Modeling of an epilepsy-related neurodevelopmental disorder caused by *de novo GNB1* missense mutations and identification of targeted treatments

Sophie Colombo[1], Emmanuel K. Ozoruonye[1,4], Sabrina Petri[1], Mu Yang[1,4], Michael J. Boland[1,2], Wayne N. Frankel[1,3] and David B. Goldstein[1,3]

[1]Institute for Genomic Medicine, [2]Department of Neurology, [3]Department of Developmental Genetics, [4]NeuroBehavior Core facility, Columbia University, New York, NY 10032 USA.

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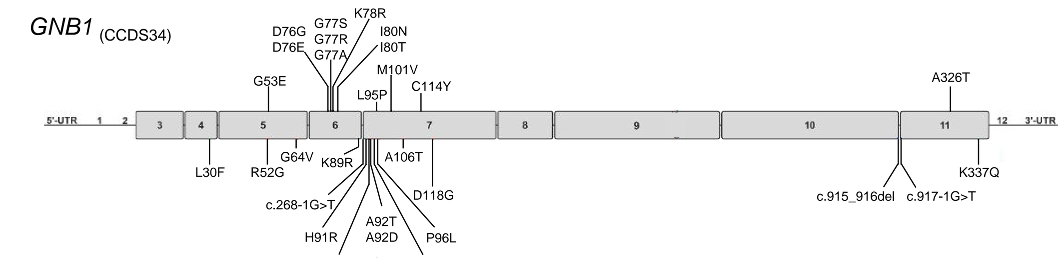
Rationale

- Molecular diagnosis via whole exome sequencing has revealed that heterozygous *de novo* mutations in *GNB1* are associated with a rare neurodevelopmental disorder characterized by the following main features:
  - developmental delay
  - hypotonia
  - seizures
  - ophthalmological defects
- 48 affected children have been identified in the last 2 years, with 29 different mutations in *GNB1* (2 frameshifts, 2 splice sites, and 25 missense) *(Petrovski et al., AJHG 2016, Steinbrucke et al., Neuroloy 2016, Brett et al., AJMG 2017, Lohmann et al., HMG 2017, unpublished).*

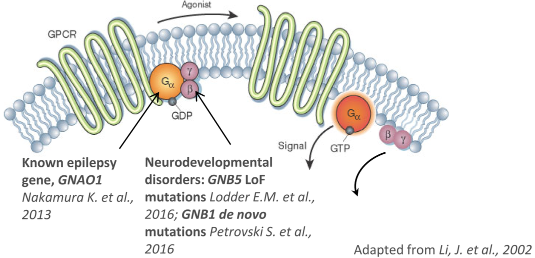
Identified *GNB1* Mutations



- Most mutations identified in the individuals were also found somatically mutated in a variety of cancers, mainly myeloid and B cell neoplasms *(Yoda et al., Nat Medicine 2015).* So far, only one child with a germline mutation was reported with both the neurodevelopmental syndrome and acute lymphoblastic leukemia *(Brett et al., AJMG 2017).*
- Our clinical study of the affected individuals' seizure characteristics suggests that *GNB1* mutations may lead to Electrical Status Epilepticus during Sleep (ESES), which is characterized by continuous (or near continuous) spike-wave discharges (SWDs) during NREM sleep. It is often associated with an encephalopathy (ESES syndrome) characterized by epileptic seizures, continuous SWDs in NREM sleep, global or selective regression of cognitive functions, and motor impairments. Many children will have absence seizures, some myoclonic seizures and others will have focal (partial) motor seizures, all of which were observed in our cohort.

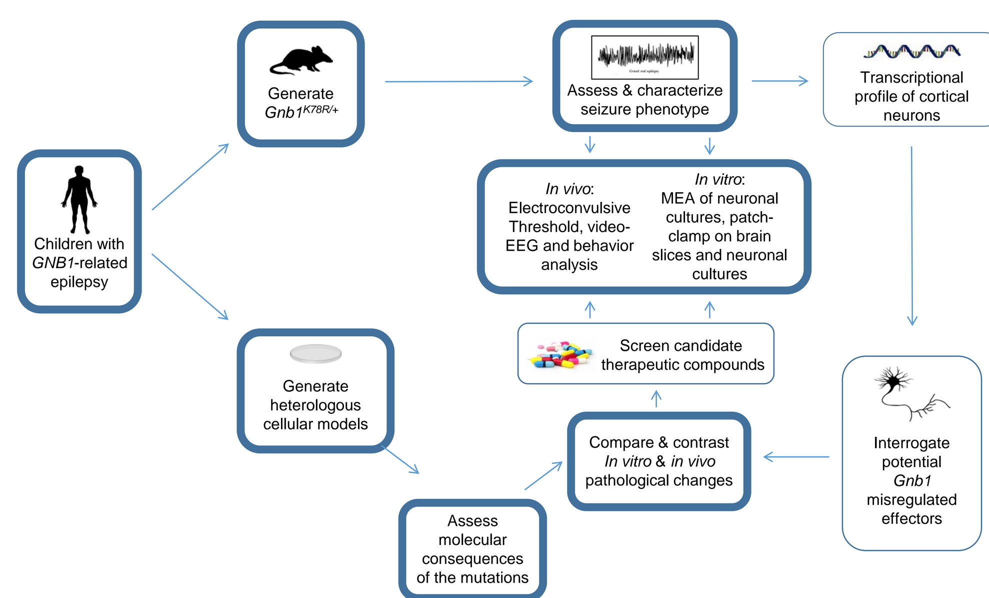
GNB1 Function

- *GNB1* encodes an ubiquitously expressed β subunit (Gβ1) of membrane bound GTPases heterotrimeric G proteins (Gαβγ) which are activated by G protein-coupled receptors (GPCRs).



- Gβγ regulate a variety of effectors and signaling pathways, such as:
  - Activation of MAPK and Pi3K pathways
  - Activation of GIRK (K^+) channels
  - Inhibition of Ca_v (Ca^{2+}) channels
  - Activation of Adenylate cyclase
  - Activation of Phospholipase Cβ
- Most of the identified mutations localize in a region that has been shown to participate to the interface of interaction of Gβ with Gα subunits and effectors like GIRK and Ca_v channels. We thus hypothesize that defective interaction with partner proteins - either increased or decreased - will affect neuronal activity.

General Approach



Acknowledgements

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The *Gnb1*^{K78R} mouse model phenocopies aspects of the human disease

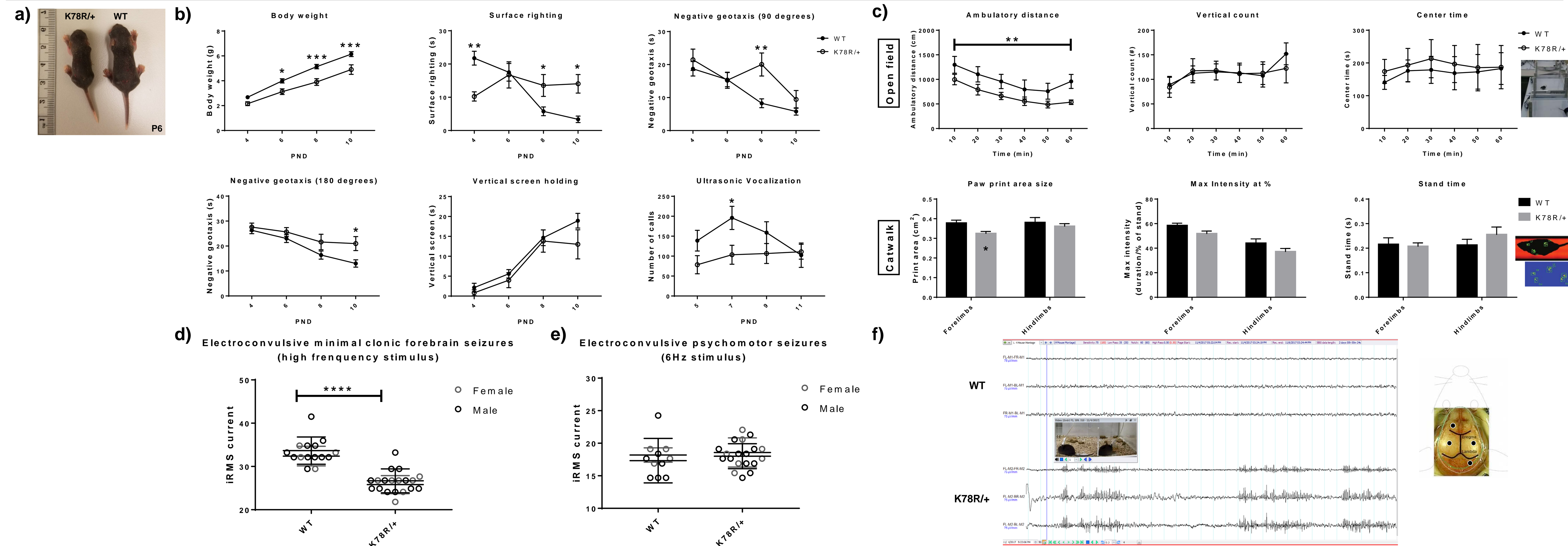


Figure 1. Heterozygous *Gnb1*$^{K78R/+}$ mice present with developmental delay, motor function deficits, susceptibility to generalized seizures and excessive SWDs. a) *Gnb1*$^{K78R/+}$ pups are smaller than WT littermates. **b)** Developmental milestones and USV testing show developmental delay of *Gnb1*$^{K78R/+}$ pups. *Gnb1*$^{K78R/+}$ pups gain weight more slowly, do not show progress in the surface righting reflex test, have delayed progression in the negative geotaxis test, and are very quiet throughout the USV test. **c)** Adult mice show hypoactivity in the open field test, while the catwalk test reveals gait anomalies. The mice seem to walk on their tiptoe for the forelimbs while they drag their hindlimbs. **d)** *Gnb1*$^{K78R/+}$ mice have a very low threshold to electroconvulsive minimal clonic forebrain seizures, compared to WT littermates but **e)** their electroconvulsive threshold in the 6 Hz psychomotor seizure model is unchanged. **f)** Preliminary video-EEG recordings of cortically implanted *Gnb1*$^{K78R/+}$ and WT littermates (N=2 each genotype) suggest that *Gnb1*$^{K78R/+}$ confers a significant number of spike-wave discharges (SWD), ranging in length from 0.5s to 15s and appear in clusters. We have not yet analyzed the relationship between SWD incidence and awareness or diurnal activity. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001 (Two-way ANOVA with multiple comparisons).

Spontaneous activity in *Gnb1*^{K78R} neurons shows aberrant firing pattern

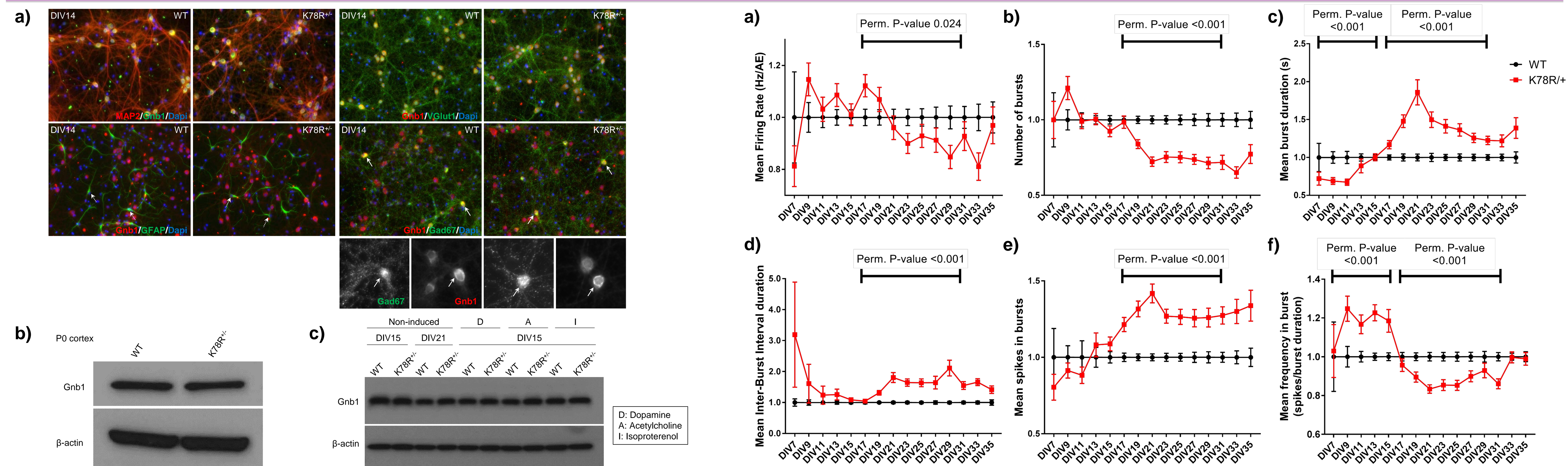


Figure 2. Normal expression and localization of Gβ1 in *Gnb1*$^{K78R/+}$ neurons. a) Immunocytochemistry on cortical primary neurons reveals somatic localization of Gβ1 in neurons (MAP2), both excitatory (VGlut1) and inhibitory (Gad67), but not in astrocytes (GFAP). No staining difference was observed between *Gnb1*$^{K78R/+}$ and WT neurons. Gβ1 is more strongly expressed in Gad67- expressing inhibitory neurons than other neuronal types (bottom right panel). **b)** Western Blot of cortex shows normal Gβ1 protein expression. **c)** Western Blot of primary cortical neurons before or after activation of GPCRs by neurotransmitters for 2 hours shows no difference in Gβ1 expression.

Figure 3. MEA reveals increased burst duration accompanied by decreased number of bursts in *Gnb1*$^{K78R/+}$ neurons. Recordings from WT and *Gnb1*$^{K78R/+}$ cortical neurons reveal a phenotype that emerges after neuronal maturation at DIV15. Data from 10 different litters averaged and normalized to WT. **a)** *Gnb1*$^{K78R/+}$ neurons tend to fire more during the developmental period of the network but slow down during the mature period compared to WT **b)** *Gnb1*$^{K78R/+}$ neurons show decreased number of bursts at maturity **c)** while they show increased mean duration of bursts **d)** and increased inter-burst intervals. **e)** *Gnb1*$^{K78R/+}$ neurons also show increased mean spikes in bursts compared to WT **f)** and decreased mean frequency of spikes in bursts, which correlates with the very long burst duration observed.

Mutations in *GNB1* affect interaction and activation of downstream effectors

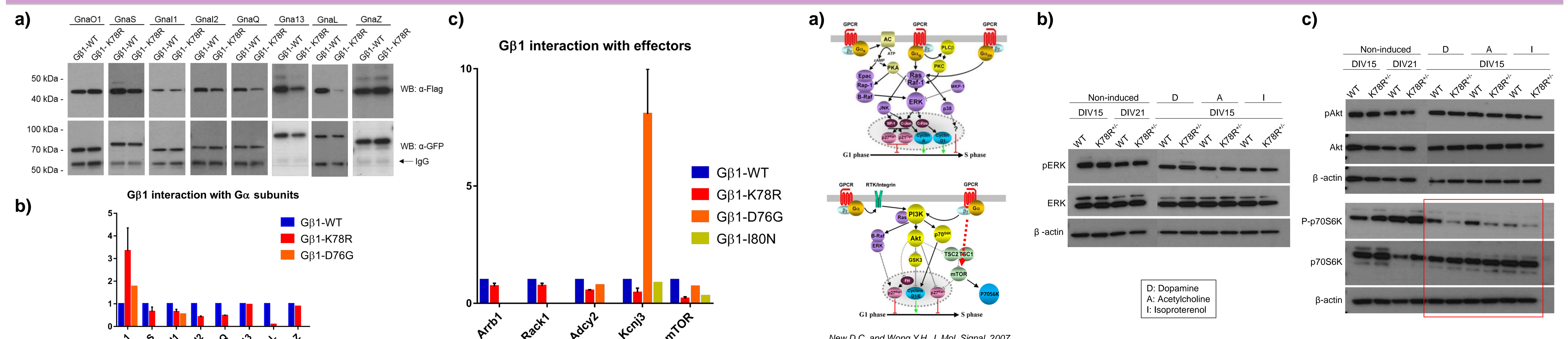
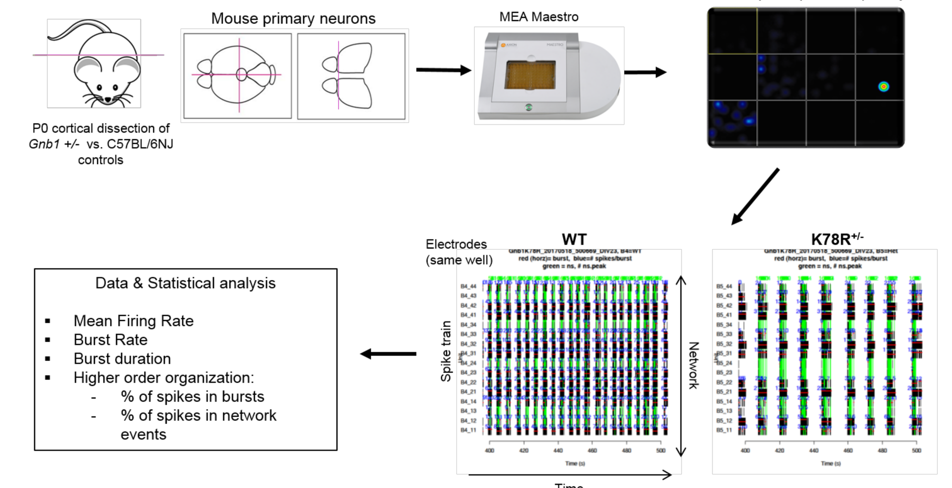


Figure 4. Gβ1 interaction with Gα subunits and downstream effectors is affected by *GNB1* mutations. a) Representative Western Blot of Co-IP between Gβ1-WT or Gβ1-K78R and Gα subunits. **b)** Quantification of the interaction between Gβ1-WT, Gβ1-K78R or Gβ1-D76G and Gα shows increased interaction with Gαo but decreased interaction with Gαs, Gαi and Gαq subunits. **c)** Quantification of the interaction between Gβ1-WT, Gβ1-K78R, Gβ1-D76G or Gβ1-I80N and β-arrestin1, Rack1, Adcy2, Kcnj3 (GIRK1) and mTOR shows variability of the interaction defects depending on the mutation. All mutations tested show decreased interaction with Adcy2 and mTOR.

Figure 5. Intracellular signaling pathway activation is affected by the K78R mutation. a) Schematics of the regulation of MAPK/Erk (top) and Pi3K/Akt/mTOR (bottom) pathways by GPCRs. **b)** Western Blot from primary cortical neurons before or after activation of GPCRs for 2 hours with neurotransmitters shows that activation of the MAPK pathway is not affected by the K78R mutation. **c)** Activation of Pi3K/Akt pathway is not affected while activation of mTOR pathway (p70S6K) is reduced in *Gnb1*$^{K78R/+}$ neurons compared to WT following GPCR activation, suggesting direct regulation of mTOR signaling by *GNB1* (red dotted arrow in **a)**).

Methods

- CRISPR/Cas9 knock-in mouse model was generated for the K78R human variant. The mutation was generated and is maintained and studied on the C57BL/6NJ strain background for results in this presentation, except for Fig.1f (BL/6NJ x FVB F1).
- Neurobehavioral tests:
  - Pups were weighed and tested on even days for developmental milestones and odd days for ultrasonic vocalizations (USV). Developmental milestones tests included surface righting reflex, negative geotaxis (90 and 180 degrees) and vertical screen holding. Pups were allowed 2 trials per tests, and time to success was recorded, up to 30 seconds which corresponded to failure. The mean of the two trials was used for analysis. For USVs pups were separated from the dam, placed in a box with an ultrasound microphone and recorded for 3 minutes (Avisoft Bioacoustics software).
  - CatWalk XT (Noldus Information Technology) is a modern automated test for gait functions and locomotion. It consists of an illuminated walled glass walkway (130 cm x 10 cm) and a high-speed camera underneath. Mice are habituated for three daily sessions before the experiment. Light is reflected and illuminates the stimulus (footprint) when downward pressure is applied. Mice are allowed to traverse the walkway as many times as needed to obtain at least 3 fluent crossings (without stopping or hesitations). Parameters automatically collected by the software include stride length, width, base of support, distance between ipsilateral prints, cadence, support formulas, step sequence, phase lags, and walking speed.
  - The open field is general test for locomotor activity. Each mouse is gently placed in the center of a clear Plexiglass arena (40 x 40 x 40 cm, Med Associates) lit with dim light (~30 lux), and is allowed to ambulate freely for 60 min. Infrared (IR) beams embedded along the X, Y, Z axes of the arena automatically track distance moved, horizontal movement, vertical movement, stereotypies, and time spent in center area.
- Seizure susceptibility was determined using electroconvulsive threshold (ECT) tests, using the Ugo Basile Electroconvulsive Device and transcorneal electrodes, as described in *Frankel, 2001 Genomics (PMID: 11414758).* High frequency ECT settings: 299 Hz, 1.6 ms pulse width, 0.2 s duration, variable current. Low frequency ECT settings: 6 Hz, 0.2 ms pulse width, 3 s shock duration, variable current. Tests were performed approximately daily in individual mice until threshold was reached. Integrated root mean square (iRMS) was calculated from stimulus parameters to describe threshold, and group means were calculated for genetic analysis.
- Spontaneous seizure events detected by video-EEG . Adult mice (6 wks or older) were implanted with electrodes for continuous EEG monitoring. Subdural cortical electrodes were implanted under general anesthesia as indicated in the image below. Signal was detected with a Grael 48 EEG amplifier and acquired on a computer using Profusion 5 software (Compumedics, USA), synchronized to a high resolution video camera.
- Activity analysis of cultured neurons from *Gnb1*^{K78R} mice utilizing Maestro MEA system (Axion BioSystems). Briefly primary cortical neurons from P0 pups were dissociated and plated on 48-well MEA plates, with each well containing 16 electrodes. Neurons were maintained in NBA/B27 media and recorded for 15 minutes every other day. Parameters including firing rate, bursting properties, and network properties were obtained using an in house program.



- Co-immunoprecipitation (Co-IP) assays were performed in HEK293 cells using flag-tagged WT or mutant *GNB1* and GFP-tagged partner proteins transfected following the Lipofectamine 3000 protocol (ThermoFisher). Proteins were pulled down using a rabbit polyclonal anti-GFP antibody that was bound to anti-rabbit magnetic beads (ThermoFisher). Western Blot was performed with anti-Flag and anti-GFP antibodies.

Summary

- Heterozygous *Gnb1*$^{K78R/+}$ mice show > 50% attrition at P0, while homozygous *Gnb1*$^{K78R/K78R}$ mice are embryonic lethal. Heterozygous *Gnb1*$^{K78R/+}$ mice present with developmental delay, motor deficits and very low threshold to minimal electroconvulsive forebrain clonic seizures. Numerous clustered and long-lasting SWDs are readily observed on video-EEG, which could be a sign of absence seizures. Overall, the K78R mouse model shares several aspects of the patient's phenotype.
- Gβ1 expression and localization is not affected by the K78R mutation. Gβ1 is expressed in the somatic region of both glutamatergic and GABAergic neurons, with a stronger expression in GABAergic neurons.
- *Gnb1*$^{K78R/+}$ cortical neurons burst less often than WT neurons, but for significantly longer period of times. The concomitant increase in inter-burst intervals suggests a longer recovery period from the bursts.
- K78R and D76G variants increase Gβ1 interaction with Gαo subunits, but decrease it with other Gα types, suggesting potential deficits in Gαo-associated GPCR signaling. K78R, D76G and I80N variants reduce interaction with Adcy2 and mTOR suggesting that these major pathways may contribute to the phenotype. The K78R variant shows defective interaction with GIRK1, while D76G shows increased interaction. The differential effects of mutations on certain interactions may contribute to the phenotypic variability observed in affected individuals.
- *Gnb1*$^{K78R/+}$ cortical neurons show defective activation of mTOR pathway following GPCR activation, while MAPK/Erk and Pi3K/Akt pathways are not affected. This suggests that Gβ1 can regulate mTOR pathway through its direct interaction with mTOR (as seen in Co-IP assay).