mordechai S, Shahor I, Romani S, Hawash-Moustafa N, Mandel H, Valente EM, Amselem S, Gershoni-Baruch R. A nonsense mutation in the human homolog of Drosophila rogdi causes Kohlschutter-Tonz syndrome. Am J Hum Genet. 2012 Apr 6;90(4):708–714. PMCID: PMC3322231

6. Uezu A, Kanak DJ, Bradshaw TWA, Soderblom EJ, Catavero CM, Burette AC, Weinberg RJ, Soderling SH. Identification of an elaborate complex mediating postsynaptic inhibition. Science. 2016 Sep 9;353(6304):1123–1129. PMCID: PMC5432043

7. Smardon AM, Diab HI, Tarsio M, Diakov TT, Nasab ND, West RW, Kane PM. The RAVE complex is an isoform-specific V-ATPase assembly factor in yeast. Mol Biol Cell. 2014 Feb;25(3):356–367. PMCID: PMC3907276

8. Smardon AM, Tarsio M, Kane PM. The RAVE complex is essential for stable assembly of the yeast V-ATPase. J Biol Chem. 2002 Apr 19;277(16):13831–13839. PMID: 11844802

9. Hnasko TS, Edwards RH. Neurotransmitter corelease: mechanism and physiological role. Annu Rev Physiol. 2012;74:225–243. PMCID: PMC4090038

10. Yan Y, Denef N, Schüpbach T. The vacuolar proton pump, V-ATPase, is required for notch signaling and endosomal trafficking in Drosophila. Dev Cell. 2009 Sep;17(3):387–402. PMCID: PMC2758249

11. Marshansky V, Futai M. The V-type H+-ATPase in vesicular trafficking: targeting, regulation and function. Curr Opin Cell Biol. 2008 Aug;20(4):415–426. PMID: 18511251

12. Hurtado-Lorenzo A, Skinner M, El Annan J, Futai M, Sun-Wada G-H, Bourgoin S, Casanova J, Wildeman A, Bechoua S, Ausiello DA, Brown D, Marshansky V. V-ATPase interacts with ARNO and Arf6 in early endosomes and regulates the protein degradative pathway. Nat Cell Biol. 2006 Feb;8(2):124–136. PMID: 16415858

13. Marshansky V, Rubinstein JL, Grüber G. Eukaryotic V-ATPase: novel structural findings and functional insights. Biochim Biophys Acta. 2014 Jun;1837(6):857–879. PMID: 24508215

14. Hosokawa H, Dip PV, Merkulova M, Bakulina A, Zhuang Z, Khatri A, Jian X, Keating SM, Bueler SA, Rubinstein JL, Randazzo PA, Ausiello DA, Grüber G, Marshansky V. The N termini of a-subunit isoforms are involved in signaling between vacuolar H+-ATPase (V-ATPase) and cytohesin-2. J Biol Chem. 2013 Feb 22;288(8):5896–5913. PMCID: PMC3581410

15. Merkulova M, Bakulina A, Thaker YR, Grüber G, Marshansky V. Specific motifs of the V-ATPase a2-subunit isoform interact with catalytic and regulatory domains of ARNO. Biochim Biophys Acta. 2010 Aug;1797(8):1398–1409. PMID: 20153292

16. Di Giovanni J, Boudkkazi S, Mochida S, Bialowas A, Samari N, Lévêque C, Youssouf F, Brechet A, Iborra C, Maulet Y, Moutot N, Debanne D, Seagar M, El Far O. V-ATPase membrane sector associates with synaptobrevin to modulate neurotransmitter release. Neuron. 2010 Jul 29;67(2):268–279. PMID: 20670834

17. Hiesinger PR, Fayyazuddin A, Mehta SQ, Rosenmund T, Schulze KL, Zhai RG, Verstreken P, Cao Y, Zhou Y, Kunz J, Bellen HJ. The v-ATPase V0 subunit a1 is required for a late step in synaptic vesicle exocytosis in Drosophila. Cell. 2005 May 20;121(4):607–620. PMCID: PMC3351201

18. Suzuki K, Tsunekawa Y, Hernandez-Benitez R, Wu J, Zhu J, Kim EJ, Hatanaka F, Yamamoto M, Araoka T, Li Z, Kurita M, Hishida T, Li M, Aizawa E, Guo S, Chen S, Goebl A, Soligalla RD, Qu J, Jiang T, Fu X, Jafari M, Esteban CR, Berggren WT, Lajara J, Nuñez-Delicado E, Guillen P, Campistol JM, Matsuzaki F, Liu G-H, Magistretti P, Zhang K, Callaway EM, Zhang K, Belmonte JCI. In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. Nature. 2016 Dec 1;540(7631):144–149. PMCID: PMC5331785

19. Treiman DM. GABAergic mechanisms in epilepsy. Epilepsia. 2001;42 Suppl 3:8–12. PMID: 11520315

20. Kubota Y, Karube F, Nomura M, Kawaguchi Y. The diversity of cortical inhibitory synapses. Front Neural Circuits. 2016 Apr 25;10:27. PMCID: PMC4842771

21. Noebels J. Pathway-driven discovery of epilepsy genes. Nat Neurosci. 2015 Mar;18(3):344–350. PMCID: PMC4852130

22. Myers CT, Mefford HC. Advancing epilepsy genetics in the genomic era. Genome Med. 2015 Aug 25;7:91. PMCID: PMC4549122

23. Berglund L, Björling E, Oksvold P, Fagerberg L, Asplund A, Szigyarto CA-K, Persson A, Ottosson J, Wernérus H, Nilsson P, Lundberg E, Sivertsson A, Navani S, Wester K, Kampf C, Hober S, Pontén F, Uhlén M. A genecentric Human Protein Atlas for expression profiles based on antibodies. Mol Cell Proteomics. 2008 Oct;7(10):2019–2027. PMID: 18669619

24. Egelhofer TA, Minoda A, Klugman S, Lee K, Kolasinska-Zwierz P, Alekseyenko AA, Cheung M-S, Day DS, Gadel S, Gorchakov AA, Gu T, Kharchenko PV, Kuan S, Latorre I, Linder-Basso D, Luu Y, Ngo Q, Perry M, Rechtsteiner A, Riddle NC, Schwartz YB, Shanower GA, Vielle A, Ahringer J, Elgin SCR, Kuroda MI, Pirrotta V, Ren B, Strome S, Park PJ, Karpen GH, Hawkins RD, Lieb JD. An assessment of histone-modification antibody quality. Nat Struct Mol Biol. 2011 Jan;18(1):91–93. PMCID: PMC3017233

25. Uhlen M, Bandrowski A, Carr S, Edwards A, Ellenberg J, Lundberg E, Rimm DL, Rodriguez H, Hiltke T, Snyder M, Yamamoto T. A proposal for validation of antibodies. Nat Methods. 2016 Sep 5;13(10):823–827. PMID: 27595404

26. Combs CA. Fluorescence microscopy: a concise guide to current imaging methods. Curr Protoc Neurosci. 2010 Jan;Chapter 2:Unit2.1. PMCID: PMC3805368

27. Dani A, Huang B, Bergan J, Dulac C, Zhuang X. Superresolution imaging of chemical synapses in the brain. Neuron. 2010 Dec 9;68(5):843–856. PMCID: PMC3057101

28. Sunkin SM, Ng L, Lau C, Dolbeare T, Gilbert TL, Thompson CL, Hawrylycz M, Dang C. Allen Brain Atlas: an integrated spatio-temporal portal for exploring the central nervous system. Nucleic Acids Res. 2013 Jan;41(Database issue):D996–D1008. PMCID: PMC3531093

29. Sharma K, Schmitt S, Bergner CG, Tyanova S, Kannaiyan N, Manrique-Hoyos N, Kongi K, Cantuti L, Hanisch U-K, Philips M-A, Rossner MJ, Mann M, Simons M. Cell type- and brain region-resolved mouse brain proteome. Nat Neurosci. 2015 Dec;18(12):1819–1831. PMID: 26523646

30. Cembrowski MS, Wang L, Sugino K, Shields BC, Spruston N. Hipposeq: a comprehensive RNA-seq database of gene expression in hippocampal principal neurons. elife. 2016 Apr 26;5:e14997. PMCID: PMC4846374

31. Zeisel A, Muñoz-Manchado AB, Codeluppi S, Lönnerberg P, La Manno G, Juréus A, Marques S, Munguba H, He L, Betsholtz C, Rolny C, Castelo-Branco G, Hjerling-Leffler J, Linnarsson S. Brain structure. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. Science. 2015 Mar 6;347(6226):1138–1142. PMID: 25700174

32. Riemann D, Wallrafen R, Dresbach T. The Kohlschütter-Tönz syndrome associated gene Rogdi encodes a novel presynaptic protein. Sci Rep. 2017 Nov 17;7(1):15791. PMCID: PMC5693994

33. Lee H, Jeong H, Choe J, Jun Y, Lim C, Lee C. The crystal structure of human Rogdi provides insight into the causes of Kohlschutter-Tönz Syndrome. Sci Rep. 2017 Jun 21;7(1):3972. PMCID: PMC5479863

34. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc. 2015 Jun;10(6):845–858. PMCID: PMC5298202

35. Seol JH, Shevchenko A, Shevchenko A, Deshaies RJ. Skp1 forms multiple protein complexes, including RAVE, a regulator of V-ATPase assembly. Nat Cell Biol. 2001 Apr;3(4):384–391. PMID: 11283612

36. Smardon AM, Nasab ND, Tarsio M, Diakov TT, Kane PM. Molecular Interactions and Cellular Itinerary of the Yeast RAVE (Regulator of the H+-ATPase of Vacuolar and Endosomal Membranes) Complex. J Biol Chem. 2015 Nov 13;290(46):27511–27523. PMCID: PMC4646003

37. Li KW, Chen N, Klemmer P, Koopmans F, Karupothula R, Smit AB. Identifying true protein complex constituents in interaction proteomics: the example of the DMXL2 protein complex. Proteomics. 2012 Aug;12(15-16):2428–2432. PMID: 22707207

38. Merkulova M, Păunescu TG, Azroyan A, Marshansky V, Breton S, Brown D. Mapping the H(+) (V)-ATPase interactome: identification of proteins involved in trafficking, folding, assembly and phosphorylation. Sci Rep. 2015 Oct 7;5:14827. PMCID: PMC4595830

39. Johnson L, Ganss B, Wang A, Zirngibl RA, Johnson DE, Owen C, Bradley G, Voronov I. V-ATPases Containing a3 Subunit Play a Direct Role in Enamel Development in Mice. J Cell Biochem. 2017 May 3;118(10):3328–3340. PMID: 28295540

40. Fassio A, Esposito A, Kato M, Saitsu H, Mei D, Marini C, Conti V, Nakashima M, Okamoto N, Olmez Turker A, Albuz B, Semerci Gündüz CN, Yanagihara K, Belmonte E, Maragliano L, Ramsey K, Balak C, Siniard A, Narayanan V, C4RCD Research Group, Ohba C, Shiina M, Ogata K, Matsumoto N, Benfenati F, Guerrini R. De novo mutations of the ATP6V1A gene cause developmental encephalopathy with epilepsy. Brain. 2018 Jun 1;141(6):1703–1718. PMCID: PMC5972584

41. Chiou S-H, Winters IP, Wang J, Naranjo S, Dudgeon C, Tamburini FB, Brady JJ, Yang D, Grüner BM, Chuang C-H, Caswell DR, Zeng H, Chu P, Kim GE, Carpizo DR, Kim SK, Winslow MM. Pancreatic cancer modeling using retrograde viral vector delivery and in vivo CRISPR/Cas9-mediated somatic genome editing. Genes Dev. 2015 Jul 15;29(14):1576–1585. PMCID: PMC4526740

42. Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR, Dahlman JE, Parnas O, Eisenhaure TM, Jovanovic M, Graham DB, Jhunjhunwala S, Heidenreich M, Xavier RJ, Langer R, Anderson DG, Hacohen N, Regev A, Feng G, Sharp PA, Zhang F. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. Cell. 2014 Oct 9;159(2):440–455. PMCID: PMC4265475

43. Viswanathan S, Williams ME, Bloss EB, Stasevich TJ, Speer CM, Nern A, Pfeiffer BD, Hooks BM, Li W-P, English BP, Tian T, Henry GL, Macklin JJ, Patel R, Gerfen CR, Zhuang X, Wang Y, Rubin GM, Looger LL. High-performance probes for light and electron microscopy. Nat Methods. 2015 Jun;12(6):568–576. PMCID: PMC4573404

44. Greenwood C, Ruff D, Kirvell S, Johnson G, Dhillon HS, Bustin SA. Proximity assays for sensitive quantification of proteins. Biomolecular Detection and Quantification. 2015 Jun;4:10–16. PMCID: PMC4822221

45. Haeussler M, Schönig K, Eckert H, Eschstruth A, Mianné J, Renaud J-B, Schneider-Maunoury S, Shkumatava A, Teboul L, Kent J, Joly J-S, Concordet J-P. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. Genome Biol. 2016 Jul 5;17(1):148. PMCID: PMC4934014

46. Michielsen ECHJ, Diris JHC, Hackeng CM, Wodzig WKWH, Van Dieijen-Visser MP. Highly sensitive immunoprecipitation method for extracting and concentrating low-abundance proteins from human serum. Clin Chem. 2005 Jan;51(1):222–224. PMID: 15613717

47. Cherry JM, Hong EL, Amundsen C, Balakrishnan R, Binkley G, Chan ET, Christie KR, Costanzo MC, Dwight SS, Engel SR, Fisk DG, Hirschman JE, Hitz BC, Karra K, Krieger CJ, Miyasato SR, Nash RS, Park J, Skrzypek MS, Simison M, Weng S, Wong ED. Saccharomyces Genome Database: the genomics resource of budding yeast. Nucleic Acids Res. 2012 Jan;40(Database issue):D700–5. PMCID: PMC3245034

48. Okada H, Uezu A, Mason FM, Soderblom EJ, Moseley MA, Soderling SH. SH3 domain-based phototrapping in living cells reveals Rho family GAP signaling complexes. Sci Signal. 2011 Nov 29;4(201):rs13. PMCID: PMC3553496

49. Uezu A, Okada H, Murakoshi H, del Vescovo CD, Yasuda R, Diviani D, Soderling SH. Modified SH2 domain to phototrap and identify phosphotyrosine proteins from subcellular sites within cells. Proc Natl Acad Sci U S A. 2012 Oct 23;109(43):E2929–38. PMCID: PMC3491470

50. Braat S, Kooy RF. The GABAA receptor as a therapeutic target for neurodevelopmental disorders. Neuron. 2015 Jun 3;86(5):1119–1130. PMID: 26050032

51. Niturad CE, Lev D, Kalscheuer VM, Charzewska A, Schubert J, Lerman-Sagie T, Kroes HY, Oegema R, Traverso M, Specchio N, Lassota M, Chelly J, Bennett-Back O, Carmi N, Koffler-Brill T, Iacomino M, Trivisano M, Capovilla G, Striano P, Nawara M, Rzonca S, Fischer U, Bienek M, Jensen C, Hu H, Thiele H, Altmüller J, Krause R, May P, Becker F, EuroEPINOMICS Consortium, Balling R, Biskup S, Haas SA, Nürnberg P, van Gassen KLI, Lerche H, Zara F, Maljevic S, Leshinsky-Silver E. Rare GABRA3 variants are associated with epileptic seizures, encephalopathy and dysmorphic features. Brain. 2017 Nov 1;140(11):2879–2894. PMID: 29053855

52. Dibbens LM, Harkin LA, Richards M, Hodgson BL, Clarke AL, Petrou S, Scheffer IE, Berkovic SF, Mulley JC. The role of neuronal GABA(A) receptor subunit mutations in idiopathic generalized epilepsies. Neurosci Lett. 2009 Apr 10;453(3):162–165. PMID: 19429026

53. Wang J, Lin Z-J, Liu L, Xu H-Q, Shi Y-W, Yi Y-H, He N, Liao W-P. Epilepsy-associated genes. Seizure. 2017 Jan;44:11–20. PMID: 28007376

54. Prasad DKV, Shaheen U, Satyanarayana U, Prabha TS, Jyothy A, Munshi A. Association of GABRA6 1519 T>C (rs3219151) and Synapsin II (rs37733634) gene polymorphisms with the development of idiopathic generalized epilepsy. Epilepsy Res. 2014 Oct;108(8):1267–1273. PMID: 25088614

55. Bromfield EB, Cavazos JE, Sirven JI. Neuropharmacology of Antiepileptic Drugs - An Introduction to Epilepsy - NCBI Bookshelf. 2006;

56. Obenaus A, Esclapez M, Houser CR. Loss of glutamate decarboxylase mRNA-containing neurons in the rat dentate gyrus following pilocarpine-induced seizures. J Neurosci. 1993 Oct;13(10):4470–4485. PMID: 8410199

57. Houser CR, Esclapez M. Vulnerability and plasticity of the GABA system in the pilocarpine model of spontaneous recurrent seizures. Epilepsy Res. 1996 Dec;26(1):207–218. PMID: 8985701

58. Sloviter RS. Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. Science. 1987 Jan 2;235(4784):73–76. PMID: 2879352

59. Morin F, Beaulieu C, Lacaille JC. Selective loss of GABA neurons in area CA1 of the rat hippocampus after intraventricular kainate. Epilepsy Res. 1998 Nov;32(3):363–369. PMID: 9839776

60. De Lanerolle NC, Kim JH, Robbins RJ, Spencer DD. Hippocampal interneuron loss and plasticity in human temporal lobe epilepsy. Brain Res. 1989 Aug 28;495(2):387–395. PMID: 2569920

61. Robbins RJ, Brines ML, Kim JH, Adrian T, de Lanerolle N, Welsh S, Spencer DD. A selective loss of somatostatin in the hippocampus of patients with temporal lobe epilepsy. Ann Neurol. 1991 Mar;29(3):325–332. PMID: 1675046

62. Mathern GW, Babb TL, Pretorius JK, Leite JP. Reactive synaptogenesis and neuron densities for neuropeptide Y, somatostatin, and glutamate decarboxylase immunoreactivity in the epileptogenic human fascia dentata. J Neurosci. 1995 May;15(5 Pt 2):3990–4004. PMID: 7751960

63. Cossart R, Dinocourt C, Hirsch JC, Merchan-Perez A, De Felipe J, Ben-Ari Y, Esclapez M, Bernard C. Dendritic but not somatic GABAergic inhibition is decreased in experimental epilepsy. Nat Neurosci. 2001 Jan;4(1):52–62. PMID: 11135645

64. Gibbs JW, Shumate MD, Coulter DA. Differential epilepsy-associated alterations in postsynaptic GABA(A) receptor function in dentate granule and CA1 neurons. J Neurophysiol. 1997 Apr;77(4):1924–1938. PMID: 9114245

65. Hirsch JC, Agassandian C, Merchán-Pérez A, Ben-Ari Y, DeFelipe J, Esclapez M, Bernard C. Deficit of quantal release of GABA in experimental models of temporal lobe epilepsy. Nat Neurosci. 1999 Jun;2(6):499–500. PMID: 10448211

66. Schossig A, Wolf NI, Fischer C, Fischer M, Stocker G, Pabinger S, Dander A, Steiner B, Tönz O, Kotzot D, Haberlandt E, Amberger A, Burwinkel B, Wimmer K, Fauth C, Grond-Ginsbach C, Koch MJ, Deichmann A, von Kalle C, Bartram CR, Kohlschütter A, Trajanoski Z, Zschocke J. Mutations in ROGDI Cause Kohlschütter-Tönz Syndrome. Am J Hum Genet. 2012 Apr 6;90(4):701–707. PMCID: PMC3322220

67. Morscher RJ, Rauscher C, Sperl W, Rittinger O. Seizures, enamel defects and psychomotor developmental delay: The first patient with Kohlschütter-Tönz syndrome caused by a ROGDI-gene deletion. Seizure. 2017 Aug;50:118–120. PMID: 28651123

68. Kim M, Jang D, Yoo E, Oh Y, Sonn JY, Lee J, Ki Y, Son HJ, Hwang O, Lee C, Lim C, Choe J. Rogdi Defines GABAergic Control of a Wake-promoting Dopaminergic Pathway to Sustain Sleep in Drosophila. Sci Rep. 2017 Sep 12;7(1):11368. PMCID: PMC5595912

69. Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR, Lein ES, Zeng H. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci. 2010 Jan;13(1):133–140. PMCID: PMC2840225

70. Taniguchi H, He M, Wu P, Kim S, Paik R, Sugino K, Kvitsiani D, Fu Y, Lu J, Lin Y, Miyoshi G, Shima Y, Fishell G, Nelson SB, Huang ZJ. A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex. Neuron. 2011 Sep 22;71(6):995–1013. PMCID: PMC3779648

71. Dimidschstein J, Chen Q, Tremblay R, Rogers SL, Saldi G-A, Guo L, Xu Q, Liu R, Lu C, Chu J, Grimley JS, Krostag A-R, Kaykas A, Avery MC, Rashid MS, Baek M, Jacob AL, Smith GB, Wilson DE, Kosche G,

Kruglikov I, Rusielewicz T, Kotak VC, Mowery TM, Anderson SA, Callaway EM, Dasen JS, Fitzpatrick D, Fossati V, Long MA, Noggle S, Reynolds JH, Sanes DH, Rudy B, Feng G, Fishell G. A viral strategy for targeting and manipulating interneurons across vertebrate species. Nat Neurosci. 2016 Oct 31;19(12):1743–1749. PMCID: PMC5348112

72. Egashira Y, Takase M, Takamori S. Monitoring of vacuolar-type H+ ATPase-mediated proton influx into synaptic vesicles. J Neurosci. 2015 Feb 25;35(8):3701–3710. PMID: 25716867

73. Gasnier B. The loading of neurotransmitters into synaptic vesicles. Biochimie. 2000 Apr;82(4):327–337. PMID: 10865121

74. Rost BR, Schneider F, Grauel MK, Wozny C, Bentz C, Blessing A, Rosenmund T, Jentsch TJ, Schmitz D, Hegemann P, Rosenmund C. Optogenetic acidification of synaptic vesicles and lysosomes. Nat Neurosci. 2015 Dec;18(12):1845–1852. PMCID: PMC4869830

75. Puranam RS, He XP, Yao L, Le T, Jang W, Rehder CW, Lewis DV, McNamara JO. Disruption of Fgf13 causes synaptic excitatory-inhibitory imbalance and genetic epilepsy and febrile seizures plus. J Neurosci. 2015 Jun 10;35(23):8866–8881. PMCID: PMC4461691

76. In vivo proximity proteomics of nascent synapses reveals a novel regulator of cytoskeleton-mediated synaptic maturation. Erin F. Spence, Shataakshi Dube, Akiyoshi Uezu, Margaret Locke, Erik J. Soderblom, and Scott H. Soderling. *Nature Communications, In-publication* 2018.

SPECIFIC AIMS

Loss-of-function mutations in ***ROGDI*** are causally linked to Kohlschütter-Tönz syndrome (KTS), an autosomal-recessive epilepsy disorder characterized by spontaneous seizures, mild to severe intellectual impairment, and amelogenesis imperfecta, a loss of the tooth enamel (OMIM #614574)[1–5]. As the function of Rogdi is not known, how the loss of Rogdi results in epilepsy is unclear. We recently performed a proteomics-based study to resolve the *in vivo* molecular composition of the inhibitory postsynaptic density (iPSD) in which we identified Rogdi at this site[6]. Moreover, 21% of the iPSD proteome was found to be genetically implicated in autism and/or epilepsy disorders in humans. These data suggest that iPSD dysfunction may be a common epileptogenic mechanism, and more specifically, that the loss of Rogdi may produce deficits in GABAergic inhibition that drives epilepsy in KTS.

Our identification of Rogdi at the iPSD suggests that it may be important for postsynaptic GABAergic neurotransmission. CRISPR-mediated tagging of endogenous Rogdi with HA epitope tags has confirmed that Rogdi localizes at the inhibitory synapse. Additionally, preliminary co-immunoprecipitation (co-IP) experiments have confirmed that Rogdi interacts with several critically important inhibitory postsynaptic proteins including Collybistin. Together, these data support the hypothesis that Rogdi functions postsynaptically. However, additional proteomics experiments in our lab have also identified Rogdi *presynaptically*. Preliminary bioinformatic analysis and co-IP experiments support this idea by demonstrating that Rogdi associates with the presynaptic vesicular machinery and may be a component of a known protein complex, the regulator of ATPase of vacuoles and endosomes (RAVE) complex. The RAVE complex modulates the function of V-ATPases[7,8], a proton pumping complex that acidifies the lumen of intracellular organelles. V-ATPases function presynaptically in the loading of synaptic vesicles with neurotransmitters[9], but may also function *postsynaptically* in receptor trafficking[10–15] and endo/exocytosis[16,17]. In summary, Rogdi is a novel synaptic protein whose loss in humans is strongly linked to epilepsy, yet it remains unclear whether and how Rogdi functions in synaptic inhibition. These preliminary data direct my main **hypotheses**: (1) Rogdi localizes at inhibitory synapses where it may function pre- and/or postsynaptically as a component of the RAVE complex and (2) loss of Rogdi causes deficits in synaptic inhibition and is sufficient to cause epilepsy in mice. I will test these hypotheses in the following aims:

Aim 1. Examining molecular links between Rogdi and the inhibitory synapse. To resolve the subcellular localization of Rogdi, I will tag the endogenous gene with epitope tags using CRISPR-mediated Homology-independent Target Integration (HITI)[18], a recently developed CRISPR-Cas9 genome editing approach. Epitope-tagged, endogenous Rogdi will be labeled by immunostaining with pre- and post-synaptic markers of excitatory and inhibitory synapses and visualized by super-resolution microscopy to unambiguously determine Rogdi's subcellular localization. Second, to determine whether Rogdi is a novel component of the mammalian RAVE complex, I will extend the HITI approach to label endogenous RAVE complex proteins and then test whether Rogdi co-IPs with these proteins. These experiments will determine whether Rogdi localizes at the inhibitory pre- and/or postsynapse and determine whether Rogdi is a component of the RAVE complex.

Aim 2. Characterizing the role of Rogdi in GABAergic synaptic transmission and epilepsy. Epilepsy is associated with deficits in GABAergic inhibition that produce neuronal hyperexcitability and spontaneous, recurrent seizures. The loss of Rogdi may produce deficits in GABAergic inhibition if its loss results in inhibitory synapse dysfunction either *(1)* postsynaptically in excitatory neurons and/or *(2)* presynaptically in inhibitory interneurons. To assess the role of Rogdi in GABAergic neurotransmission and to determine if the loss of Rogdi is sufficient to cause epilepsy, I have developed a new conditional Rogdi knockout (KO) mouse. Using this mouse in combination with cell-type selective adeno associated viruses (AAVs) and mouse Cre-genetics I will dissect the role of Rogdi in postsynaptic and presynaptic GABAergic function. Sparse knock out of Rogdi in either excitatory or inhibitory neurons will allow for selective recordings from KO neurons receiving input from wildtype neurons (postsynaptic effects) or recordings from wildtype neurons receiving input from KO neurons (presynaptic effects). To assess the role of Rogdi in seizures, our *Rogdi* conditional knockout mice will be crossed with CMV-Cre mice to generate a conventional *Rogdi* KO mouse line. These mice will be monitored for spontaneous seizures in early life. These tests will determine if the loss of *Rogdi* in mice results in impaired synaptic transmission and KTS-like phenotypes *in vivo*.

I anticipate that the results of these aims will provide fundamental insights into the *molecular- to cellular-level mechanisms* by which the loss of *ROGDI* contributes to the pathogenesis of a human epilepsy disorder. As such, these studies can be expected to reveal new insights into fundamental processes of normal inhibitory synapse function, which are currently poorly understood.

SIGNIFICANCE

Deficits in synaptic inhibition are strongly implicated in human epilepsies as mutations in GABA receptor genes are associated with numerous human epilepsy disorders and pharmacological blockade of inhibition invariably results in seizures or epilepsy[19]. However, the molecular mechanisms which govern synaptic inhibition in health and disease are not well understood. Thus, the proposed work is **highly significant** as it will address *fundamental questions regarding the poorly understood mechanisms regulating synaptic inhibition, and it will shed light on how inhibitory synapse dysfunction may lead to a human epilepsy disorder*.

Cortical inhibitory synapses form predominately on the dendritic shaft or soma[20]. This has occluded the biochemical purification of inhibitory synapses. Consequently, there was limited knowledge regarding the molecular composition of the inhibitory synapse as well as the mechanisms by which disruption of inhibition drives brain pathologies. The iPSD proteome[6] provides a prospectus for a deeper understanding of synaptic physiology that was previously confined to the excitatory PSD. Furthermore, these findings provide a rationale for interrogating the links between epilepsy and novel inhibitory synapse proteins.

Rogdi, a protein strongly associated with human epilepsy, was one of the novel proteins we discovered at the inhibitory postsynapse. Preliminary data discussed below direct the hypothesis that Rogdi is the mammalian ortholog of a yeast protein of known function, Rav2. In yeast, Rav2 functions as part of a three-protein complex, the regulator of ATPase of vacuoles and endosomes or **RAVE complex** which regulates the reversible assembly of the V-ATPase and thereby modulates its function. Although V-ATPase function and components of the RAVE complex have been established to be conserved in mammalian cells, a Rav2 homolog has not been identified. I propose that Rogdi is the final missing piece of this tripartite complex and aim to test the conservation of the RAVE complex in mammalian neurons. Thus, the successful completion of this proposed work will represent a **fundamental step** towards demonstrating *the function of Rogdi, a synaptic epilepsy candidate protein of unknown function, and the function of the RAVE complex, a poorly understood protein complex in mammalian cells.*

INNOVATION

Conceptual Innovation. While the excitatory postsynapse has been a major focus of molecular neuroscience for the last three decades, the proposed work to study a novel protein of the inhibitory synapse is ***conceptually innovative*** and is expected to shed light on mechanisms of synaptic inhibition and potential disease etiology.

The realization that the iPSD is ***more complex than previously appreciated*** and is a ***locus of disease burden*** emphasizes the fundamental importance of an understanding of synaptic inhibition. Genomic studies are rapidly accumulating evidence implicating various genes in epilepsy disorders[21,22]. Although the field of epilepsy has focused on the role of GABAergic deficits in the pathogenesis of epilepsy, little beyond the role of GABA receptors and ion channels in producing these deficits is known. To begin to address these questions, I will utilize a new conditional *Rogdi* KO mouse in combination with a ***conceptually innovative optogenetics approach*** to dissect the potential role of Rogdi inhibitory pre- and postsynaptic function.

Technical Innovation. Herein I proposed to use homology-independent targeted integration (**HITI**), a ***technically innovative*** CRISPR-Cas9 based genome editing approach that enables the labeling of endogenous proteins[74]. HITI does not rely upon protein-specific antibodies and thereby overcomes several limitations of antibodies such as unsuspected cross-reactivity to other proteins and insufficient application-specific validation[23–25]. Our preliminary data demonstrate the feasibility of this approach as we have employed HITI to tag and visualize a growing number of proteins *in vitro* and *in vivo*.

Combining CRISPR based gene editing approaches with super-resolution microscopy techniques such as stimulated emission depletion microscopy or **STED** is ***technically innovative*** and enables the high acuity resolution of endogenous cellular structures. The resolution of confocal microscopy is limited by the diffraction limit of light and thus has a lateral resolution of ~200 nm[26]. As the pre- and postsynapse are separated by only ~20 nm[27], confocal microscopy cannot accurately resolve these structures. Thus, super-resolution techniques such as STED are required. Here, I propose to use HITI and STED microscopy to analyze the localization of endogenous Rogdi. This approach is ***technically superior*** to more commonly employed approaches utilizing overexpression and/or confocal microscopy as it does not rely upon protein overexpression and has the optical resolution to precisely resolve synaptic structures.

I will also utilize the ***technically innovative HITI approach*** to determine whether Rogdi associates with endogenous RAVE complex proteins. In the absence of validated antibodies for a protein of interest, HITI enables the tagging of endogenous genes and thereby avoids the confounds of protein overexpression, but also overcomes limitations inherent with working with large genes whose coding sequences may be prohibitively large for overexpression.

APPROACH

In this application, I will focus on **Rogdi,** a protein of *unknown function* whose gene is strongly linked to a human epilepsy disorder, Kohlschütter-Tönz syndrome. *In situ* hybridization[28], mouse brain proteomics[29], and single-cell RNA-seq[30,31] indicate that *Rogdi* is highly expressed in the mouse hippocampus—putatively in the dentate gyrus and CA1 regions. This expression pattern is consistent with the potential role of Rogdi in regulating hippocampal excitability and may be pertinent to the seizures and intellectual impairments observed in KTS individuals. I have therefore chosen to focus on the hippocampus as a model system throughout this proposal.

To ensure robust and unbiased analyses, all data will be collected blind to condition/genotype and both sexes will be included throughout the experimental design.

Aim 1. Examining molecular links between Rogdi and the inhibitory synapse.

Rogdi localizes to inhibitory synapses. *I hypothesize that (1) Rogdi localizes at inhibitory synapses and (2) is a component of the RAVE complex*. Rogdi was identified in the Collybistin iBioID proteome. Subsequently, we have confirmed that Rogdi can complex with InSyn1 and Collybistin, key protein components of the iPSD (**Fig.1a**). Our preliminary data demonstrate that endogenous Rogdi localizes at inhibitory synapses *in vitro* (**Fig.1b**). In agreement with this, a recent study observed punctate, endogenous Rogdi immunofluorescence in the dendrites of cultured neurons; a staining pattern indicative of a postsynaptic localization[32]. However, the same authors also found that overexpression of GFP-tagged Rogdi colocalized with presynaptic markers in axons of mature cultured hippocampal neurons. Thus, it remains unclear if Rogdi resides and functions presynaptically, postsynaptically, or at both locations. To address this question, I will first investigate the localization of endogenous Rogdi using HITI and STED.

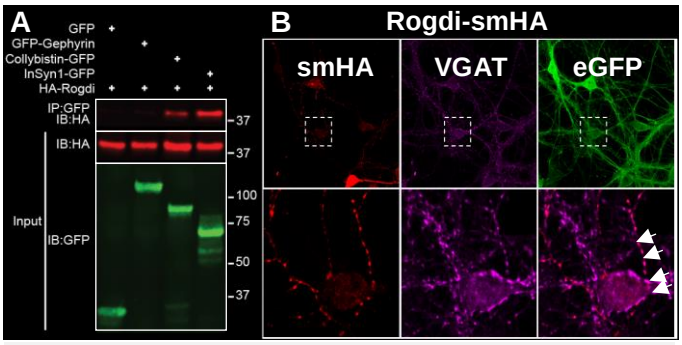


Fig.1 ***Rogdi localizes to the inhibitory synapse. A***. HA-Rogdi immunoprecipitates with InSyn1-GFP and Collybistin-GFP. ***B***. HITI smHA-tagged, endogenous Rogdi colocalizes with Vgat (inhibitory synapse marker) in DIV13 hippocampal neurons.

Rogdi is a component of the RAVE complex. Evidence also supports the hypothesis that Rogdi is a component of the RAVE complex. Querying databases for known protein domains in Rogdi revealed a leucine zipper domain (PF10259) that is found in Rogdi proteins across numerous species, including the Rav2 protein in yeast. Alignment of Rogdi's recently determined crystal structure[33] with the predicted structure of yeast Rav2 using Phyre2[34] suggests a strikingly similar overall domain organization and tertiary structure (**Fig.2a**). In yeast, Rav2 is a component of a three-protein complex, the RAVE complex[7,8] composed of Rav1, Rav2, and Skp1p[35]. The RAVE complex associates with and coordinates the assembly of V-ATPases in the plasma membrane of cellular organelles. Loss of the core RAVE scaffolding protein, Rav1 or its binding partner Rav2 impairs V-ATPase function in yeast[7,35,36]. In humans, Rav1 is encoded by the orthologous DMXL1, DMXL2, and WDR7 genes. AP-MS proteomics studies have identified Rogdi in complex with Dmxl2[37] and a V-ATPase subunit[38], supporting the existence of a mammalian RAVE complex composed of Dmxl1/2, Wdr7, and Rogdi.

Finally, loss of *ROGDI* in KTS causes amylogenesis imperfecta (AI; loss of tooth enamel) and epilepsy. The V-ATPase α3 subunit is expressed in ameloblasts, a cell type that is responsible for the formation of the tooth enamel. Loss of the V-ATPase α3 gene results in AI in mice[39]. If Rogdi acts as Rav2 in facilitating the assembly of the V-ATPase complex, loss of Rogdi could be expected to compromise V-ATPase function, and this may be the underlying mechanism by which AI occurs in KTS. Furthermore, in humans *de novo* mutations in *ATP6V1A* were identified in patients with developmental encephalopathy and epilepsy[40]. Thus, a loss of V-ATPase function caused by a loss of *ROGDI* may underly two hallmark features of KTS—AI and epilepsy.

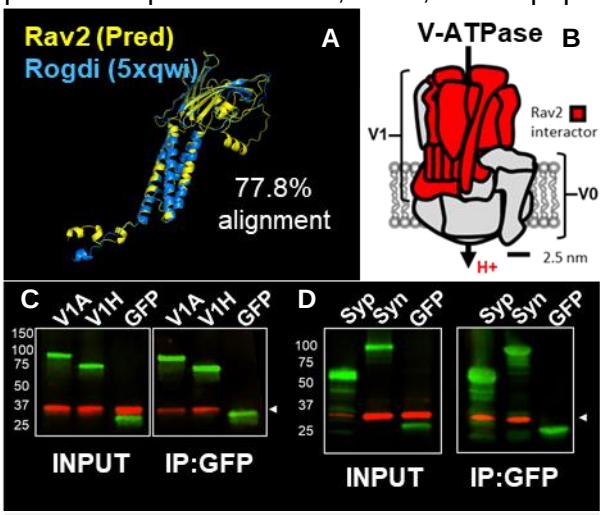


Fig.2 ***Rogdi and the RAVE complex. A.*** Structural alignment of the Rav2 and Rogdi proteins. ***B.*** Schematic of the V-ATPase protein complex. Rav2 interactors in red. ***C.*** Rogdi-HA immunoprecipitates with GFP-tagged V_1 complex proteins V1A and V1H as well as with ***D***. GFP-tagged components of the synaptic vesicle Synaptophysin (Syp) and Synapsin (Syn).

Despite the apparent conservation of the RAVE complex function from yeast to humans, no homolog of the Rav2 gene has been identified in mammals. The above evidence provides preliminary support for my hypothesis ***that Rogdi represents the mammalian ortholog of Rav2.***

Aim 1. Research design. The proposed research relies upon CRISPR-Cas9 to knock-in epitope tags to the endogenous mouse *Rogdi*, *Dmxl1, Dmxl2,* and *Wdr7* loci. We have modified an AAV virus to direct expression of CRISPR guide RNAs (AAV-U6-gRNA). Transduction of neurons derived from a *Cas9* (*H11-Cas9;* Jax #028239) knock-in mouse[41], or delivery of this virus *in vivo*, induces the CRISPR-mediated cutting of genomic or foreign DNA[42].

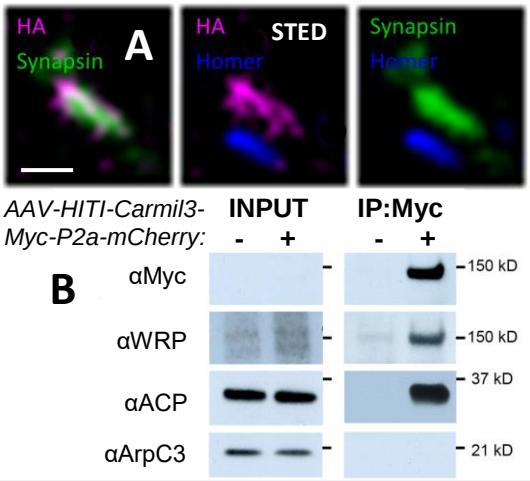


Fig.3. ***HITI tagging for STED and Co-IP.*** ***A.*** STED immunostaining of an endogenous Synaptophysin-HA immunopositive excitatory synapse. ***B.*** Example co-IP of HITI-tagged, endogenous Carmil3-Myc and detection of Wrp and actin capping protein (ACP) in the immunoprecipitate.

SubAim1a: Determining the localization of Rogdi. To visualize the endogenous localization of Rogdi, I will employ HITI (*see innovation*) to insert DNA encoding HA epitope tags (spaghetti monster HA, smHA[43]) into the last coding exon of Rogdi. This technique utilizes endogenous Rogdi expression and overcomes a major concern with recombinant protein overexpression—that overexpression may result in aberrant protein localization. I chose to target the C-terminus of Rogdi because *(1)* our preliminary data demonstrate that C-terminally tagged Rogdi is stably expressed in cultured cells. Furthermore, *(2)* work in yeast demonstrates that a C-terminally tagged Rav2 transgene can functionally replace the endogenous Rav2 gene[36]. Cultured neurons will be prepared from the hippocampi of post-natal day 0 (P0) *H11-Cas9* mice, and AAV virus will be added to direct cutting of the *Rogdi* genomic DNA (AAV-Rogdi-gRNA), and a viral donor vector (AAV-HITI-smHA) that will direct homology-independent insertion of smHA (**Fig.1b**). Neurons will be cultured according to standard procedures and fixed on day *in vitro* (DIV)15. Endogenous Rogdi will then be visualized by immunostaining with a high-affinity HA antibody (Rat monoclonal 3F10). The synaptic localization of Rogdi will be interrogated by co-staining for the presynaptic marker Synapsin (Synaptic Systems; 106104) and excitatory or inhibitory post-synaptic markers Homer or $GABA_AR\gamma2$ (Synaptic Systems; 106002 and 224003), respectively. We have demonstrated the feasibility of this approach in preliminary experiments in which the presynaptic protein Synaptophysin was tagged with HA using HITI and visualized using STED (**Fig.3a**). ***Expected outcomes:*** I expect to identify the endogenous localization of Rogdi. I predict that Rogdi will localize at inhibitory synapses (identified by $GABA_AR\gamma2$ staining). Using STED, I will be able to determine whether Rogdi localizes at the presynapse (colocalizes with Synapsin) or postsynapse (colocalize with $GABA_AR\gamma2$) or may localize at both locations. If Rogdi localizes at the inhibitory synapse, a loss of Rogdi may impair inhibitory synapse function. Therefore, I will test this hypothesis in my second aim by interrogating specific measures of pre- and postsynaptic function. ***Potential issues and solutions:*** The work in this aim will be conducted with the expert guidance of Dr. Lisa Cameron in the Duke Light Microscopy Core Facility (*see letter of support*). Our preliminary data support the feasibility of this approach, however visualizing low abundance, endogenous proteins is challenging. One alternative approach is to use proximity ligation assay (PLA) as a means of enhancing the signal[44]. One concern with any CRISPR approach is potential off-target cutting that could introduce HITI epitope tags into unintended genomic sites. We minimize this potential by selecting guide RNAs with minimal off-targets using CRISPOR[45]. Additionally, each cell event is independent—thus we will note non-consensus labeling if observed. Using a commercial antibody against Rogdi is another alternative, however, we have yet to validate these antibodies.

SubAim1b: Demonstrating that Rogdi interacts with mammalian RAVE complex proteins. I will determine whether Rogdi associates with the mammalian RAVE complex proteins Dmxl1, Dmxl2, and Wdr7 by HITI tagging these genes with Myc-epitope tags and immunoprecipitating these proteins and their associated complexes from mouse brain tissue. P0 *H11-Cas9* mice will be co-injected with gene-specific AAV CRISPR guide RNA viruses targeting *Dmxl1*, *Dmxl2*, and *Wdr7* and a universal HITI Myc donor vector. After 28 days, these mice will be sacrificed, their brains processed for co-immunoprecipitation (co-IP), and finally the presence of Rogdi in the Myc-immunoprecipitated complexes will be determined by immunoblotting using one of three commercially available antibodies against Rogdi. Preliminary testing of these three antibodies demonstrates that they can detect the recombinant Rogdi protein and detect a major band of the correct size by western blot of whole brain lysate. Here the HITI approach is necessitated by the large size of Dmxl1/2 (336-338 kDa) which occludes the possibility of overexpressing these proteins. Moreover, available antibodies for Rogdi, Dmxl1/2,

and Wdr7 have all been produced in rabbit, confounding the simultaneous immunodetection of these proteins using available antibodies. Thus, HITI will enable me to address a question which is otherwise extremely difficult. ***Expected outcomes:*** I expect to determine whether Rogdi associates with Dmxl1, Dmxl2, and Wdr7 *in vivo*. Several AP-proteomics studies have provided initial evidence that the RAVE complex in mammalian cells consists of Dmxl1/2 and Wdr7. These studies have also identified Rogdi. I will address the potential synaptic deficits associated with loss of Rogdi in my second aim. ***Potential issues and solutions:*** I will immunoprecipitate Dmxl1/2 and Wdr7 and probe for Rogdi by western blotting. The feasibility of this approach has been demonstrated by recent work in our lab in which endogenous, Myc-tagged Carmil3 was immunoprecipitated from mouse brain. Western blotting of the immunoprecipitate confirmed the association of Carmil3 with Wrp and actin Capping Protein (**Fig.3b**)[76]. An alternative to using the Myc tag may be to use the Halo tag system. HITI can be used to tag the Dmxl1/2 and Wdr7 genes with the Halo tag protein. As the Halo protein forms a covalent bond with its chemical ligand, this approach is well suited for immunoprecipitation of low abundance proteins[46]. Dr. Michael Tadross, an expert in the engineering of the Halo tag system, will be an excellent resource if this approach is required (*see letter of reference*). Another alternative approach is to immunoprecipitate V-ATPase subunits and probe for Rogdi. As yeast Rav2 binds five of the eight V_1 V-ATPase subunits (**Fig.2b**) (Saccharomyces Genome Database[47], SGD:S000002610), Rogdi is expected to co-IP with V_1 complex proteins. Our preliminary data indicate that Rogdi associates with two V_1 V-ATPase proteins, demonstrating the potential for Rogdi to associate with the V-ATPase complex in mouse brain (**Fig.2c**). Yet a final alternative approach may be to use iBioID and proteomics to analyze the Rogdi interactome. Our lab has extensive experience performing proteomics using this approach[6,48,49,76].

Aim 2. Characterizing the role of Rogdi in GABAergic synaptic transmission and epilepsy.

Deficits in GABAergic inhibition cause epilepsy.

I hypothesize that loss of Rogdi produces deficits in synaptic inhibition and spontaneous seizures. Genetic[50–54], pharmacological[55], anatomical[56–62], and electrophysiological[63–65] evidence all demonstrate that the loss of GABAergic inhibition is a critical aspect of the pathology of epilepsy, yet the molecular mechanisms which govern synaptic inhibition in health and disease are still not clear. Our novel Rogdi mouse represents an opportunity to study the pre- and postsynaptic mechanisms of synaptic inhibition.

Loss of Rogdi may cause deficits in synaptic inhibition. Genetic mutations that underlie human epilepsies provide unique opportunities to understand the molecular mechanisms that drive seizure susceptibility, as the underlying mechanism of epileptogenesis can be traced to dysregulation of a single gene. Homozygous loss-of-function mutations in *ROGDI* cause KTS[1–3,5,66,67]. We identified Rogdi at the iPSD, therefore I hypothesize that loss of Rogdi causes deficits in synaptic inhibition which may drive the KTS epilepsy phenotype. In support of Rogdi's role in GABAergic neurotransmission, a recent study in Drosophila found that *Rogdi* is required in GABAergic neurons for normal sleep[68]. To assess the role of Rogdi in GABAergic neurotransmission I will perform whole-cell patch clamp electrophysiology of *Rogdi* KO neurons receiving input from WT neurons (postsynaptic effects) and in WT neurons that receive input from *Rogdi* KO GABAergic neurons (presynaptic effects). The cell-type specificity of Rogdi knockout will be controlled with cell-type selective AAVs and mouse Cre-genetics—restricting the KO of Rogdi to excitatory or inhibitory neurons. To assess the role of Rogdi in epilepsy and seizure susceptibility I will monitor *Rogdi* conventional KO mice for seizures.

Aim 2. Research Design. The goal of this aim is to evaluate the function of Rogdi in pre- and postsynaptic GABAergic inhibition and to determine if the loss of Rogdi is sufficient to cause seizures. It relies upon a novel conditional KO mouse that we have developed (**Fig.4**). This mouse has LoxP sites inserted into introns

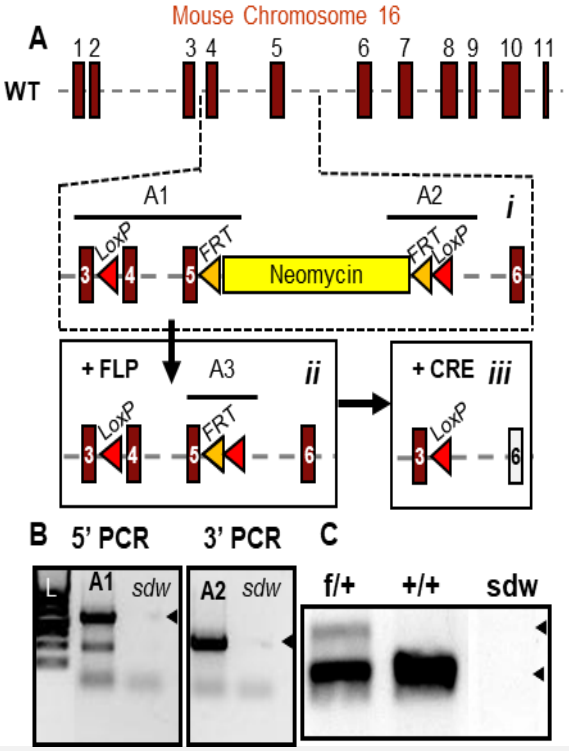


Fig.4. ***Development of a Rogdi KO mouse.*** ***A.*** Overview of the Rogdi genomic locus ***i.*** The Rogdi targeted allele in which LoxP sites were inserted into introns 3 and 5. Confirmation of the targeted allele was accomplished by amplifying across these target sites (A1 and A2, ***B***). ***ii.*** Removal of the neomycin cassette was accomplished by crossing founder mice to Flp mice. Germline transmission of the floxed allele was confirmed by PCR (A3, ***C***). ***iii.*** Cre-recombinase mediates removal of exons 4 and 5 and results a premature stop codon in exon 6.

three and five. Cre recombinase induces excision of the fourth and fifth exons and results in a premature stop codon in exon six. As nonsense mutations throughout the *ROGDI* gene cause KTS, including nonsense mutations in exons 6 and 7 that result in truncation of the Rogdi protein[1,5], the Rogdi floxed allele is predicted to result in complete loss-of-function after Cre recombination. We recently obtained germline transmission of the *Rogdi* floxed allele (**Fig.4c**).

SubAim 2a: Evaluating the role of Rogdi in postsynaptic function. Loss of Rogdi may result in neuronal hyperexcitability if it functions <u>postsynaptically at inhibitory synapses on excitatory neurons.</u> I will assess the cell-autonomous, postsynaptic function of Rogdi in excitatory neurons using my conditional Rogdi KO mouse and cell-type selective AAVs (*see overview of approach in* **Fig.5a**). Neurons will be cultured from *Rogdi$^{f/f}$:Ai14* mice. The *Ai14* mouse expresses tdTomato in a Cre-dependent manner and will provide a reliable marker for KO cells[69]. On DIV10, dilute AAV-CamkIIα-Cre virus will be added to induce sparse KO of Rogdi in excitatory neurons. Patching onto KO (tdTomato+) cells, which receive predominantly WT inputs, can be used to evaluate the postsynaptic function of Rogdi. Because KO is sparse, WT (tdTomato-) cells within the same coverslip can be used as a negative control. Whole cell patch clamp of pharmacologically isolated miniature inhibitory postsynaptic currents (mIPSC) and AMPA-mediated miniature excitatory postsynaptic currents (AMPA-mEPSCs) will be performed on DIV16 according to standard procedures. ***Expected outcomes:*** I expect that the analysis of isolated mIPSC frequency and amplitude in *Rogdi* KO neurons, compared to control neurons, will reveal the select role of Rogdi in GABAergic inhibition. Perturbations in amplitude may reflect altered levels of postsynaptic $GABA_A$ receptors, while changes in the mIPSC frequency may reflect an altered number of postsynaptic sites. These results will represent a significant step forward in understanding the mechanistic basis by which human *ROGDI* loss-of-function mutations result in increased seizure susceptibility. Analysis of mEPSCs will determine whether effects are specific to inhibitory synapses or whether loss of *Rogdi* also alters synaptic excitation. ***Potential issues and solutions:*** If Rogdi functions postsynaptically in GABAergic neurons, the global balance of excitation and inhibition may also be perturbed if loss of Rogdi results in an <u>*increase in inhibition onto interneurons that provide input to excitatory neurons.*</u> However, such a gain-of-function seems unlikely. To evaluate the postsynaptic role of Rogdi in GABAergic neurons, the approach described below in Aim2b could be modified by patching onto *Rogd$^{f/f}$:Gad2-Cre*+ neurons. Our lab has established the electrophysiology protocols required in this aim, but an alternative approach to achieve sparse postsynaptic knockout of Rogdi in excitatory neurons may be to use biolistic transfection of hippocampal slice cultures, as we have previous published[6]. Rogdi may function in early synaptic development, here an attempt to avoid this confound is made by adding Cre after the majority of synaptic development in culture. However, if no effect is observed by addition of Cre on DIV10, I will explore adding Cre earlier (e.g. on DIV0) in an attempt to identify the developmental window in which Rogdi is required. Expert help in the execution of the electrophysiology experiments will be obtained from members in my lab who have extensive experience performing electrophysiology as well as my committee member Dr. Nicole Calakos, who is an expert in electrophysiology (*see letter of support*).

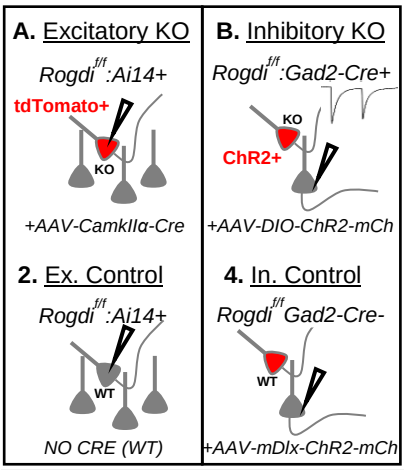


Fig.5. ***Overview of the proposed electrophysiology assays to assess postsynaptic (A) and presynaptic (B) function***. For description, see text. Also, see *Vertebrate Animals* for a detailed description of mouse husbandry.

SubAim 2b: Evaluating the role of Rogdi in presynaptic function. If Rogdi functions <u>presynaptically at inhibitory synapses</u>, its loss may result in a <u>decrease in inhibitory output onto excitatory cells</u>. To evaluate the presynaptic function of Rogdi in GABAergic neurons, neurons from Rogdi$^{f/f}$:Gad2-Cre+ mice will be sparsely seeded into Rogdi$^{f/f}$:Cre- (WT) neuronal cultures at the time of plating (*for overview of approach see* **Fig.5b**). The Gad2-Cre mouse expresses Cre broadly in interneurons of the cortex and hippocampus and will restrict KO of Rogdi to these cells[70]. On DIV10, Cre-dependent channelrhodopsin-2 (ChR2; AAV-DIO-ChR2-mCherry) virus will be added for expression of ChR2-mCherry in Gad2+ interneurons. As a control, cultures from Rogdi$^{f/f}$:Gad2-Cre- littermates will be transduced with AAV-mDlx-ChR2-mCherry[71] for optical control of WT interneurons. In this way, optically evoked measures of presynaptic function can be obtained by patching onto WT (dark) neurons that are nearby inhibitory KO (*Rogdi$^{f/f}$:Gad2-Cre*+) neurons. Short term plasticity as well as additional presynaptic parameters such as release probability, size of the readily releasable pool (RRP), and vesicle replenishment rate can be calculated by fitting the cumulative evoked current response to 20Hz optical stimulation. As proof-of-concept we have evaluated the deficits in presynaptic function associated with presynaptic loss of Arpc3, a major

regulator of the cytoskeleton (**Fig.6**). Presynaptic loss of Arpc3 results in alterations in short term synaptic plasticity, in conjunction with changes in release probability and synaptic vesicle replenishment rate (**Fig.6c-e**). ***Expected outcomes:*** In neurons, the V-ATPase establishes a proton driving force for the loading of synaptic vesicles with neurotransmitters[72,73]. If Rogdi is a component of the RAVE complex, V-ATPase function is expected to be impaired, and this may produce deficits in the loading of synaptic vesicle (SVs) with GABA at inhibitory synapses. Moreover, as fully acidified SVs exhibit greater release probability[74], loss of Rogdi may affect the size of the RRP and/or vesicle replenishment rate. ***Potential issues and solutions:*** Our lab has established the electrophysiology protocols required in this aim using sparse seeding of floxed allele neurons into WT cultures and recording of optically evoked responses in WT neurons (*see* **Fig.6**). We will extend this approach to measure evoked inhibitory currents. An imbalance in excitation and inhibition may also result from an *increase in glutamatergic neurotransmission caused by a gain-of-function at the excitatory presynapse.* In order to evaluate the presynaptic function of Rogdi at excitatory synapses, we may modify the approach in Aim2a to knockout Rogdi in executory neurons and record evoked currents in WT cells (*e.g.* **Fig.6**). Rogdi may be required for early presynaptic development. Temporal control of Rogdi KO may be achieved by addition of Cre after the majority of synaptic development *in vitro*. However, this approach would sacrifice the spatial precision of the proposed sparse knockout approach. Another alternative may be to utilize hippocampal cultures from Gad2-CreER (Jax #010702) mice treated with low doses of 4-hydroxy tamoxifen to achieve sparse KO.

SubAim 2c: Role of Rogdi in seizures. Individuals with homozygous loss of *ROGDI* suffer from spontaneous recurrent seizures beginning early in life. The mean onset of epilepsy in individuals with KTS is approximately 6 months. I will cross our *Rogdi*$^{f/f}$ mouse to CMV-Cre mice to create a constitutive *Rogdi* KO mouse. This model will most closely recapitulate the human genetics of KTS. Rogdi$^{+/-}$ (Heterozygote), Rogdi$^{+/+}$ (WT), and Rogdi$^{-/-}$ (KO) mice will be monitored daily by video recording for behavioral seizures in the month following birth. ***Expected outcomes:*** As homozygous loss of *ROGDI* causes seizures in human's I predict that homozygous loss of *Rogdi* in mice will be sufficient to produce seizures. I also expect to identify AI in these mice. ***Potential issues and solutions:*** Work associated with this aim will be done in close collaboration with Dr. James McNamara (*see letter of support*), an expert in the study of the molecular mechanisms of epileptogenesis. One possible confound is the mice will not develop *de novo* seizures but may instead have an elevated propensity to develop seizures. Video-EEG recordings may be made to quantify epileptic brain activity, and altered seizure threshold may be determined by comparing the current required to induce focal electrographic seizures in Rogdi$^{+/-}$ and Rogdi$^{-/-}$ mice[75].

***In conclusion*,** the combined results from Aims 1 and 2 can be expected to uncover the function of Rogdi at inhibitory synapses. This will increase our knowledge of how inhibitory synapses work and mechanisms of how their dysfunction leads to epilepsy. If time allows other phenotypes in the Rogdi KO mice can be evaluated, including histological analysis of tooth enamel relevant to AI. These studies may further inform the potential role of Rogdi as a component of the RAVE complex.

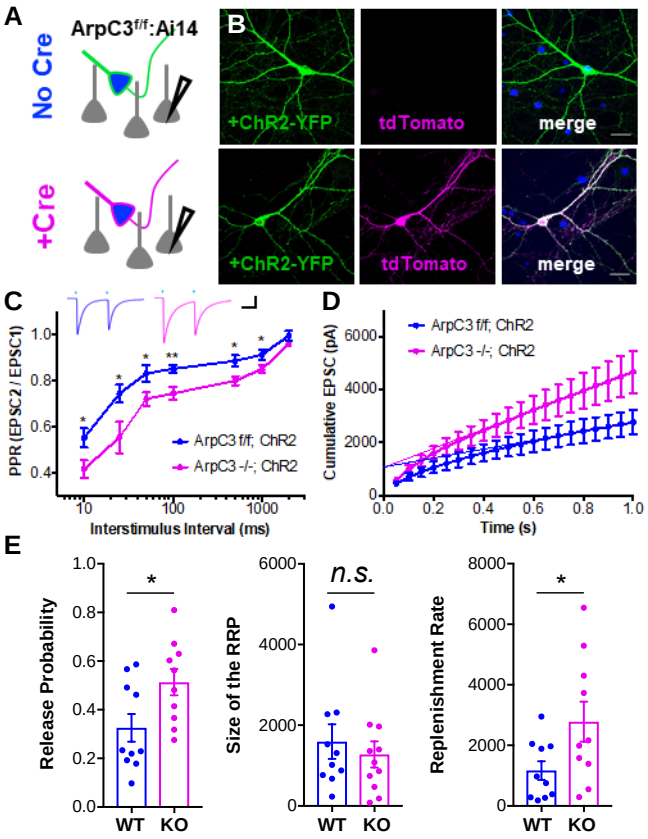


Fig.6. ***Electrophysiological assessment of presynaptic function***. ***A.*** Experimental design to evoke release from *ArpC3* KO terminals. Hippocampal neurons were cultured from *ArpC3*$^{f/f}$*:Ai14* mice and electroporated with hSyn-ChR2-eYFP. AAV-hSyn-Cre was added on DIV10, inducing in sparse deletion of *ArpC3* and expression of tdTomato. Whole-cell recordings were conducted on DIV16 from WT neurons while evoking release from WT or KO terminals using 1ms pulses of 488nm light. ***B.*** Immunostaining of a WT (top) and KO (bottom) neuron. ***C.*** Presynaptic loss of *Arpc3* reduces paired pulse ratio. ***D.*** Evoked EPSCs from 20Hz light stimulation. **E.** From the fitted curve in D., several synaptic parameters can be calculated: release probability, size of the readily releasable pool (RRP), and replenishment rate.

VERTEBRATE ANIMALS

1. Description of the proposed use of animals: The Soderling laboratory has already generated or acquired all of the mouse lines necessary to complete the experiments proposed in this application. To interrogate the function of Rogdi we generated a *Rogdi* conditional knockout allele by CRISPR-mediated Homology Independent Target Integration (HITI). Briefly, mouse embryonic stem cells were electroporated with Cas9 protein and CRISPR guide RNAs targeting the third and fifth intronic regions of the mouse Rogdi genomic locus and a ~3kb donor DNA template designed to replace this region. The donor DNA contains loxP sites inserted into the third and fifth introns designed to mediate removal of exons 4 and 5 as well as an attB/attP-flanked PGK-FRT-Neo-polyA cassette. Correctly modified stem cells were selected for using neomycin, confirmed by PCR and genomic sequencing, and injected into a pseudo pregnant female mouse. Transmission of the *Rogdi* floxed allele into founder mice from this dam was confirmed by PCR. A *Rogdi*$^{floxed\text{-}FRT\text{-}neo/+}$ founder mouse was then crossed to a Flp+ mouse (B6.129S4-Gt(ROSA)26Sortm2(FLP*)Sor/J, **Jax #012930**) to mediate removal of the neomycin selection cassette leaving exons 4 and 5 floxed. The genotype of the resulting Rogdi$^{f/+}$ mice were confirmed by PCR. These mice were used to generate two parallel lines of mice: *(1)* Rogdi$^{f/+}$ mice were crossed to C57Bl/6J (**Jax #000664**) for stock breeding and maintenance of the floxed allele; *(2)* a male Rogdi$^{f/+}$ mouse was crossed to a CMV-Cre (B6.C-Tg(CMV-cre)1Cgn/J, **Jax #006054**) female mouse to mediate removal of the *Rogdi* allele. Rogdi$^{f/+}$ and Rogdi$^{+/-}$ mice will be maintained as heterozygotes by crossing to C57Bl/6J mice for several generations prior to beginning experiments. *Since the generation of the Rogdi conditional allele has already been completed, these mice are not included in the totals below.*

Aim 1a: Sub Aim 1a studies the localization of Rogdi at synapses. To accomplish this aim we have acquired the *H11-Cas9* mouse (B6J.129(Cg)-*Igs2*$^{tm1.1(CAG\text{-}cas9*)Mmw}$/J, **Jax #028239**) which expresses Cas9 in all tissues. *Cas9* mice have been maintained by crossing to C57Bl/6J and bred to homozygosity. I will establish two breeding cages to generate homozygous Cas9 pups (4 total mice). I will culture neurons from these pups and add virus to mediate HITI labeling of Rogdi. A typical litter of six pups is more than enough to make a 24-well plate of cultured neurons and perform staining for my two conditions. Approximately three litters will be required to perform this experiment in triplicate (18 total mice).

Aim 1b: Cas9 pups will be injected with AAV virus to label Dmxl1, Dmxl2, and Wdr7. A fourth group of mice will be injected with control virus. After injection of virus, pups will be allowed to mature for 28 days, and sacrificed for biochemistry. Based on our previous work, five pups per condition should be sufficient for the completion of this work (20 mice total). Two additional Cas9 breeding pairs have been requested to generate these mice (4 mice total).

Aim 2: Sub Aims 2a and 2b study the functional role of Rogdi using electrophysiology. Six additional C57Bl/6J mice are requested for maintenance of the Rogdi$^{f/+}$ and Rogdi$^{+/-}$ lines. Matings to generate the pups required for electrophysiology require two parallel mating strategies each consisting of three iterative matings, described below. The expected time to generate these pups is thus ~24 weeks. A conservative estimate then is that these pups will become available for experiments in the second year of my award.

Aim 2a: *Rogdi*$^{f/+}$ mice will be crossed to *Ai14* mice to generate Rogdi$^{f/+}$:Ai14$^{tdTomato+/-}$ mice (expected 25% of offspring). The offspring of these matings will be interbred to generate Rogdi$^{f/f}$:Ai14+ (Rogdi$^{f/f}$:Ai14$^{tdTomato+/-}$ and Rogdi$^{f/f}$:Ai14$^{tdTomato+/+}$) mice (expected 18.75% of offspring). These Rogdi$^{f/f}$:Ai14+ mice will be used to generate Rogdi$^{f/f}$:Ai14+ pups (expected 50-75% of offspring). In order to account for technical variability between cultures and a patch success rate of 70%, the proposed electrophysiology experiments will be done in duplicate. I will require 3 mice per group to complete these experiments (KO and control; 12 total mice). The estimated numbers of Rogdi$^{f/+}$, Ai14, Rogdi$^{f/+}$:Ai14+, and Rogdi$^{f/f}$:Ai14+ mice requested below reflect the required number of pups and the expected number of correct genotype offspring in a litter.

Aim 2b: The Rogdi$^{f/+}$ mouse will be bred to Gad2-Cre (B6J.Cg-Gad2tm2(cre)Zjh/MwarJ, Jax **#028867**) mouse to generate Rogdi$^{f/+}$:Gad2-Cre+ mice (expected 25% of offspring). Het-Het matings will be established between these mice to generate Rogdi$^{f/f}$:Gad2-Cre+ mice (expected 18.75% of offspring). Rogdi$^{f/f}$ mice will be generated by interbreeding Rogdi$^{f/+}$ mice (expect 25% of offspring). These mice will then be bred to Rogdi$^{f/f}$:Gad2-Cre+ mice to generate Rogdi$^{f/f}$:Gad2-Cre (50%) and Rogdi$^{f/f}$:Gad2-Cre- (50%) pups for electrophysiology. A total of 12 pups will be required for electrophysiology experiments. The requested number of mice below reflects the final number of required pups and the expected number of correct genotype offspring in a litter.

Aim 2c: Rogdi knockout mice will be evaluated for behavioral seizures. Rogdi$^{+/-}$ mice will be interbred to generate Rogdi wild-type (Rogdi$^{+/+}$), heterozygous (Rogdi$^{+/-}$), and homozygous (Rogdi$^{-/-}$) knockout mice. Based on all available human genetic and clinical studies, we suspect that heterozygote Rogdi$^{+/-}$ mice will be phenotypically normal, and we will not have trouble breeding these mice. To generate 5 mice per condition, I anticipate that 4-5 litters will be required. Two Rogdi$^{+/-}$ breeding pairs will be established to generate these mice (4 total mice).

Equal numbers of male and female mice will be used for each experimental condition.

Aim	Genotype	Description	Mice per Group	Number of Groups	Total Mice
Aim 1a	*H11-Cas9*	Cas9 Breeders	2	1	4
		Cas9 pups for HiUGE labeling Staining of excitatory synapses Staining of inhibitory synapses	9	2	18
Aim 1b	*H11-Cas9*	Cas9 Breeders	2	1	4
		Cas9 pups for HiUGE labeling and Co-immunoprecipitation	5	4	20
Aim 2a	*C57Bl/6J*	C57Bl/6J breeders for maintenance of *Rogdi*$^{f/+}$ and *Rogdi*$^{+/-}$ lines	3	2	6
	Rogdi$^{f/+}$	*Rogdi*$^{f/+}$ breeders for crossing to *Ai14*	2	1	2
	Ai14	*Ai14* breeders for crossing to Rogdi$^{f/+}$	2	1	2
	Rogdi$^{f/+}$*:Ai14+*	*Rogdi*$^{f/+}$*:Ai14+* mice for generation of *Rogdi*$^{f/f}$*:Ai14+* mice.	2	3	6
	Rogdi$^{f/f}$*:Ai14+*	*Rogdi*$^{f/f}$*:Ai14* breeders for generating pups for electrophysiology.	2	3	6
	Rogdi$^{f/f}$*:Ai14+*	Rogdi$^{f/f}$:Ai14+ pups for electrophysiology.	6	2	12
Aim 2b	*Rogdi*$^{f/+}$	Rogdi$^{f/+}$ breeders for generation of Rogdi$^{f/f}$ mice and crossing to Gad2-Cre mice.	2	4	8
	Gad2-Cre$^{Cre+/-}$	Gad2-Cre mice for crossing to Rogdi$^{f/+}$	2	1	2
	Rogdi$^{f/+}$*:Gad2-Cre+*	*Rogdi*$^{f/+}$*:Gad2-Cre+* mice for crossing to Rogdi$^{f/f}$ for generation of pups for electrophysiology	2	1	2
	Rogdif/f:Gad2-Cre+ and Rogdif/f:Gad2-Cre-	*Pups for electrophysiology*	6	2	12
Aim 2c	*Rogdi*$^{+/-}$	Rogdi$^{+/-}$ breeders for generation of Rogdi$^{+/+}$, Rogdi$^{+/-}$, and Rogdi$^{-/-}$ mice	4	1	4
		Rogdi$^{+/+}$, Rogdi$^{+/-}$, and Rogdi$^{-/-}$ mice for seizure assessment.	5	3	15
Total Projected Mice for Breeding and All Experiments = 123					

2. Justification for the use of animals: The mouse is an excellent model system to study neurobiology, particularly when assessing synaptic protein composition and function. The species was chosen because synaptic gene and protein expression in mice is similar to expression in humans. The use of the mouse model is required because neuronal subtype-specific synaptic content and how it is modified in the context of development, experience, or disease cannot be faithfully studied in cultured cell lines. Thus, the mouse is an excellent model for the study of synaptic proteins and genetics of human disorders.

3. Veterinary care: Duke University is fully accredited by the American Association for Accreditation of Laboratory Animal Use. The mice for these studies will be housed at the Genome Science and Research Building II (GSRB II) at Duke University Medical Center, under the direction of John Norton, DVM, PhD. All facilities are fully accredited by the AAALAC (Animal Welfare Assurance number A3195-01) and meet NIH standards as set forth in the "Guide for Care and Use of Laboratory Animals" (DHHS). This barrier facility provides a pathogen-free environment. All animals are housed in micro-isolator cages. Each rack has a cage of sentry mice that is exposed to bedding from the other cages in the room. Sentry mice are examined serologically on a monthly basis. Mice are well-fed, hydrated, and have bedding changed weekly. All mice are cared for by a full-time veterinary staff, in accordance with USDA, State, Federal Animal Welfare Act, and the USPHS guide for the Care and Use of Laboratory Animals. The facilities are also overseen by a staff of veterinarians and vet technicians, who examine all cages on a weekly basis to ensure proper care and handling of the animals as well as to identify any animals with noticeable disease or signs of distress. Cages containing animals with compromised health are marked to alert the investigator, and the veterinary staff then monitors these animals closely. In addition, we personally check our cages every morning, seven days a week. Animals that become sick will be promptly euthanized according to the corresponding guidelines (see below).

4. Minimizing Distress: Genotyping by toe clip will be performed at P7 to minimize pain and stress for the animals under aseptic conditions. For viral injections, P0 pups will be anesthetized and monitored carefully post-injection for body temperature, respiratory rate, response to stimulus, and signs of distress. To minimize discomfort and stress during euthanasia, mice will be euthanized with isoflurane overdose, followed by decapitation, in accordance with AALAC protocols.

5. Methods of euthanasia: Standard methods of euthanasia either using a CO2 chamber or inhalation of anesthetics (Isoflurane) will be used for all adult mice, and death will be assured by a secondary method. Decapitation will be used for euthanasia of newborn pups. These methods are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

SELECT AGENT RESEARCH

No select agents will be used in the conduct of this research.

RESOURCE SHARING PLAN

Data sharing plan:

Data generated by this proposal will be published once complete. These publications will be shared via PubMed Central, which is freely accessible to all researchers. Additionally, we will provide PDF copies to requesting scientists. Code and raw data used in the analysis of data will be published on relevant repositories online or made available by request. The protocols we develop will be published on our lab website (http://soderlinglab.cellbio.duke.edu/) in order to benefit the broader research community.

Reagent sharing plan:

All reagents generated in this study (mice, virus and cDNA constructs) will be made available at the completion of this study. Our laboratory has a history of sharing many of our reagents, including mice, with labs both within US and internationally. Additionally, we will deposit non-commercial plasmids with Addgene for the distribution to outside investigators.

AUTHENTICATION OF KEY RESOURCES

Authentication of Key Biological and/or Chemical Resources

The proposed studies will utilize both *in vitro* and *in vivo* mouse model systems. A *Rogdi* mouse model has been established by the Duke Mouse Transgenic core facility and verified independently by our own lab. The culture of mouse neurons will be performed according to well-established protocols. Viruses will be produced using established methods and authenticated cell lines.

Authentication of DNA constructs and antibodies

All DNA samples created in our lab or acquired from other labs will be sequenced to confirm the construct is accurate. Any antibodies acquired from commercial sources or other labs will be evaluated using necessary controls. This would include staining cells that are knocked down for the given gene, use of mouse mutants, and identification of specific bands by western analysis.

Authentication of Mouse Strains

The *Rogdi* conditional knockout mouse strain for this project was developed by Duke's Transgenic Mouse Facility. Additional mouse strains for this project have been purchased from Jackson Laboratories. Any mutant or transgenic mouse strains will be genotyped before use to confirm the relevant mutation or transgene. Mouse strains will be maintained on appropriate genetic backgrounds. Records of mouse breeding will be kept in an online database (mLIMs) to ensure accurate breeding history for all animals used, and to maintain organization of the mouse colony so that appropriate genotypes are bred. Antibodies coupled with westerns or immunofluorescence, or qRT PCR, will be used where relevant to verify protein/RNA alterations in a given mutant.

RESPECTIVE CONTRIBUTIONS

I developed the questions, hypotheses, and research approach for this proposal with guidance from my mentor Dr. Scott Soderling. Dr. Soderling and I worked together to develop a training plan that is tailored to my proposed research, goals, and future aspirations. The iterative process of preparing this grant began in a scientific writing course I took in my second year of graduate school at Duke University. In this course, I prepared a NRSA F31 style research proposal. Intense discussion and review by my colleagues and mentors continually refined the proposal in this period. The result, a mock F31, provided the basis for my qualifying exam which I successfully defended in May 2018. I performed the final construction and editing of the research strategy with help from Dr. Soderling in the Fall of 2018.

I will lead the execution of the research with guidance from Dr. Soderling and my thesis committee and select collaborators. My thesis committee, which is composed of Dr. Michael Tadross, MD, PhD, Dr. Nicole Calakos MD, PhD, and Dr. James McNamara, MD. Combined, my committee has expertise in all aspects of the proposed research including: neuronal imaging, protein biochemistry, electrophysiology, epilepsy, and mouse behavior. Moreover, my committee consists of several MDs with experience in translating science from bench to beside. Thus, my committee will be an invaluable resource as I interrogate the molecular mechanisms by which loss of Rogdi causes a human epilepsy disorder. Dr. McNamara especially will be an excellent resource as he is an internationally recognized expert in the research of epilepsy. I rotated in Dr. McNamara's lab to learn how to surgically implant mice with electrodes for video-EEG recordings and become exposed to epilepsy research using mice. I consulted Dr. McNamara during the development of the proposed research and Dr. McNamara will continue to provide his support during the execution of the epilepsy associated experiments in my proposal (*see letter of support*).

The work in this proposal will rely upon expertise and equipment in Duke's core facilities. Dr. Lisa Cameron of the Duke Light Microscopy Core Facility will provide support for the proposed super resolution imaging experiments. Our lab has numerous on-going projects and collaborations with Duke's core facilities and this work will be a continuation of these successful relationships.

Finally, my lab will continue to be a great resource during the remainder of my PhD. I hold regular, weekly meetings with Dr. Soderling for informal discussion of on-going experiments and preliminary results. Our lab also holds weekly lab meetings with regular opportunities to discuss my results. I will build on the expertise and experiences of other graduate students and postdoctoral fellows in the Soderling lab who have strong backgrounds in molecular biology, genome editing, *in vivo* imaging, electrophysiology, and mouse behavior.

SELECTION OF SPONSOR AND INSTITUTION

Sponsor: After completing my undergraduate studies in molecular, cellular, and developmental biology at the University of Washington, I sought out research technician positions in labs across the United States. My research up to this point had focused on the cellular and molecular mechanisms by which Type 2 diabetes mellitus is ameliorated after bariatric surgery, as well as how diabetes promotes atherosclerosis and heart disease. Although I have always been interested in understanding the mechanisms of normal cellular function as well as dysfunction in disease, I had no research experience in neuroscience. Thus, I found the research of Dr. Scott Soderling at Duke University particularly exciting, but daunting, as his lab aims to better understand the cellular and molecular mechanisms of brain function and dysfunction. Although I was intimidated by the challenge of trying to understand the complexity of the brain, I found Dr. Soderling's lab to be a welcoming environment in which I was able to quickly immerse myself. In a short period of time, I was able to contribute significantly towards a paper that we published in *Science* in 2016, which has formed the foundation for the work proposed here.

I chose Dr. Soderling as my mentor because of the positive experiences I had while working in his lab as a research technician. I believe that Dr. Soderling values his students learning and professional development very highly and is committed to excellence in these pursuits. Science is often hard work, and I believe Dr. Soderling leads by example, working hard to balance his administrative duties with the personalized mentorship and guidance of his students. I admire these traits and hope to embody them as a future mentor. In addition to my positive working relationship with Dr. Soderling and other lab members, I enjoyed the creative freedom I experienced in Dr. Soderling's lab and believed that this would be a great environment for my graduate studies.

Institution: I decided to attend Duke University after working at Duke for two years as a research technician. This experience was paramount in making the decision to stay at Duke for graduate school. While working at Duke, I experienced firsthand its exciting and supportive research environment. Duke's Neurobiology graduate student program is replete with intelligent and hardworking students and faculty that tackle a diverse range of research topics across all scales of neuroscience. Moreover, Duke's close scientific community encourages collaboration between labs and disciplines, and I believe this is essential for successful and productive science. Moreover, I found that the graduate student program was committed to finding a productive balance between time spent in the class room and lab. Finally, I found Durham, NC an excellent place to live as it is a quickly growing community, yet affordable, and has easy access to variety of activities within the state and along the east coast. For these reasons, I chose to apply to Duke University's neurobiology graduate program. I was accepted and began my graduate studies in the Fall of 2016.

TRAINING IN THE RESPONSIBLE CONDUCT OF RESEARCH

Duke University requires that all biomedical PhD students participate in a rigorous and highly applicable program to train students in the responsible conduct of research (RCR). Students are required to participate in three different courses that compromise a total of 18 hours of RCR credit as described here. I have completed 12 credit hours of RCR training through Duke's Ethics Retreat and I plan to complete the remaining 6 credit hours in 2019.

Format: All students must complete 18 credit hours and complete a written assignment for each course. Credit is formally tracked by the university registrar. The three courses that make up the 18 credit hours are described here:

1) I attended the required First-Year Ethics Retreat at the start of my PhD program in September 2016. This three-day weekend retreat includes both formal lectures and small group discussions led by a variety of biomedical faculty from many different departments. Completion of this course provided 12 credit hours in RCR training.
2) Next summer I plan to attend the required Third-Year RCR course. This course consists of an on-site, one-day retreat that covers both professional development and reviews of the topics covered in the First-Year Retreat. Completion of this course will provide me with 4 credit hours in RCR training.
3) In order to complete my RCR credit requirement, I will participate in one additional RCR forum during my graduate tenure, likely during my fourth or fifth year. This forum will consist of a one-hour lecture and a 1.5-hour small group discussion. Completion of this forum will provide me with the remaining 2 credit hours in RCR training needed to complete my 18 credit hour requirement.

Subject Matter:

1) Ethics Retreat: Topics covered on the retreat include scientific integrity, profession obligations of a scientist, ethical research on animals, publication and authorship, data acquisition and management, avoiding misconduct, handling of misconduct allegations at Duke, as well as interactive sessions.
2) Third Year Course: Students will complete Individual Development Plans and participate in career interest break-out groups covering topics such as academia, teaching, biotech, pharmaceutical industries, etc. in the morning session. The afternoon session will consist of lectures that cover NIH- recommended topics and extend the information provided during the First-Year Ethics Retreat.
3) RCR Forums: The topics of these forums change annually but include such topics as data management, career development, biases in academia, and copywrite issues. The faculty conducting these forums varies with the topic and includes faculty both within and outside the biomedical field.

Faculty Participation: The Ethics Retreat and Third Year RCR course are run by research faculty from biomedical departments at Duke, including the Neurobiology department. Faculty members give lectures and participate in small group sessions. The RCR forums are put together by faculty members throughout the Duke graduate schools according their expertise.

Duration of Instruction:

1) Ethics Retreat: 12 credit hours
2) Third Year Course: 4 credit hours
3) RCR Forums: 2 credit hours

Frequency of Instruction: RCR training must occur at least once every four years, as mandated by the NIH requirement. The Ethics Retreat is attended at the beginning of the first year, the Third Year Course is taken at the end of the third year, and the RCR Forums are taken before the end of the fourth year.

A. DOCTORAL DISSERTATION AND RESEARCH EXPERIENCE:

Early experiences in Science: Undergraduate research [Fall 2012 – May 2014]
I studied molecular, cellular, and developmental biology at the University of Washington. Inspired by my studies of the human gastrointestinal system in an anatomy course, I joined the Surgical Outcomes Research Center (SORCE) as a research assistant. Our group in SORCE used the Ossabaw pig as a model system to investigate the physiological changes which underlie weight loss and the normalization of diabetes following bariatric surgery. Raised apart from domestication on an island off the coast of Georgia, the Ossabaw pig exhibits a remarkably human-like pathogenesis of obesity and diabetes when fed a high-fat diet. I collected blood draws during oral and intravenous glucose tolerance testing and administered perioperative care to the animals. My efforts culminated with the independent analysis of data gathered in the lab for a research project which I presented to SORCE faculty and staff. This research experience instilled in me a deep desire to learn more about the molecular mechanisms which control the physiology of complex biological systems.

To satisfy this interest, I joined the lab of Dr. Karen Bornfeldt (University of Washington) where I continued researching diabetes, but with a focus on how insulin resistance, a hallmark of both type 1 and type 2 diabetes, contributes to heart disease. In the Bornfeldt lab, I performed immunoblotting for inflammatory markers from macrophages isolated from atherosclerotic plaques. As an undergraduate researcher in the Bornfeldt lab, I gained more independence than I previously experienced. This research experience provided the basis for a manuscript which I prepared to satisfy the requirement for an honors degree in molecular biology at the University of Washington. In the Bornfeldt lab, I progressed from simply collecting samples, to processing them, and finally developing new ideas about the data I generated.

Seeking to yet further develop my skills at the bench and independence in the laboratory, in May of 2014 I accepted an offer from Dr. Scott Soderling at Duke University to become a research technician in his laboratory. Having completed my requirements for graduation, I elected to graduate a quarter early and move across the country to Durham, North Carolina to join the Soderling lab.

Driving Innovation in Science: Research technician experience [May 2014 – June 2016]
Before joining the Soderling lab, I had no experience in neurobiology. Despite this lack of experience, I launched myself into the lab's research. Working in the Soderling lab has revealed my ultimate passion for neurobiology.

Not long before I arrived at Duke University, Dr. Akiyoshi Uezu, a postdoctoral researcher in the Soderling lab, adapted a chemicogenetic-based technique to label the proteomes of non-membrane bound cellular compartments as they exist *in vivo*. We used this approach, termed *in vivo* proximity based biotin identification (iBioID), to identify the proteome of the inhibitory postsynaptic density (iPSD). I contributed to this project by performing much of the basic molecular biology that was required as well as performing the analysis of the iBioID proteomics data set.

Our discovery of the iPSD proteome revealed a new challenge—verifying the functional importance of novel iPSD proteins. To overcome this challenge, I developed several new tools which are now widely used in the Soderling lab. I became the first in our lab use CRISPR-Cas9 genome editing technology to knock-down proteins of interest, and thereby asses their function. Using CRISPR-Cas9 we demonstrated that depletion of two new iPSD proteins resulted in deficits in GABAergic inhibition. Next, to demonstrate the functional importance of these findings *in vivo,* I adapted an adeno associated virus (AAV) strategy to knockout these proteins *in vivo*. This body of work was published in the journal *Science*—on which I am joint second-author. In just two years, by driving the innovative use of several new technologies in our lab, I helped bridge a fundamental gap in knowledge in molecular neuroscience by identifying an iPSD proteome. The tools I initiated work on in the Soderling lab have revolutionized the lab's research and has thrust our lab into the forefront of the application of CRISPR genome editing, iBioID, and AAV technologies to study novel synaptic proteins. These experiences motivated me to apply to graduate school at Duke University, where I am now working towards a Ph.D. in Neurobiology.

Studies of synaptic function: Early graduate school research experiences [September 2014 – May 2018]
The synaptic proteome is composed of a complex network of proteins that includes neurotransmitter receptors, their submembrane scaffolds, and associated signaling proteins. An emerging realization in neuroscience has been that many brain disorders result from genetic variation in synaptic proteins. Yet, an understanding of the basic cellular mechanisms which drive these diverse pathologies are still largely missing. To overcome these challenges, proteomics has emerged as a powerful tool to identify the proteomes of specific subcellular organelles and quantify changes in the brain proteome in disease states. As a graduate student at Duke, I have spearheaded several collaborative efforts to apply two different proteomic approaches towards generating an understanding of the mechanisms of synapse function and dysfunction.

Soderling Lab: Current evidence demonstrates that neurodevelopmental disorders are genetically heterogenous, but also exhibit phenotypic overlap. Thus, we and others have hypothesized that some degree of convergence at the level of synaptic dysfunction may contribute to the phenotypic overlap observed in these disorders. To test this hypothesis, I collaborated with the proteomics core facility at Duke to assemble a targeted-proteomics library enabling the quantification of 342 synaptic proteins which are genetically implicated in human neurodevelopmental disorders. I have applied this library to quantify changes in synaptic protein abundance from two tissues in five different mouse models of autism. This project has proven incredibly challenging, but immensely rewarding. In order to learn more about targeted-proteomics I attended an advanced course at the University of Washington in Seattle. Here I learned how to use analysis tools for targeted-proteomics that I have brought back to Duke. To grapple with the large and complex data sets that we have generated, I have independently learned computer programming in Matlab and R. Using these tools, I am beginning to uncover common molecular signatures across different autism mouse models. This research has given me the opportunity to present at multiple Cell Biology and Neurobiology departmental retreats as well as at the Society for Neuroscience meeting in San Diego and Washington DC. *I hope to publish this body of work in 2019.*

McNamara Lab: Among the complex network of proteins significantly enriched at the inhibitory postsynapse, I found that 21% of the iPSD proteome was genetically implicated in autism and/or epilepsy disorders in humans or mice. Although a loss of synaptic inhibition is broadly implicated in epilepsy, little is known about the molecular mechanisms of postsynaptic GABAergic inhibition. Thus, I decided to rotate in the lab of Dr. James McNamara, a leader in the research of temporal lobe epilepsy, to learn more about epilepsy research.

The McNamara lab has discovered how an episode of prolonged seizures can induce lifelong epilepsy through activation of the brain-derived neurotrophic factor (BDNF) receptor TrkB. To better understand the molecular mechanisms by which activation of TrkB can transform the normal brain into an epileptic brain, the McNamara lab has utilized proteomics to quantify changes in excitatory synaptic protein abundance following an episode of seizures. This work led to the identification of proteins whose synaptic expression is dependent upon TrkB activation and are candidates for transforming the brain from normal to epileptic. To further test this idea, I utilized the lab's proteomics and RNA microarray data as well as bioinformatic analysis of the TrkB-BDNF signaling pathway to design a targeted-proteomics library to quantify 300 synaptic proteins whose expression is regulated by activation of TrkB. Additionally, while rotating in the McNamara lab I learned how to surgically implant electrodes into the mouse brain for recording epileptic brain activity. During my rotation I collaborated extensively with Duke's proteomics core facility in the development of this assay and analysis of preliminary data. We expect that the results of these experiments will inform the molecular mechanisms by which activation of TrkB transforms the brain from normal to epileptic.

Caron Lab: The Caron lab has developed an antipsychotic compound for treatment of schizophrenia. Although the drug is known to act on the β-Arrestin signaling pathway, the downstream signaling events by which the drug acts are not fully understood. Furthermore, the drug is known to act as an antagonist in one brain region and an agonist in another. Therefore, in collaboration with the Caron lab, I have sought out to identify the mechanism of action of this drug using proteomics. To answer this question, we prepared synaptic brain fractions from wild-type and β-Arrestin knock-out animals that were treated with drug or vehicle control. This experimental design will enable us to identify synaptic proteins whose expression is dependent upon the drug and β-Arrestin activation. We analyzed these samples using quantitative tandem-mass tag proteomics. Using this approach,

we hope to better understand the mechanism of action of this compound. Analyzing this data has required me to master R programming. Additionally, in order to learn about the statistical methods required to analyze this data I have initiated discussions and collaborations with biostatisticians at Duke, the University of North Carolina Chapel Hill, and Emory.

Thesis Dissertation [May 2018 – current]

I have begun my third year as a graduate student in the Neurobiology Graduate Training Program at Duke University. I am interested in the molecular mechanisms of synaptic transmission and the synaptic basis of human brain disorders and have thus chosen to join the research laboratory of Dr. Scott Soderling. The Soderling lab has extensive experience investigating the synaptic basis of neurodevelopmental brain disorders and molecular function of synapses. Including recent work that has sought to interrogate the specific subcellular proteomes of specialized neuronal compartments including excitatory synapses, inhibitory synapses, the axon initial segment, gap junctions, and the tripartite synapse. Thus, the Soderling lab is among scientific leaders in understanding the specific molecular machinery which operates at specialized neuronal compartments to coordinate all aspects of neurotransmission and is an ideal place for the completion of my thesis work.

As a graduate student in Duke's Department of Neurobiology, I am immersed in a rich environment that supports my scientific development through coursework, research rotations, weekly seminars, and many opportunities to present and communicate science. During my first year, I took the courses "Concepts in Neurobiology I & II" and "Neurobiology of Disease" which introduced me to the broad range of subdisciplines of neuroscience. During the beginning of my second year, I gave a seminar to Neurobiology faculty, and took additional courses in grant writing and quantitative neuroscience. These experiences will help develop my speaking ability, grantsmanship, and quantitative and computational neuroscience skills. These core experiences are supplemented with participation in numerous graduate student led journal clubs in which I will present and discuss current neuroscience literature with my peers. During my first year as a graduate student, I rotated in the labs of Dr. Henry Yin, and Dr. James McNamara. These rotations gave me foundational exposure to experimental techniques in mouse behavior and optogenetics, as well as established personal relationships on which I plan to build collaborations in the future. In the McNamara lab, I learned about a mouse model of epilepsy and developed my surgical skills which will be very useful for my proposed research.

Our discovery of an iPSD proteome has provided the foundation for my thesis work. Many of the proteins we identified at the iPSD have not been characterized to function at the inhibitory synapse, including several proteins of unknown function. Since working on this project, I have been fascinated with the challenge of understanding these proteins. Rogdi, one of the iPSD proteins we identified, was an intriguing candidate protein because although it is strongly linked to a human epilepsy disorder, its function is almost completely unknown. I wanted to develop a comprehensive understanding of whatever could be learned about the Rogdi protein. To this end, I have read all the published clinical and scientific papers on Rogdi or its associated syndrome, Kohlschütter-Tönz syndrome, including contacting Dr. Alfried Kohlschütter in order to obtain his original published description of the disorder. I have studied Rogdi's amino acid sequence, protein structure, and expression in the body and brain in order to gain insight into the function of Rogdi. One particularly productive approach that I have employed is gene co-expression analysis. Using multiple datasets from mouse brain proteomics and RNAseq, this analysis demonstrated that Rogdi is highly correlated with proteins associated with the V-ATPase. These preliminary data and bioinformatic analysis provide a strong foundation for the proposed work to investigate Rogdi's function.

My thesis committee consists of distinguished professors in neurobiology with expertise in areas relevant to my thesis work including Dr. James McNamara (epilepsy), Dr. Nicole Calakos (electrophysiology), and Michael Tadross (molecular neurobiology, pharmacology, and biomedical engineering). I will meet with this committee annually in formal meetings and have also established close working relationships with each member that enable me to discuss ideas and problems with them as needed. In May of 2018 I took and passed my qualifying exam. The work proposed in my NRSA F31 is a continuation of the dissertation research that I defended in this exam. I hope that upon the completion of the proposed research I will be able to defend my PhD in neurobiology.

B. TRAINING GOALS AND OBJECTIVES:

Meetings and presentations

I have attended several annual and biannual retreats held by Duke's Neurobiology and Cell Biology departments. I will continue to attend these meetings in the future and present posters at these meetings. I have also attended the annual Society for Neuroscience meeting, where I presented my research, and I plan on continuing to attend these meetings. In the future, I will also present my research at seminars held by the Cell Biology and Neurobiology departments. I will also attend national and international conferences to present my research. These experiences will not only help hone my scientific presentation skills but also will expose me to other scientists in my field and present great opportunities to get feedback on my research. I also plan to write a scientific review in 2019 with Dr. Soderling, which will further expose me to the scientific field and further prepare me for a future career as a postdoctoral scholar upon completion of my thesis. ***An NRSA F31 will provide me with funding to attend these meetings which will be a critical aspect of my career development.***

Mentorship

The experiences that I have highlighted above demonstrate my commitment to developing productive collaborations within labs at Duke University and beyond. While a research technician and graduate student, I have mentored a Duke undergraduate student as well as a Duke first-year graduate student. During the period of this award I hope to mentor 1-2 more undergraduate students. ***An NRSA F31 will enable me to continue to pursue collaborations with other labs, advise students, and develop my mentorship abilities.***

Technical goals for fellowship training

In the past several years I have established a strong foundation in molecular neuroscience. These skills include cloning, cell culture, culture of dissociated neurons, hippocampal slice culture, immunohistochemistry, immunocytochemistry, immunoblotting, proteomics, and bioinformatics. I wish to build on this foundation of skills and my proposed research closely aligns with my training goals. In the completion of the proposed research I will utilize super-resolution imaging, electrophysiology and mouse behavior. I will also learn patch clamp electrophysiology. The attainment of this reward will be a critical step towards a successful career in science. ***An NRSA F31 will enable me to enrich my technical skillset and continue to pursue cutting-edge***, ***innovative science.***

Future and Career Goals

After earning my Ph.D. in neurobiology, my long-term goal is to become a faculty member teaching neuroscience and leading my own research program. I will continue to develop and apply innovative technologies to understand the mechanisms of synaptic transmission in health and disease. As a leader, I will build a collaborative, and inclusive environment in which I will attempt to foster curiosity while supporting the rigorous scientific investigation. I will promote interdisciplinary training and collaboration to tackle fundamental problems in neurobiology. ***An NRSA F31 will provide a strong foundation for the ultimate completion of my aim to become a principle investigator.***

C. ACTIVITIES PLANNED UNDER THIS AWARD:

Below I have outlined the major activities I plan to complete during the period of the award:

Activity Outline						
Award Year	**Research**	**Coursework**	**Seminar**	**Mentoring**	**Meetings**	**Professional Development**
1	80%	5%	5%	2%	3%	5%
2	80%	*NA*	10%	*NA*	3%	5%
3	75%	*NA*	10%	*NA*	*NA*	15%

Description of Activities by Year:

Year 1

Research: *see Research Timeline and Description of Research Activities*, below.

Coursework: Teaching assistant in neurobiology
Seminar: Attendance of weekly neurobiology seminars given by invited speakers, and weekly seminars given by Duke neurobiology students. Attendance of weekly cell biology seminars by students and post-docs.
Mentoring: I previously mentored a graduate student and undergraduate student. I will continue to mentor 1 undergraduate student per school year.
Meetings: Attendance of one International neuroscience conference and one local meeting such as the Triangle Chapter's Society for Neuroscience meeting.
Professional development:

Year 2

Research: see *Research Timeline and Description of Research Activities*, below.
Coursework: None.
Seminar: Attendance of weekly neurobiology seminars given by invited speakers, and weekly seminars given by Duke neurobiology students. Presentation at either Duke Neurobiology or Duke Cell biology In-House seminars.
Mentoring: I previously mentored a graduate student and undergraduate student. I will continue to mentor 1 undergraduate student per school year.
Meetings: International neurodevelopment conference and a local meeting such as the Triangle Chapter's Society for Neuroscience meeting
Professional development:

Year 3

Research: Dedicated to complete experimental aims
Coursework: None.
Seminar: Attendance of weekly neurobiology seminars given by invited speakers, and weekly seminars given by Duke neurobiology students. Presentation at either Duke Neurobiology or Duke Cell biology In-House seminars.
Mentoring: In the final year of this reward I will not mentor a student in order to make additional time for professional development.
Meetings: See professional development.
Professional development: In the final year of this award I hope to finalize my research for publication and defense of my thesis. I hope to attend several national conferences in order to network and find a post-doctoral research lab.

Research Timeline

The majority of my time (75-80% per year) will be dedicated towards the completion of the experimental aims of this proposal. I plan to accomplish most of the biochemistry and imaging associated with Aim 1 in the first year of this award. In this time, I will be breeding my *Rogdi*$^{f/+}$ mice to *Gad2-Cre* and *Ai14* mice. The mice required for experiments should become available in the second year of my award. I will then perform the electrophysiology experiments to evaluate pre- and postsynaptic function. I have spread these activities over years 2 and 3, as I anticipate additional time will be required to acquire the required electrophysiology skills. Finally, I will complete the assessment of seizures in Rogdi KO mice.

Aim	Objective	Y1	Y2	Y3
Aim 1	Reagent generation.	X		
	HiUGE and STED imaging of Rogdi-smHA	X		
	HiUGE and IP of Myc-tagged Dmxl1, Dmxl2, and Wdr7	X		
Aim 2	Mouse husbandry	X	X	X
	Postsynaptic and Presynaptic electrophysiology		X	X
	Evaluation of seizures in Rogdi KO mice		X	X

CONCURRENT SUPPORT DESCRIPTION

During the first three years of my training (2015-2018) I was supported by the Duke Neurobiology graduate student program. However, this funding ended in June 2018, which is prior to the start of Fellowship funding for this proposal. Consequently, I am requesting three years of NRSA funding. I will have no additional concurrent funding during the period of this award.

SPONSOR STATEMENT

A. Research Support Available for the Applicant's Training Experience

Current Funding

Source	Number	Title	PI	Dates	Direct costs
NIH-NIMH	R01-MH103374	Molecular, Synaptic, and Circuit Basis for Schizophrenia-related Endophenotypes	Scott Soderling	02/14-01/19	1,250,000
NIH-NINDS	R01-NS102456	Analysis of Neuronal Inhibitory Synaptic Proteins Associated with Brain Disorders	Scott Soderling	09/17-08/22	1,696,587
NIH-NIMH	R01-MH111684	Molecular Analysis of Developmental Brain Disorders Associated with Synaptic Pathology	Scott Soderling	09/17-08/22	1,745,732
NIH-NIDA	R01-DA047258	New Proteomic and Genome Engineering Approaches to Decipher Astrocyte Function at Synapses	Scott Soderling	09/18-08/21	1,356,199

B. Sponsor's Previous Fellows/Trainees

One of the greatest legacies of a successful scientific career is the rigorous training and success of the next generation of scientists. Early in my own career (as a senior postdoc and new Assistant Professor) I realized that effective mentoring is a skill that, like other skills in science, must be actively developed. Thus, I took advantage of several programs aimed at building and honing mentoring skills of scientists, including an HHMI mentoring course and Duke Faculty Development courses for early stage Assistant Professors. Over the course of my career, I have now mentored eleven graduate students and seven postdoctoral fellows in my laboratory (past and current). My graduate students have been highly successful in garnering competitive external support, being awarded six scholarships or fellowships, including National Science Foundation Awards and NIH National Research Service Awards. Graduate students from my laboratory have won numerous awards for talks and presentations at scientific meetings and have published ten scientific articles in the past twelve years. I have also mentored 37 students as a member of their thesis committee and oversee the Cell Biology graduate program as the Director of Graduate studies (currently 57 students). I have also been highly involved with the Duke Medical Scientist Training Program (MSTP), participating in interviews, MSTP mentoring thesis committees, research opportunity lectures, and programmatic fair luncheons for student advising. Additionally, in my current role as Interim Chair I also help mentor junior faculty in the Department. These have provided a rich and diverse background from which I bring substantial mentoring experience and success to this proposed fellowship. Some representative past and current mentees from my laboratory, which are now in both academic and industry positions include:

Name	**Position**	**Current Position**
Hirokazu Okada	Postdoctoral Fellow (2008-12)	Instructor, Swiss Federal Institute of Technology
Thomas Newpher	Postdoctoral Fellow (2010-12)	Associate Professor of the Practice, Duke Univ. (*Awarded Fellowship in Fundamental & Translational Neuroscience)*
Il Hwan Kim	Postdoctoral Fellow (2009-16)	Assistant Professor, Dept. of Neuroscience, University of Tennessee Memphis (*Awarded NARSAD Young Investigator Award)*
Tetsuya Takano	Postdoctoral Fellow(2017-)	(*Awarded JSPS Research Fellowship for Young Scientists, Elected Executive Council Member Japanese Society for Neurochemistry)*

Frank Mason	Received PhD 2011	Research Faculty, Vanderbilt University
Lauren Burianek	Received PhD 2015	Postdoctoral Fellow, Tufts (*Awarded NSF*)
Rohit Ramnath	Received PhD 2016	Project Manager, Nuventra Pharma Sciences
Erin Spence	Received PhD 2018	Project Manager, Nuventra Pharma Sciences (*Awarded NIH F31 Fellowship)*
Shataakshi Dube	PhD candidate (2016-)	(*Awarded a Hemsley Scholarship and a National Science Graduate Research Fellowship)*
Jamie Croucher	MD, PhD candidate (2017-)	(*Awarded NIH F30 Fellowship)*

C. Training Plan, Environment, and Research Facilities

Introduction.

Since Tyler Bradshaw joined my laboratory, I have been working with him on his intellectual development, scientific concepts, experimental skills, as well as grant writing and data presentation skills. These include one-on-one meetings in which research design, data interpretation, troubleshooting and new ideas are discussed. The training program for Tyler is designed to expose him to a broad array of experimental approaches and a rich diversity of career experiences within the context of a highly focused research project. I am absolutely convinced Tyler will become a successful scientist as he progresses in his career training towards an independent research position of his own. This training proposal represents a project and training experience that is carefully designed to nurture a well-rounded scientist training experience through opportunities in the two departments I am jointly appointed in: the Neurobiology and Cell Biology Departments at Duke. Of note, Tyler successfully passed his preliminary examination on May 22nd of 2018 by defending this research proposal to his thesis committee (see below *Mentoring Plan* for members of his committee).

Mentoring Plan.

To facilitate the mentoring process, Tyler and I spend a considerable amount of time (1 hour per week scheduled, ~1 hour informal) discussing experiments, protocols, and results. These discussions encompass both the conceptual and specific details of the experiments. Our informal conversations, which generally occur on a daily basis, are extremely valuable. They help us perform the best experiments possible and keep me engaged in the progress of his project. It also gives me the opportunity to show Tyler how to evaluate and analyze data on an as needed basis. Additionally, Tyler meets yearly with his graduate committee to present his work, discuss his research direction, and highlight his training progress. His committee consists of a range of experts in translational and basic neuroscience research; Dr. James McNamara, a physician-scientist who is an internationally recognized expert on the molecular mechanisms of epileptogenesis (Neurology, MD, PhD), Dr. Nicole Calakos, a physician-scientist whose work focuses on molecular mechanisms of plasticity in relation to basal ganglia movement disorders (Neurology, MD, PhD), and Dr. Michael Tadross, an Assistant Professor whose laboratory pioneered the use of Drugs Acutely Restricted by Tethering (DART) to study cellular and circuit mechanisms of behavior and psychiatric disorders (Biomedical Engineering, MD, PhD). Together, my mentoring along with that of Tyler's scientific advisors, will provide him with a strong foundation in basic and translational neuroscience, making him an extremely competitive applicant for a competitive research postdoctoral fellowship and future independent position.

Lab meeting (once a week).

In addition to our informal discussions, we also have lab meetings where Tyler and other members of the laboratory present their research progress. A crucial aspect of these presentations is the ability to discuss work freely and offer a dedicated environment where Tyler and his colleagues can solicit feedback from each other. Consistent with Tyler's inquisitive and engaged personality, he often asks questions related to the presentations. These meetings occur three weeks a month, with one week each month being a joint lab meeting held with Dr. Cagla Eroglu (http://www.cellbio.duke.edu/cagla-eroglu) in the Cell Biology Department.

The Eroglu lab is primarily interested in the cellular neuroscience of glia, and the interests of her lab closely align with our own. Because of this, our joint meetings tend to be very energetic with robust discussion. Importantly, through these meetings students are exposed to the ideas and questions of two different labs, broadening their viewpoints. Further, as Dr. Eroglu is not a member of Tyler's committee these joint lab

meetings provide an additional means for Tyler to communicate his research progress to a broader faculty audience.

Together, these lab meetings (lab-specific and joint) help ensure a rigorous and well-rounded graduate school experience for Tyler.

Opportunities to Present Data and Research Progress.

Tyler also takes part in parallel opportunities to present his research and gather feedback through my affiliations with the Cell Biology Department and Neurobiology Departments. The Cell Biology Department sponsors a weekly "In House Symposium," which consists of student and post-doc presentations to the department. The In House series is its own unique training opportunity. Because of the diverse research interests of the department, a successful presentation must contain an engaging introduction that explains the research topic, methodologies, type of data obtained, analysis, and conclusions to a broad audience—this is a fundamental skill for successful science communication. Participation in the In House Symposium by the Junior and Senior Faculty is very high and written feedback is provided by the audience for student presentations. Similarly, Tyler will also give an oral presentation once per year in the Department of Neurobiology's Student Seminar lecture series. These presentations are followed by a half-hour oral feedback session from faculty and peers, as well as summarized written comments and critiques. Tyler has already presented his research in the form of posters at the Cell and Neurobiology retreats and will continue to do so on an annual basis. . Now that Tyler's project is starting to generate results, he will also be attending national and international meetings on a yearly basis to present his work. In the past, Tyler attended the 2016 and 2017 Annual Society for Neuroscience meetings and presented posters on his research there.

Translational Opportunities in Neuroscience.

The Department of Neurobiology also hosts a Ruth K. Broad Lecture Series in Translational Neuroscience once a month during the academic calendar, which allows students to meet and have lunch with world-renowned neuroscientists (*https://www.neuro.duke.edu/seminars/ruth-k-broad-seminars*). This series is organized so that graduate students get maximum exposure to outside experts and leading scientists who are conducting cutting-edge translational neuroscience research. For each visit (typically 10 per year), students have lunch with the speaker where they can ask scientific and career-oriented questions. This gives the students an opportunity to directly interact with outside leaders in the field in a variety of informal and formal settings- an invaluable experience for their careers. Tyler actively participates in this training opportunity and has used these experiences to directly influence his current research proposal.

Additional course work and training opportunities.

In addition to the required coursework for Neurobiology, Tyler has or will take several supplementary classes to enhance his research training. These include Responsible Conduct in Research seminars (to be completed in summer 2019) and a quantitative and statistical approaches to neuroscience course (NEUROBIO735, completed Spring 2018). Tyler has also completed a course in Seattle, Washington on mass spectrometry theory, experimental design, and analytical methods used in proteomic research (completed July 2017). These experiences have been invaluable in giving him an exposure to statistics, quantitative neurobiology, bioinformatics, and computer programing.

One important additional component of Tyler's research will be to master the approaches necessary for the functional analysis of Rogdi based on his published proteomic discoveries. To facilitate this aspect of his research career development, I have an established electrophysiology suite with patch clamp recording rigs as well as multi-electrode arrays, both of which are configured for cutting-edge optogenetics. Tyler will have full access to the equipment and has rapidly established a positive working collaboration with one of my senior students whose expertise is electrophysiology. I have supported Tyler in organizing training sessions to develop expertise to independently pursue his patch clamp recording experiments for functional characterization of Rogdi.

In summary, these courses, which will be completed before the start of Fellowship funding (with the exception of Responsible Research Conduct seminars), will provide Tyler with all of the necessary theoretical knowledge and practical skills required for successful completion of the proposed research. I will continue to support Tyler by providing him with contacts for collaboration, material to conduct his experiments, and advice to guide him in experimental planning, and to further improve his critical thinking and mentoring abilities in this, as well as other lines of his experimental design.

Research and mentoring skills and career goals.

It is my goal and ambition that the environment in my lab promotes, through example and close mentoring, personal responsibility for research and excellence. Tyler's project, as detailed in the research and design section (which he has written himself with my guidance), will teach him a wide range of skills such as molecular biology (including cutting-edge CRISPR-based genome editing methods we have developed and for which he is a co-author on a submitted manuscript), protein purification and novel proteomics approaches, neuronal imaging (including super-resolution imaging), mouse husbandry and genetics, and electrophysiology. He will also learn to think critically about his research and identify how it addresses translational neuroscience questions. Additionally, Tyler has and will continue to have opportunities to supervise undergraduate and/or rotating graduate students in the laboratory. I will serve as the "ex officio" by discussing with Tyler about the experimental design for these students, while Tyler will teach the actual techniques and help them grasp the underlying concepts. This will give Tyler critical experience in mentorship, which is required for a successful career in science. These skills will further his career aspirations to continue in neuroscience research as a postdoctoral fellow and ultimately an independent scientist.

Research environment, facility, and equipment.

The training environment and institutional commitment for Tyler's research training at Duke University are excellent. I have approximately 1,900 sq. feet of laboratory space, including a separate cell culture room, an electrophysiology suite, and a dedicated microscopy room with newly acquired optical sectioning capability using structured illumination microscopy. Tyler is provided a 6ft. long bench with an adjacent desk in my laboratory. Our mice are housed in a dedicated mouse colony located in an adjacent building to our main laboratory space. The colony is maintained as a biological barrier facility with a well-trained and well-managed staff who attend to the daily care of the mice. Within the barrier zone we have a procedure room in which we have the necessary equipment mouse surgery, including a surgical scope, stereotactic frames, high-speed drills for skull thinning, and a BSL-2 hood for viral injections. All the necessary equipment needed for the proposed experiments are available in my laboratory or through the Duke Microscopy and Proteomics Cores. Through the Light Microscopy Imaging Facility (LMCF), we have direct access to confocal and a newly acquired super-resolution STED microscope that are adjacent to my laboratory and are managed by a professional staff of scientists, one of whom was trained in my laboratory. Furthermore, my colleague and friend, Dr. Nicole Calakos, who is on Tyler's committee, also has extensive experience in electrophysiology. This further provides a rich environment for Tyler to master electrophysiology (with oversight from other electrophysiologists in my lab), and other concepts of cellular and translational neuroscience. Tyler will also benefit from my collaboration network, which will allow him to receive additional intellectual and technical training, as well as support from my collaborators, including Drs. Marc Caron (Duke; see letter of reference), Fan Wang (Duke), Richard Mooney (Duke), Ryohei Yasuda (Max Planck Florida), Henry Yin (Duke), Yong-hui Jiang (Duke) and Benjamin Philpot (UNC).

Relationship of the proposed research training to the applicant's career.

Tyler is a highly motivated student who strongly desires to pursue a position as an independent scientist at an academic medical institution in the field of neuroscience. Tyler's primary goal with this F31 proposal is to obtain the research skills that will lay the foundation for his future career as an independent investigator studying basic mechanisms underlying GABAergic synapse function. His research training and thesis proposal is carefully crafted to facilitate this career goal. This proposal is highly innovative and will tackle the basic and translational importance of inhibitory synapses within the brain.

Normal brain function requires a proper balance between neurons that excite or inhibit neuronal circuits. This balance is established during a critical developmental period in children during the first weeks after birth. Abnormalities in this development can result in reduced inhibition and elevated excitation of neurons associated with many of the most devastating childhood neurodevelopmental disorders. These include *autism spectrum disorders (ASD)* (≈45,000 US births every year), *epilepsy* (children under 2 years of age are particularly susceptible), *neonatal hyperekplexia* (Stiff-Baby syndrome), and *intellectual disability* (nearly 220,000 US births annually). While extensive research has focused on the analysis of excitatory glutamatergic neurons and their synapses, inhibitory synapses and the molecular mechanisms that modulate them remain understudied. This is in large part because, in contrast with the excitatory neuronal synapses, the purification and analysis of inhibitory synapses using classical strategies is intractable. Thus, the biochemical nature of inhibitory interneuron synapses had largely eluded neuroscience. This basic lack of knowledge posed an unmet need and critical barrier to understanding how inhibitory synapses function, and how they are

associated with neurodevelopmental disorders. The research proposed by Tyler, follows on his work as a technician to utilize a highly innovative approach to specifically label, purify, and discover using mass spectrometry, the synaptic machinery responsible for inhibitory synapses. This work, which aims to reveal the internal workings of inhibitory synapse development and function, as well how molecular perturbations associated with epilepsy may affect GABAergic function is the focus of Tyler's thesis.

The results of this proposal should directly impact Tyler's next stage of training. Tyler and I have spoken at length about his career ambitions, and I believe the proposed F31 research training plan will provide an ideal foundation for his transition into a postdoctoral fellowship. This future post-doctoral training will provide him with dedicated time to expand upon his current research and submit a career development award to the NIH. Consequently, the proposed project is a fundamental stepping-stone to his ultimate career goal of establishing an independent laboratory that investigates the molecular mechanisms of neurodevelopment disorders arising from dysfunctional GABAergic synapses. Because this proposal is technically and conceptually innovative with clear relevance to translational neuroscience, I anticipate its completion will serve as strong foundation upon which Tyler will build a successful career.

D. Number of Fellows/Trainees to be Supervised During the Fellowship.

In my laboratory, in addition to Tyler, I currently supervise four pre-doctoral students, four postdoctoral fellows, and three technical staff. This provides a rich, rigorous, and highly supportive intellectual environment for Tyler's training. Tyler also has extensive interactions with other graduate students within the Neurobiology and Cell Biology training programs who have different backgrounds in research skills and training.

E. Applicant's Qualifications and Potential for a Research Career.

Tyler is the ideal candidate for this research training grant. He is highly motivated and completely dedicated to a career as an independent scientist conducting competitive neuroscience research in an academic setting. He has an excellent academic record and research progress at Duke thus far, including co-authored publications from my laboratory. Furthermore, he is very enthusiastic about conceptual and technical development, as well as learning and practicing mentorship (he has already successfully mentored rotating graduate students). He is showing great promise in every area necessary for a successful career in basic neuroscience research.

Tyler has been my most productive student at this early stage of his graduate work and has excelled during his time in my lab. He is a remarkably curious and intelligent student. He reads the literature both avidly and critically, is able to integrate diverse scientific disciplines, and thinks very carefully and thoughtfully about his research. I very much enjoy our back-and-forth discussions of his research, especially because Tyler is fearless in his pursuit of new research ideas and directions for his project. The idea that Rogdi may be a novel component of the RAVE complex is an idea that is his entirely (in fact I had never even heard of the RAVE complex until he proposed his idea)! He is also very hard-working and is remarkably adept in learning new techniques. Since joining my lab, Tyler has helped to established a novel genome engineering method, Homology-independent Universal Genome Engineering, to label and manipulate endogenous proteins in high-throughput. This is a cutting-edge approach that was rapidly pioneered and mastered by Tyler, in collaboration with a postdoc in my laboratory who has helped teach him some of the necessary techniques. This new approach will help Tyler analyze endogenous Rogdi to tease out its molecular functions in ways that were not possible even a year ago. I am very impressed that he has established and validated these methods in a relatively short time frame. Tyler is now in a position to discover, analyze, and test the molecular basis of Rogdi function *in vivo*, and to specifically identify how its loss may contribute to epilepsy. I strongly believe this work will have important and potentially paradigm-shifting discoveries for the field.

Tyler has not only proven himself to be an excellent researcher, but he also has a well-documented ability to think critically about scientific theory and practice. Tyler arrived at Duke with an excellent academic record from his undergraduate university (University of Washington) and has a strong record of productivity during his two-year research technician position (co-second author on a publication in Science). He joined the Neurobiology graduate program in the fall semester of 2016 and has excelled in his classes throughout his first two years of graduate school (3.94 GPA). My colleagues who have had the privilege of teaching him during have been impressed by the level of critical thinking and thoughtfulness he exhibits when discussing and reading the scientific literature. In lab, he has continued to demonstrate a high level of critical thinking and

enthusiasm for learning about his field. He displays an avid interest in the ever-changing field of neuroscience, and often has highly relevant papers to share during our weekly meetings.

This thirst for knowledge has quickly provided Tyler with the background necessary to make a link between the molecular mechanisms regulating inhibitory synapses with neurodevelopmental disorders such as epilepsy. Tyler generated a proposal of detailed experiments to thoroughly investigate this process both *in vitro* and *in vivo*. Previously, my laboratory had investigated the regulation of excitatory neuronal synapses broadly using similar approaches. Tyler, however, wanted to be able to investigate this process in a more selective manner, by leveraging Cre-transgenic lines to specifically analyze Rogdi, including its potential role presynaptically in GABAergic neurons. Based on his reading of the literature, he quickly realized that she could take advantage of existing GAD Cre lines for his project. This realization on his part proved critical, as deleting Rogdi in these interneuron populations will allow him to further test its potential role in GABA release that he will test using optogenetics in cultured neurons.

Tyler also has great promise as a mentor. One of Tyler's great strengths is his remarkable attention to the literature and experimental details. He is clearly one of the most well-read lab members I have ever mentored, and I believe this a strong reflection on his passion for research and attention to detail. I anticipate his skills will allow him to effectively mentor an undergraduate student, without negatively impacting his own progress, in the future once he has further established his project in the lab.

During the process of working with Tyler on this NRSA application, I have been impressed with his scientific writing skills, which are essential to becoming a scientist heading a competitive neuroscience research lab. I am confident that the support this research training grant provides will propel Tyler into the forefront of research linking inhibitory synaptic dysregulation with brain disorders and I am excited to be a part of his evolution from a graduate student to an independent investigator conducting exceptional neuroscience research.

In summary, I also serve as the Director of Graduate Studies for the Cell Biology graduate program, and thus I am very familiar with the range of graduate students we have in our programs at Duke. Tyler is certainly among the brightest and most dedicated graduate students of our graduate program. I would rank him without reservation in the top 1-2% of our graduate students. His long-term commitment to academic research, his self-motivation, persistence, intelligence, productivity, and independent learning capability make him an ideal candidate for the support from an NIH training fellowship. I consider it an honor to provide him with mentorship and training for the development of his career.

DUKE UNIVERSITY MEDICAL CENTER
Nicole Calakos, M.D., Ph.D.

Professor of Neurology and Neurobiology
Chief, Movement Disorder Division

November 30, 2018

Dear Tyler,

I would like to affirm my great interest and availability to provide support for your electrophysiological experiments characterizing the synaptic effects of Rogdi on inhibitory neurotransmission. The progress you have shown in your thesis committee meetings and your F31 application are extremely encouraging and show great potential to make highly novel insights regarding mechanisms of inhibitory synaptic regulators. The reagents you have at your disposal will also be ideal to elegantly discern both pre and post synaptic contributions. As our laboratories, Soderling and Calakos, are nearby and have frequent interactions on several projects, I see no obstacles to ensuring your success. Best wishes on your application.

Sincerely,

Nicole Calakos, MD PhD
Professor
Neurology, Neurobiology and Cell Biology

Box 2900 • Durham, NC 27710-0001 • tel (919) 684-2423 • fax (919) 684-6514 • nicole.calakos@dm.duke.edu

Light Microscopy Core Facility
DUKE UNIVERSITY AND DUKE UNIVERSITY MEDICAL CENTER

December 3, 2018
Lisa A. Cameron, Ph.D.
4215 French Family Science Center
124 Science Drive
Durham, NC 27708

Dear F31 NRSA Review Committee,

I write to you as the Director of the Light Microscopy Core Facility (LMCF) at Duke University in support of Tyler Bradshaw's F31 NRSA proposal titled "*Interrogating the role of the novel synaptic protein Rogdi in GABAergic inhibition and epilepsy*". My core staff and I would be glad to offer training, technical support and advice for Tyler's proposed project which will utilize super-resolution microscopy to analyze the localization of the Rogdi protein.

The Duke LMCF core facilities include a wide range of confocal, and conventional fluorescence microscopes including a Leica SP8 STED (STimulated Emission Depletion) microscope designed for super-resolution imaging. Equipment is available to trained users 24 hours a day, every day of the year. Tyler has already completed training required for the use of the STED system along with several other confocal microscopes in our core, We are happy to provide Tyler access to any of our microscopes in addition to any training and technical support that is required to acquire and analyze imaging data that will allow him to investigate the functional role of Rogdi in the brain. The Rogdi protein has been found to be associated with the human epilepsy disorder and localizes to synapses in the mouse brain. In order to resolve whether the protein localization is pre- or post-synaptic, STED imaging is required, as confocal microscopy has not been able to accurately resolve its location.

We look forward to working with Tyler on this project.

Sincerely,

Lisa A. Cameron, Ph.D.
Director, Light Microscopy Core Facility
Duke University and Duke University Medical Center

DUKE UNIVERSITY MEDICAL CENTER
Department of Neurobiology
James O. McNamara, MD
Duke School of Medicine Professor of Neurosciences
Professor, Department of Neurology
Professor, Department of Pharmacology and Cancer Biology

November 29, 2018

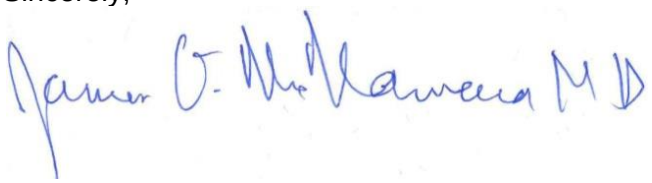
F31 Review Panel

Dear Colleagues:

I write to formally articulate my strong support for the research project outlined by Tyler Bradshaw. I have a career long focus on mechanisms of epileptogenesis. This focus dovetails nicely with Tyler's project focused on susceptibility to epilepsy in the Rogdi knockout mouse. I have reviewed his preliminary data and discussed his experimental approaches. I will review his results and mentor him as the project develops. This includes access to our expertise in implementing various animal models of epilepsy and their assessment with long term video EEG recording.

Tyler is a highly promising young scientist and I look forward to working with him on this project.

Sincerely,



James O. McNamara, M.D.
Duke School of Medicine Professor of Neurosciences

Description of Institutional Environment and Commitment to Training

Duke's Neurobiology Graduate Training Program is a well-established, collaborative, and interdisciplinary research program with the goal of training students who will lead the field of neuroscience through positions in research-oriented institutions. The Neurobiology program provides broad training in the structure and function of the brain, from molecules to cognition, and provides a particularly strong group of faculty with expertise in the areas of research that pertain to Tyler Bradshaw's project. Tyler's mentoring team, made up of Drs. Scott Soderling (mentor and project Sponsor), James McNamara (thesis committee chair), Nicole Calakos (thesis committee member), and Michael Tadross (thesis committee member), are all excellent molecular neuroscientists committed to training the next generation of researchers. Dr. McNamara has particularly strong record in epilepsy research. The facilities in Dr. Soderling's laboratory are ideally suited for the experimental and analytic work required for completion of Tyler's project. Tyler will have access to all of the necessary facilities and resources necessary for successfully completing his Ph.D., including frequent interaction with fellow researchers, excellent support from faculty experts, necessary equipment and expertise required for his research project, and career development opportunities through the Duke Graduate School.

Additional Education Information

Neurobiology Graduate Training at Duke is an interdepartmental, interdisciplinary program that has its home in the Department of Neurobiology with Steve Lisberger as Chair and Jörg Grandl as Director of Graduate Studies. The program currently comprises 50 faculty members and, counting students matriculating in 2018, 64 predoctoral students. The Neurobiology Training Program faculty members come from 15 departments across the Duke campus and have diverse interests in molecular and developmental neuroscience, synaptic physiology, biophysics, cellular neuroscience, computational neuroscience, systems and behavioral neuroscience, neurology, psychiatry, and pediatrics.

In their first year, students perform 2-3 required lab rotations and take a series of fundamental courses. They affiliate with a lab at the end of spring or summer of the first year and take their preliminary exam by the end of the Spring semester of the second year. Of students who entered in 2007-2012, over 83% completed their PhD with an average time to degree of 5.6 years. Of the 84 students enrolled since 2012, 24% completed their PhD, 72% are still in the program, and 4% voluntarily withdrew or were awarded a terminal master's degree.

In the first year, the required courses start with **Neuroscience Bootcamp (NBI 751)**, a two-week immersive lecture, discussion, and laboratory course for graduate students in the Neurobiology Graduate Program and the Cognitive Neuroscience Admitting Program. Bootcamp (1) provides a common knowledge base of neuroscience fundamentals; and (2) demystifies the tools of the discipline by providing hands-on experience with common techniques Lab experiences include optogenetic interrogation of neural circuits, molecular genetic analysis of neurons and functional brain imaging in humans. After **Bootcamp**, the students take **Concepts in Neuroscience I and II (NBI 719 and 720).** This series introduces graduate students to the basic principles underlying Neuronal Excitability, Synaptic Transmission and Plasticity, Sensory Transduction, and the Cell Biology of the Synapse in the Fall. It continues in the Spring with topics that include Neuroanatomy, Learning and Memory, Sensorimotor integration, Circuit Development, and Sensory Perception and Cognition. In January, students take **Neurobiology of Disease (NBI 762),** a 4-week long seminar that covers: ALS, Alzheimer's, CNS neoplasms, epilepsy, multiple sclerosis, Parkinson's disease, retinitis pigmentosa, and stroke. It considers the key features of the disease, the etiology and pathogenetic mechanisms of the disease, animal models, and current and potential therapies.

Additional course requirements include **Journal Club (NBI 726),** where first and second year neurobiology graduate students meet to discuss recent research published by an invited **Neurobiology Seminar Series** speaker from an outside institution. The Journal club serves to prepare the students for the seminar as well as the following lunch (open to all students and postdoctoral fellows) where they can directly interact with and learn more about the scientist's research program. Also, in the first and second years, they take **Student Seminar (NBI 790S),** where they prepare and present seminars to students and faculty on topics of broad interest in neurobiology. In the second year, students also take a required grant writing course (**NBI 710 – Scientific Writing**) in the fall semester and a quantitative and statistical methods course in the spring semester (**NBI 735 – Quantitative approaches in Neurobiology or NBI 733 – Experiment Design & Statistics**). In the second through fourth years, students are also free to take **additional "advanced" elective courses** selected from a fairly broad menu, including several special topics partial-credit courses led by experts in the Neurobiology department. They have a very basic teaching requirement that usually is satisfied by serving as **Teaching Assistant** in the Concepts courses or in one of the "advanced" courses.

For the **preliminary exam**, students prepare an NRSA-like document on their proposed thesis research. They are examined by a faculty committee for 1.5-2 hours on the area of their proposed research, the specifics of the proposed research, and their general knowledge in Neurobiology. After satisfactory completion of the preliminary exam, students form their thesis committee, frequently constituted mainly or entirely by the preliminary exam committee.

We monitor our students' progress regularly and carefully. This is possible because our program is fairly small (~64 students altogether) so that we are able to provide personalized attention to each student. In the first two years, they meet bi-annually with the Director of Graduate Studies or Lindsey Glickfeld, who serves as a graduate advisor. Each lab rotation is discussed and documented with written report to the Director of Graduate Studies. As students progress toward the preliminary exam, they meet once or twice with their prelim committee. After the thesis committee is formed, students meet at least once a year with the thesis committee. They provide a scientific presentation and engage in supportive discussion of their accomplishments and goals. Written documentation of the meeting, including the committee's advice on how to proceed are collected yearly and maintained by the Department of Neurobiology.

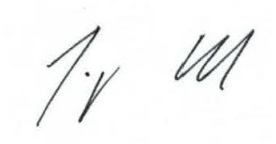
More importantly, we have a strong informal structure for mentoring our students. The Chair and Director of Graduate Studies have open-door policies and they and other faculty form strong mentoring relationships with the students. Interaction is frequent, collegial, supportive, and intense. Finally, the Fall and Spring meetings of the Program's Steering Committee ends with an evaluation of the progress of all students in the program.

Our students complete 16 hours of instruction **Responsible Conduct in Research** (RCR) credit within the first three years of graduate school. This begins at the start of the first year with a 12-hour Academic Integrity and Research Ethics Retreat, a two-day event held at Duke's Marine Lab Campus in Beaufort, North Carolina, followed by an additional 4 hours of RCR retraining at the end of the third year to satisfy the NIH's policy of RCR re-training every 4 years. In addition to the retreat, all students attend at least one 2-hour RCR Forum during their PhD training. These forums address pertinent issues in the ethical conduct of research, laboratory management, animal experimentation, and authorship through a combination of expert faculty lectures and small group discussions.

All third year students are required to generate **Individual Development Plans (IDPs)** in advance of the RCR re-training event. Our students use an IDP template based on the FASEB IDP as a guide to examining their skills and motivations. In addition, the University offers venues for students to be exposed to alternative careers including a half-day symposium held every other year that is open to all students and a yearly **Neurobiology Retreat** in which one of the keynote speakers is from outside of academic research.

Tyler Bradshaw entered the Duke Neurobiology Graduate Training Program in August 2016. He completed his rotations, all courses, and passed his preliminary exam in May 2018. He has completed twelve hours of RCR training and made very strong progress on his thesis research, as outlined in the narrative of his application.

This document is furnished by:



Jörg Grandl
Associate Professor of Neurobiology, Director of Graduate Studies, Duke Neurobiology,
grandl@neuro.duke.edu