NPRL2 Inhibition of mTORC1 Controls Sodium Channel Expression and Brain Amino Acid Homeostasis

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1 NPRL2 Inhibition of mTORC1 Controls Sodium Channel Expression and Brain Amino Acid Homeostasis.

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28 **Abstract:**

29 Genetic mutations in nitrogen permease regulator-like 2 (NPRL2) are associated with a wide spectrum of
 30 familial focal epilepsies, autism, and sudden unexpected death of epileptics (SUDEP), but the
 31 mechanisms by which NPRL2 contributes to these effects are not well known. NPRL2 is a requisite
 32 subunit of the Gap Activity Toward Rags 1 (GATOR1) complex, which functions as a negative regulator of
 33 mammalian Target Of Rapamycin Complex 1 (mTORC1) kinase when intracellular amino acids are low.
 34 Here we show that loss of NPRL2 expression in mouse excitatory glutamatergic neurons causes seizures
 35 prior to death, consistent with SUDEP in humans with epilepsy. Additionally, the absence of NPRL2
 36 expression increases mTORC1-dependent signal transduction and significantly alters amino acid
 37 homeostasis in the brain. Loss of NPRL2 reduces dendritic branching and increases the strength of
 38 electrically stimulated action potentials in neurons. The increased action potential strength is consistent
 39 with elevated expression of epilepsy-linked, voltage-gated sodium channels in the NPRL2-deficient
 40 brain. Targeted deletion of NPRL2 in primary neurons increases the expression of sodium channel
 41 *Scn1A*, whereas treatment with the pharmacological mTORC1 inhibitor called rapamycin prevents *Scn1A*
 42 upregulation. These studies demonstrate a novel role of NPRL2 and mTORC1 signaling in the regulation
 43 of sodium channels, which can contribute to seizures and early lethality.

44 **Significance Statement:** NPRL2 is a requisite subunit of the epilepsy-linked GATOR1 complex that
 45 functions as a negative regulator of mTORC1 kinase when intracellular amino acids are limited. Here we
 46 report the generation and characterization of a new neurological model of GATOR1-dependent
 47 mTORopathy, caused by the loss of NPRL2 function in glutamatergic neurons. Loss of NPRL2 increases
 48 mTORC1 signal transduction, significantly alters amino acid homeostasis in the brain, and causes SUDEP.
 49 In addition, loss of NPRL2 increases the strength of electrically stimulated action potentials and the
 50 expression of epilepsy-linked sodium channels. These data reveal an unanticipated link between

51 intracellular amino acid signaling by NPRL2 and a novel mTORC1-dependent regulation of sodium-
52 channel expression in epilepsy.

53 **Introduction:**

54 Nitrogen permease regulator-like 2 (NPRL2) is a requisite subunit of Gap Activity Toward Rags 1
55 (GATOR1), an evolutionarily conserved complex that is comprised of three proteins called NPRL2, NPRL3,
56 and DEPDC5 (DEP Domain Containing 5). Recent genomic studies have identified mutations in all
57 subunits of GATOR1 that collectively represent approximately 10% of the epileptic and autistic
58 population (Baldassari et al., 2019; Dibbens et al., 2013; Ricos et al., 2016). Mutations in the DEPDC5
59 have also been suggested to be a causal factor for sudden unexpected death of epileptics (SUDEP) in
60 humans (Nascimento et al., 2015). Individuals with GATOR1 mutations can present with focal cortical
61 dysplasia, with defects in cortical lamination and the presence of dysmorphic neurons (Cen et al., 2017;
62 Kabat and Król, 2012; Scerri et al., 2015; Weckhuysen et al., 2016). These malformations have been
63 found to occur through a two-hit genetic mechanism (Ribierre et al., 2018), confounding the specific
64 GATOR1-dependent mechanisms that contribute to neurological dysfunction.

65 GATOR1 functions as a negative regulator of mTORC1 (mammalian Target Of Rapamycin Complex 1), a
66 dynamic protein kinase that controls cellular growth, protein translation, and metabolic processes in
67 cells (Bar-Peled et al., 2013; Liu and Sabatini, 2020). mTORC1 activity is controlled by distinct upstream
68 regulatory protein complexes comprised of GATOR1 and TSC1/2, which function as GTPase activating
69 proteins toward small GTP-binding proteins on the lysosomal surface called RAGs and Rheb, respectively
70 (Efeyan et al., 2013; Meikle et al., 2007; Sancak et al., 2010). The interaction of mTORC1 with GTP-
71 bound RAG and Rheb contributes to the activation of its kinase function (Laplane and Sabatini, 2009).
72 Mutations in either GATOR1 or TSC1/2 are generally classified as mTORopathies, which present with
73 overlapping neurological hallmarks, including seizures and autism spectrum disorders (Griffith and

74 Wong, 2018; Talos et al., 2012). These similarities suggest an important role for strict mTORC1
75 regulation in the control of brain function (Curatolo et al., 2015; Specchio et al., 2020).

76 When intracellular amino acids are limited, GATOR1 represses mTORC1-dependent signaling and
77 reduces the anabolic consumption of amino acids (Bar-Peled et al., 2013; Bourdeau Julien et al., 2018;
78 Shen et al., 2019). Previous loss-of-function studies have shown that NPRL2 is necessary to maintain
79 intracellular amino acids homeostasis by regulating the expression of genes important for metabolism of
80 amino acids, including glutamine and glutamate (Chen et al., 2017; Dutchak et al., 2018; Dutchak et al.,
81 2015). While intracellular glutamate functions as a building block for general protein synthesis, it can
82 also contribute to cellular energy metabolism through mitochondrial anapleurosis or function as an
83 excitatory neurotransmitter of glutamatergic neurons (Zhou and Danbolt, 2014).

84 Within the brain, glutamine contributes to the synthesis of both excitatory and inhibitory
85 neurotransmitters in glutamatergic or GABAergic neurons, respectively. The metabolism of these
86 neurotransmitters is highly inter-dependent within distinct cell populations, comprising the glutamine-
87 glutamate/GABA cycle to adequately supply neurotransmitters within the central nervous system. This
88 cycle describes the shuttling of glutamine from astrocytes to glutamatergic or GABAergic neurons for
89 neurotransmission, and subsequent re-uptake in astrocytes for glutamine regeneration (Schousboe et
90 al., 2013). Alterations in this cycle are frequently observed in epileptic patients and have been
91 associated with defects in astrocyte glutamine synthesis, or changes in glutaminase and glutamate
92 dehydrogenase (Eid et al., 2016). These metabolic links to epilepsy prompted us to investigate the role
93 of NPRL2 in glutamatergic neurons, which utilize glutamate as an excitatory neurotransmitter. While
94 the contribution of other GATOR1 proteins, namely DEPDC5, have been explored in the GABAergic
95 neurons in zebrafish (Swaminathan et al., 2018), the role of NPRL2 in glutamatergic neurons and its
96 contribution to the mammalian brain has not been investigated.

97 Here we report the generation and characterization of a new neurological model of GATOR1-dependent
 98 mTORopathy, caused by the loss of NPRL2 function in glutamatergic neurons. Loss of NPRL2 is sufficient
 99 to increase mTORC1 signaling despite an intact TSC1/2 complex, significantly alter amino acid
 100 homeostasis in the brain and cause seizures prior to early death. In addition, loss of NPRL2 increases the
 101 strength of electrically stimulated action potentials and the expression of epilepsy-linked sodium
 102 channels (Kaplan et al., 2016; Lakhan et al., 2009). These data reveal an unanticipated link between
 103 intracellular amino acid signaling through GATOR1 and a novel mTORC1-dependent regulation of
 104 sodium channel expression.

105 **Materials and Methods:**

106 **Animal Experiments**

107 NPRL2 nKO mice were generated by crossing *Nprl2*^{loxP/+} mice previously described (Dutchak et al., 2015),
 108 with *Vglut2-ires-Cre* females mice (Vong et al., 2011) to generate glutamatergic neuron-specific
 109 heterozygous animals. The expression of Cre-recombinase in these mice is regulated by an internal
 110 ribosomal entry site that was created using a knock-in strategy targeting the *Vglut2* gene. Heterozygous
 111 animals were backcrossed to C57/B6 for at least 3 generations, with subsequent crosses with *Nprl2*^{loxP/+}
 112 and *Nprl2*^{loxP/loxP} animals, resulting in control mice (*Nprl2*^{loxP/+} and *Nprl2*^{loxP/loxP}), NPRL2 nHET mice
 113 (*Nprl2*^{loxP/+}; *Vglut2-ires-Cre*) and NPRL2 nKO mice (*Nprl2*^{loxP/loxP}; *Vglut2-ires-Cre*) that were used for these
 114 studies. Genotypes of all animals were determined by PCR, using the following primers: *Nprl2* (forward)
 115 5'-CTCAGGTTCTACGCAGTGACTTC-3', *Nprl2* (reverse) 5'-CATGGCGCTGTCTGGATCC-3', *Nprl2* (knockout) 5'-
 116 CAGGCTTCATACTTCTACCCTC-3', *Vglut2* (forward) 5'-AAGAAGGTGCGCAAGACG-3', *Vglut2* (Cre-reverse)
 117 5'-ACACCGGCCTTATTCCAAG-3', *Vglut2* (wt-reverse) 5'-CTGCCACAGATTGCACTTGA-3'. Video recordings
 118 were captured using a Sony FDR-AX53 camcorder, with the mother present in the cage. All animal
 119 experiments were approved by the Université Laval Committee on Ethics and Animal Research.

120 Immunohistochemistry

121 Tissue sections were prepared from P16 animals that were perfused with 4% (wt/vol) formaldehyde and
122 immersion fixed in 4% formaldehyde for 16 hours. Floating sections were immunostained with primary
123 antibodies overnight at 4°C using a 1:500 antibody dilution shown in Table 1. Alexa Fluor-conjugated
124 secondary antibodies (Invitrogen) were incubated for 2 hours at room temperature prior to washing and
125 mounting. Three animals per each genotype were analyzed using confocal microscope.

126 Western Blot Analysis

127 Tissue were homogenized in lysis buffer containing of 150 mM sodium chloride, 50 mM sodium fluoride,
128 100 μ M sodium orthovanadate (pH 10.0), 50 mM sodium pyrophosphate tetrabasic, 10 mM β -
129 glycerophosphate, 5 mM EDTA, 5 mM EGTA, 10 mM HEPES (pH 7.4) and 0.5% Triton X-100
130 supplemented with complete anti-protease cocktail (Roche). Lysates were pelleted by centrifugation.
131 Cleared lysates, representing the soluble fraction, and the insoluble membrane fractions were boiled in
132 1X Laemmli sample buffer and separated on SDS-PAGE for Western blotting using antibodies listed in
133 Table 1.

134 Golgi Staining

135 NPRL2 nKO and littermate controls were used for Golgi staining, using the manufacturer's protocol of
136 the FD Rapid GolgiStain™ kit (FD Neurotechnologies, Inc.). 10 cortical neurons (layers IV-V) from three
137 independent animals per genotype were traced and analyzed using NeuroLucida 360 (MBF Biosciences,
138 Williston, VT). Sholl analysis was performed starting at 10 μ m from the soma with 5 μ m intervals as
139 previously described (Sephton et al., 2014).

140 Slice Preparation for Electrophysiology

141 Mice were first anesthetized with isoflurane. The brain was then quickly dissected and placed in the
 142 cutting chamber filled with an ice-cold NMDG-artificial cerebrospinal fluid (aCSF) solution containing (in
 143 mM): 1.25 NaH₂PO₄, 2.5 KCl, 10 MgCl₂, 20 HEPES, 0.5 CaCl₂, 24 NaHCO₃, 8 D-glucose, 5 L-ascorbate, 3
 144 Na-Pyruvate, 2 Thiourea, 93 NMDG (osmolarity adjusted with sucrose to 300–310 mOsmol/l) and pH
 145 adjusted to 7.4 with HCl 10N. Coronal slices (250 μ m) were then cut with a vibratome (VT2000; Leica) to
 146 obtain complete sections containing lateral habenula. Slices were placed in a 32°C oxygenated NMDG-
 147 aCSF solution for 10 minutes before incubation for one hour at room temperature in HEPES-aCSF
 148 solution (in mM): 1.25 NaH₂PO₄, 2.5 KCl, 10 MgCl₂, 20 HEPES, 0.5 CaCl₂, 24 NaHCO₃, 2.5 D-glucose, 5L-
 149 ascorbate, 1 Na-Pyruvate, 2 Thiourea, 92 NaCl, 20 Sucrose (osmolarity adjusted to 300–310mOsmol/l at
 150 pH 7.4) and finally transferred into a recording chamber on the stage of an upright microscope (Zeiss)
 151 where it was perfused at a rate of 3–4 ml/min with artificial cerebrospinal solution (aCSF in mM): 120
 152 NaCl, 5 HEPES, 2.5 KCl, 1.2 NaH₂PO₄, 2 MgCl₂, 2 CaCl₂, 2.5 glucose, 24 NaHCO₃, 7.5 sucrose. The aCSF in
 153 the perfusion chamber was kept at 32°C. All solutions were aerated with 95% O₂ and 5% CO₂. (Ting et
 154 al,2018).

155 **Whole cell patch clamp recordings**

156 A water immersion x60 objective and a video camera (Zeiss, Germany) were used to visualize neurons in
 157 LHb. Whole-cell patch clamp recordings were performed under current clamp with an Axopatch 200B
 158 amplifier (Molecular Devices, San Jose, CA) using borosilicate patch electrodes (3–7 M Ω resistance).
 159 Pipettes were filled with an intracellular patch solution containing (in mmol/l): 130 K-Gluconate, 5 KCl,
 160 10 HEPES, 2.5 MgCl₂, 4 Na₂-ATP, 0.4 Na₃-GTP, 10 Na-phosphocreatine, 0.6 EGTA and 0.2% Biocytin (pH
 161 7.35). Signals were filtered at 5 kHz using a Digidata 1500A data acquisition interface (Molecular
 162 Devices, San Jose, CA) and acquired using pClamp 10.6 software (Molecular Devices, San Jose, CA).
 163 Pipette and cell capacitances were fully compensated. The pH and osmolarity were adjusted to 7.3 and
 164 285–290 mOsm/L, respectively. Data were analyzed offline using clampfit 10.6 software. Neurons were

165 selected randomly from the LHb of prepared slices. 1-2 minutes after obtaining whole cell configuration,
166 the resting membrane potential (RMP) was recorded in current clamp mode right after whole cell
167 configuration had been obtained. Rheobase current was assessed by applying depolarizing current steps
168 (from -20 to 100 pA, 5 pA increments and 1 s duration) and it was calculated as the minimum current
169 needed to elicit the action potential. To examine evoked firing properties, depolarizing current steps (-
170 20 to +100 pA, 20 pA increments and 300 ms duration) were applied to the cells. Action potentials (APs)
171 generated during this period were counted and we obtained the number of spikes and frequency of
172 firing.

173 **Metabolite Analysis**

174 The cortex of NPRL2 nKO and littermate controls were harvested from living P16 animals showing no
175 physical signs of stress, by cervical dislocation, rapid dissection, and snap freezing in liquid nitrogen.
176 Samples were stored at -80°C until processing. One hemisphere was subjected to metabolite extraction
177 using the Absolute/DQ® p180 kit (Biocrates Life Sciences), using the manufacturer's protocol. Samples
178 were analyzed on a SCIEX 5500 QTRAP by The Analytical Facility for Bioactive Molecules, The Hospital for
179 Sick Children, Toronto, Canada.

180 **Primary Neuron Cultures**

181 Primary cortical neurons were prepared from P0-P1 *Nprl2^{fl/fl}* mice using previously described methods
182 (Nault and De Koninck, 2010). Dissociated neurons were grown in neurobasal medium supplemented
183 with B27 (Thermo fisher), L-Glutamine (Fisher Scientific) and penicillin/streptomycin (Thermofisher). On
184 DIV4 cultures were treated with 5µM AraC (Sigma) and either control or CRE expressing lentivirus. On
185 DIV7 cells were treated with 10nM rapamycin or DMSO (vehicle) for 24 hours and harvested in TRIzol
186 (Thermofisher) for RNA isolation.

187 **Quantitative RT-PCR Analysis**

188 Total RNA from tissues or cells was extracted using TRIzol® (Invitrogen), following the manufacturers
 189 protocols. Primer were selected to span exon-exon junctions where possible. RNA extracts were
 190 treated with DNase (Roche) and the High Cap cDNA Reverse Transcription kit (Applied Biosystems) was
 191 used for cDNA synthesis. Quantitative RT-PCR reactions contained 25 ng cDNA, 150 nM of each primer
 192 pair, and 5 uL SYBR GreenER (Invitrogen). All reactions were performed in triplicate on the
 193 QuantStudio™ 5 Real-Time PCR system (Applied Biosystems). Relative mRNA levels were calculated
 194 using the comparative threshold cycle method using U36B4 as the internal control. qRT-PCR primer
 195 sequences used in this study are listed in Table 2.

196 **Statistical Analysis**

197 Statistical analysis was performed by two-tailed Student's t test using Microsoft Excel 2016. A p-value of
 198 < 0.05 was considered significant.

199 **Results:**

200 **Loss of NPRL2 in Glutamatergic Neurons Causes Seizures and Early Lethality**

201 To determine the function of NPRL2 in glutamatergic neurons, we crossed our genetically engineered
 202 *Nprl2*-floxed mice with *Vglut2-ires-Cre* females mice to generate *Nprl2^{loxP/loxP}; Vglut2-ires-Cre^{+/WT}* (NPRL2
 203 nKO) mice (Dutchak et al., 2015; Vong et al., 2011). We selected this Cre-driver because it is widely
 204 expressed early in developing glutamatergic neurons, permitting us to investigate early onset epileptic
 205 phenotypes. To verify NPRL2 deletion, we performed western blot analysis targeting NPRL2 and other
 206 protein subunits of GATOR1, NPRL3 and DEPDC5, in cerebral protein extracts from WT and NPRL2 nKO
 207 animals. NPRL2 expression was reduced by 50% in the NPRL2 nKO compared to control, indicating a
 208 targeted knockout of NPRL2 in the VGLUT2 neuron population (Fig. 1A, 1B). Consistent with previous
 209 observations, loss of NPRL2 did not alter NPRL3 or DEPDC5 expression in the NPRL2 nKO (Dutchak et al.,
 210 2015). Animals were born at the expected Mendelian ratios; however, NPRL2 nKO mice did not live

beyond 20 days after birth (Fig. 1C). We measured the body mass of NPRL2 nKO mice at P16 and found a small but significant reduction in both males and females compared to littermate controls (Fig. 1D). To investigate the cause of death, we performed 24-hour video recordings of control and NPRL2 nKO littermates, with their mother present. Control mice were unremarkable, whereas all NPRL2 nKO mice began a series of seizures approximately 3 hours before the start of the 12-hour light cycle on the day of their death. Over the next 2 to 4 hours, both tonic and tonic-clonic seizures were observed, lasting 1.6 ± 0.6 minutes and 5.6 ± 1.4 minutes, respectively (Extended Data Fig. 1-1). NPRL2 nKO pups died following a tonic-clonic seizure lasting more than 5 minutes. These observations show that NPRL2 function in glutamatergic neurons is essential for viability, despite the presence of an intact TSC1/2 complex.

NPRL2 contributes to dendritic branching and soma size of neurons

Previous studies have shown that deletion of the GATOR1 subunit called DEPDC5, using a pan-neuronal Cre driver, reduces dendritic branching of neurons (Yuskaitis et al., 2018). To determine if the loss of NPRL2 alters dendritic branching in our model, we performed Golgi staining and Sholl analysis of layer IV-V cortical neurons in the NPRL2 nKO and littermate controls at P16 (Fig. 2A). We observed a significant decrease in the number of intersections and cumulative area of NPRL2 nKO neurons, compared to control (Fig. 2B, 2C). We also performed 3D reconstruction of the cell bodies to determine if the loss of NPRL2 increased the soma size, consistent with established pro-growth effects of mTORC1. We observed the soma of NPRL2 nKO neurons were 1.5-fold larger compared to control (Fig. 2D). No gross morphological differences were observed in the NPRL2 nKO brain compared to controls at P16 (*data not shown*). These data support NPRL2 as a critical regulator of dendritic branching and neuron size *in vivo*.

Loss of NPRL2 alters intrinsic electrophysiological properties in neurons

234 Mutations in NPRL2 are associated with familial forms of epilepsy (Baldassari et al., 2019; Ricos et al.,
 235 2016; Weckhuysen et al., 2016). To determine if the loss of NPRL2 alters the intrinsic passive and active
 236 neuronal electrophysiological properties, we prepared acute brain slices encompassing the lateral
 237 habenula (LHb), which contain exclusively VGLUT2-positive neurons, from NPRL2 nKO and control
 238 littermates. To determine neuronal firing properties, evoked firing activity was evaluated by injecting
 239 depolarizing current steps. We observed no significant difference in the number of action potentials
 240 (AP) (Fig. 3A, 3B), or instantaneous firing frequency (Fig. 3C) elicited by progressive depolarizing current
 241 steps. However, the electrophysiological properties of the first AP evoked by each depolarizing step
 242 showed significantly greater AP amplitude (Fig. 3D, 3E), and reduced rise time (Fig. 3F) in the NPRL2 nKO
 243 neurons compared to littermate controls. No significant difference was observed in the first AP decay
 244 slope, resting membrane potential, or rheobase current (Fig. 3G, 3H, 3I). Consistent with the larger size
 245 of the NPRL2 nKO neurons (Fig. 2D), a significant increase in capacitance was observed in these cells
 246 compared to control (Fig. 3J). These data suggest that loss of NPRL2 increases sodium-dependent
 247 depolarization step of the AP.

248 **NPRL2 controls mTORC1 signal transduction and neurometabolic homeostasis**

249 NPRL2 is an essential protein subunit of the GATOR1 complex, which functions as a negative regulator of
 250 mTORC1 when intracellular amino acids are limited. To determine if NPRL2 is necessary to repress
 251 mTORC1 activity our model, we performed immunohistochemistry and confocal microscopy on sections
 252 from NPRL2 nKO and control animals at P16, stained with phosphorylated ribosomal protein S6 (P-S6), a
 253 downstream marker of mTORC1 activity, and VGLUT2 (Fig. 4A, 4B, Extended Data Fig. 4-1). Increased
 254 levels of P-S6 in VGLUT2-positive brain regions were observed in NPRL2 nKO compared to control,
 255 suggesting a failure to repress mTORC1 signaling. We also co-stained sections with NeuN, GFAP and P-
 256 S6 to determine if the effect on P-S6 was cell autonomous. We observed no increase in P-S6 staining in
 257 cells co-stained with GFAP in the NPRL2 nKO compared to control, consistent with a cell autonomous

258 effect (Extended Data Fig. 4-2). Western blot analysis of protein extracts showed a significant increase
 259 in P-S6 abundance in the NPRL2 nKO compared to WT (Fig. 4C, Extended Data Fig. 4-1), consistent with
 260 histological observations. We also observed a trend of increased phosphorylation of S6-kinase, and a
 261 small reduction in total AKT and phosphorylated AKT (S473) in the NPRL2 nKO. These data suggest that
 262 the regulation of mTORC1 through NPRL2 is necessary to prevent hyperactive mTORC1 signaling in
 263 glutamatergic neurons despite an intact TSC1/2 complex.

264 mTORC1 activity is implicated in diverse cellular processes that impact the regulation of metabolic
 265 homeostasis within cells. To determine if the loss of NPRL2 in glutamatergic neurons contributes to
 266 metabolic defects in the brain, metabolites were extracted and quantified using targeted small molecule
 267 mass spectrometry. NPRL2 nKO extracts showed a significant reduction in the abundance of all amino
 268 acids except glutamate, aspartate and leucine, compared to littermate controls (Fig. 5A). Remarkably,
 269 the abundance of glutamine was 60% lower in the NPRL2 nKO brain extracts, compared to littermate
 270 controls. We also observed a significant decrease in dopamine, but no change in serotonin, histamine,
 271 creatinine or taurine (Fig. 5B). The abundance of carnosine was significantly reduced, while putrescine
 272 was significantly increased in the NPRL2 nKO brain (Fig. 5B). Since increased levels of putrescine are
 273 associated with cell death, we measured the expression of genes that are transcriptionally induced by
 274 neuronal cell death, including *Bax*, *Dapk2*, *Dapk3*, and *Pcna*, but found no significant change at P16
 275 (Extended Data Fig. 5-1) (Chiang et al., 2001; Takao et al., 2006). No significant differences were
 276 observed in the abundance of measured acylcarnitines, sphingolipids, lysophosphatidylcholines
 277 (Extended Data Fig. 5-2), nor phosphatidylcholines between NPRL2 nKO and control extracts.
 278 Collectively, these data show an important function of NPRL2 in regulating amino acid homeostasis in
 279 brain.

280 **NPRL2-dependent mTORC1 activity controls sodium channel expression**

281 The electrophysiological characteristics of the NPRL2 nKO neurons suggested a dysregulation of sodium
 282 ion channel expression could contribute to the increased amplitude of the action potential. We used
 283 quantitative RT-PCR to measure the expression of sodium channels in the brains of P16 NPRL2 nKO and
 284 littermate controls. We observed a significant upregulation of *Scn1A*, *Scn1B*, *Scn2A* and *Scn2B* in the
 285 NPRL2 nKO compared to control, but no change in *Scn3A*, *Scn3B*, *Scn4B* or *Scn5A* (Fig. 6A). Expression of
 286 potassium channels: Kv1.1, Kv1.3, Kv1.4 and Kv2.2, were not significantly changed; whereas Kv 1.6 was
 287 induced 1.2-fold (Extended Data Fig. 6-1). We next performed western blot analysis on membrane
 288 fractions and observed a significant increase in SCN1A protein expression in the NPRL2 nKO, compared
 289 to internal control (Fig. 6B).

290 We next hypothesized that mTORC1 activation contributes to the expression of sodium channels in
 291 neurons. We generated primary neuron cultures from *Nprl2*^{loxP/loxP} animals and infected them with
 292 either Cre-expressing lentivirus or negative control virus. Neurons were treated at DIV 7 with
 293 rapamycin, an inhibitor of mTORC1 kinase activity, and quantitative RT-PCR was used to measure the
 294 expression of *Nprl2* and *Scn1A*. Treatment of *Nprl2*^{loxP/loxP} neurons with Cre-expressing lentivirus
 295 produced a >90% reduction of *Nprl2* and a significant up-regulation of *Scn1A*, compared to control
 296 infected neurons (Fig. 6C). Moreover, rapamycin treatment blocked the upregulation of *Scn1A* in NPRL2
 297 knockout neurons (Fig. 6C). These observations support an important role of NPRL2 and mTORC1
 298 activity in the regulation of *Scn1A* in neurons.

299 **Discussion:**

300 Human mutations in NPRL2 are associated with a spectrum of neurological disorders, including autism,
 301 epilepsy, and SUDEP. Here we show that loss of NPRL2 in glutamatergic neurons causes early lethality
 302 following seizures, increases the strength of electrically stimulated action potentials, and significantly
 303 alters amino acid homeostasis in the brain. Remarkably, the onset of seizures in the NPRL2 nKO mice

304 occurred shortly before the start of the light-cycle, consistent with observations of GATOR1-dependent
 305 nocturnal seizures in humans (Korenke et al., 2016; Picard et al., 2014). These circadian differences
 306 between human and mouse observations could be due to their diurnal versus nocturnal nature. While
 307 human genomic sequencing studies have identified a greater numbers of disease-linked mutations in
 308 the larger GATOR1 subunit called DEPDC5 (Baldassari et al., 2019) compared to NPRL2, our data show
 309 that strict loss-of-function mutations in NPRL2 cause early lethality and may be under-represented as a
 310 genetic risk factor for SUDEP. As previous studies have showed global deletion of NPRL2 results in
 311 embryonic lethality and defective fetal liver hematopoiesis (Dutchak et al., 2015), the mechanisms that
 312 underlie epilepsy-linked NPRL2 somatic mutations may elicit distinct changes in the GATOR1 structure
 313 that impair cell-type specific mTORC1 regulatory functions. Collectively, these data support genetic
 314 screening of GATOR1 mutations during risk assessments for SUDEP.

315 GATOR1 functions as an evolutionarily conserved intracellular amino acid-signaling complex that has a
 316 critical function to maintain intracellular amino acid homeostasis (Bar-Peled et al., 2013; Chen et al.,
 317 2017; Dutchak et al., 2018; Dutchak et al., 2015). Previous studies have shown that defects in NPRL2
 318 cause significant changes in cellular glutamine and glutamate homeostasis, and alter the expression of
 319 metabolic enzymes that control these reactions (Dutchak et al., 2018). Here, we show that deletion of
 320 NPRL2 from glutamatergic neurons causes a 60% reduction in brain glutamine levels, but an insignificant
 321 decrease in glutamate levels. These data suggest that the absence of NPRL2 in glutamatergic neurons,
 322 the high demand of glutamate to support neuronal functions, including synaptic transmission and
 323 protein translation, can be met by increased metabolic supply of glutamine from astrocytes (Schousboe
 324 et al., 2014) or potential rewiring of secondary metabolic pathways (Chen et al., 2017). Since the
 325 glutamate-glutamine cycle relies on glutamine from astrocytes to support neural populations and
 326 prevent neurotransmitter depletion, these data suggest that the ability of astrocytes to regenerate
 327 glutamine is insufficient when NPRL2 is impaired in neurons (Eid et al., 2016; Schousboe et al., 2013).

328 Subsequently, these metabolic deficiencies may contribute to the changes in excitatory glutamate
 329 signaling associated with seizures (Eid et al., 2016; Schousboe et al., 2014), or by substrate deficiencies
 330 in the inhibitory GABAergic system (Swaminathan et al., 2018).

331 We also identified other amino acids deficiencies, including arginine, which may represent homeostatic
 332 mechanism to downregulate mTORC1 activity in the brain (Chantranupong et al., 2016). We did not
 333 detect any change in creatinine or taurine in the NPRL2 nKO, indicating these secondary pathways of
 334 energy metabolism are not affected. However, the increase in putrescine indicates a neurodegenerative
 335 metabolic process may be stimulated prior to death, whereas no changes in genetic markers of
 336 apoptosis were observed in living mice at P16 (Chiang et al., 2001; Takao et al., 2006). No changes in
 337 brain acylcarnitines, sphingolipids, lysophosphatidylcholines or phosphatidylcholines were observed.
 338 Collectively, these data support NPRL2 as a specific regulator of amino acids and their metabolic
 339 pathways in the brain.

340 Early studies showed patients with activating mutations in mTORC1 signaling presented with focal
 341 cortical dysplasia and neurodevelopmental cortical malformations (Lim et al., 2015; Mirzaa et al., 2016),
 342 with recent evidence supporting a novel two-hit mosaic mutation mechanism as the etiology of the focal
 343 cortical dysplasia (Ribierre et al., 2018). While no gross morphological differences were apparent in the
 344 NPRL2 nKO brain, layer IV-V cortical neurons of the NPRL2 nKO mice had larger cell bodies, with reduced
 345 dendritic branching, similar to previous reports when DEPDC5 is deleted using a pan-neuronal Cre-driver
 346 (Yuskaitis et al., 2018). These data support a critical role for strict mTORC1 regulation by the GATOR1
 347 amino acid signaling pathway in early neuronal development.

348 Our data show that loss of NPRL2 in glutamatergic neurons is sufficient to increase mTORC1 signal
 349 transduction, despite in an intact TSC1/2 complex (Bourdeau Julien et al., 2018). Previous studies have
 350 shown that mTORC1 is localized to the axons and dendrites of neurons (Takei et al., 2004; Terenzio et

351 al., 2018), but its biological impact has not been clearly defined. Our data support an important link
352 between hyperactive mTORC1 and changes in the electrical activity of the neurons by affecting the
353 expression of sodium channels. Our electrophysiological analysis shows that loss of NPRL2 is sufficient
354 to increase the strength of evoked APs, with a significantly greater amplitude and faster rise time during
355 the depolarization current step. Consistent with these changes in electrophysiological parameters, we
356 observed a significant upregulation of *Scn1a*, *Scn1b*, *Scn2a*, and *Scn2b* in the brain of the NPRL2 nKO
357 mice. Our loss-of-function studies in primary neuronal cultures also show consistent up-regulation of
358 *Scn1a* expression when NPRL2 is deleted, consistent with a cell autonomous effect. Moreover, the
359 upregulation of *Scn1a* expression is prevented by treatment with rapamycin, the pharmacological
360 inhibitor of mTORC1. As genetic studies have identified more than 700 mutations in genes that code for
361 voltage-gated sodium channels in epilepsy (Kaplan et al., 2016), and reports of GATOR1 and *Scn1a*
362 mutations contributing to temporal epilepsy (Baldassari et al., 2016), our data now implicate the NPRL2
363 and mTORC1 regulatory pathways in controlling *Scn1a* expression.

364 Multiple mutations in several voltage-gated sodium channels have been linked to human epilepsy
365 (Menezes et al., 2020). For example, the gain-of-function T226M mutation in *Scn1a* has been found in
366 children with developmental and epileptic encephalopathy (Berecki et al., 2019) and gain-of-function
367 mutations in *Scn2a* have been shown to elicit seizures, behavioral arrest, and behavioral abnormalities
368 in mice (Kearney et al., 2001). The phenotype of our NPRL2 nKO model is consistent with the
369 upregulation of *Scn1A* causing a gain-of-function effect. In contrast, loss-of-function *Scn1A* mutations
370 are associated with genetic epilepsy with febrile seizures plus (GEFS+) and Dravet syndrome, consistent
371 with decreased activity of inhibitory GABAergic neurons (Escayg and Goldin, 2010; Ruffolo et al., 2018).
372 Future research investigating the contribution of voltage-gated sodium channels in distinct neuronal
373 sub-types and brain regions will be of interest for targeted pharmacological therapies.

374 Genomic studies have shown that 8% of patients carrying DEPDC5 mutations present with depression
375 (Baldassari et al., 2019). Our metabolite analysis of the NPRL2 nKO brain show significantly lower levels
376 of the neurotransmitter dopamine, which is linked to depression (Dunlop and Nemeroff, 2007).
377 Intriguingly, animal studies have shown that dopamine levels are decreased in rat models expressing
378 epilepsy linked *Scn1a*-mutations (Ohmori et al., 2014), providing a functional link between the
379 overexpression of *Scn1A* and reduced dopamine levels in the *Nprl2* nKO model. Collectively, these
380 observations suggest a novel mechanism linking mTORC1 and the expression of sodium channels and
381 the mesolimbic dopamine reward pathway.

382 In summary, we have shown that the NPRL2 expression in glutamatergic neurons is essential to prevent
383 seizures prior to death at P20, and contributes to the regulation of amino acid homeostasis and sodium
384 channel expression in the brain. While the impact of altered amino acid metabolism in the brain
385 remains under investigation, our findings represent a novel link between cellular metabolic regulation
386 and neuronal activity. We conclude that NPRL2 functions as an important regulator of mTORC1 activity
387 that controls the expression of epilepsy-linked *Scn1a* expression to regulate the strength of neuronal
388 action potentials.

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- 520

521 **Figure Legends:**

522 Figure 1. Loss of NPRL2 expression in glutamatergic neurons causes early lethality. A) Western blot
523 analysis of NPRL2, NPRL3, DEPDC5 and GAPDH from brain protein extracts (n=3 per group). B)
524 Quantification of Western blot signal intensities relative to GAPDH (n=6 per genotype). Error bars
525 represent the mean $\pm$ SD. C) Survival curve of NPRL2 nKO and wild-type littermate controls (n=12/40,
526 respectively). All NPRL2 nKO animals die 20 days after birth. D) Body mass of NPRL2 nKO and littermate
527 controls (male n=7/16 and female n=7/6, respectively). (Extended Data Fig. 1-1). Error bars represent
528 the mean $\pm$ SEM. *a*, $p<0.05$; *c*, $p<0.001$.

529 Figure 2. NPRL2 regulates dendritic branching of cortical neurons. A) Representative images of
530 Neurolucida tracing of the dendrites of layer IV-V cortical neurons in NPRL2 nKO and littermate controls
531 (n=30 neurons/3 mice per genotype). B) Sholl analysis show a reduced number of intersections, and C)
532 reduced cumulative area of dendrites in the NPRL2 nKO compared to littermate controls at P16. D)
533 Soma volume of NPRL2 nKO versus littermate controls. Scale bar = 50 μ m. *a*, $p<0.05$; *c*, $p<0.001$.

534 Figure 3. Electrophysiological properties of NPRL2 nKO neurons. A) Representative tracings of the
535 response samples of control (black) or NPRL2 nKO (red) neurons and protocol of injected depolarization
536 steps. B) The number of action potentials elicited by different current steps and their C) frequency. D)
537 Example trace of the first action potential from control (black) or NPRL2 nKO (red). First action potential
538 E) amplitude, F) rise time and G) decay slope evoked by different depolarizing steps. H) Resting
539 membrane potential, I) rheobase current, and J) capacitance measurements from NPRL2 nKO and
540 controls. NPRL2 nKO=23 neurons/3 mice; Control=25 neurons/3 mice. *a*, $p<0.05$; *b*, $p<0.01$.

541 Figure 4. Loss of NPRL2 increases mTORC1 signaling. Representative image of immunohistochemical
542 staining of P-S6 (Ser240/244) and VGLUT2 in the A) hippocampus and B) cortex of NPRL2 nKO and

control at P16. C) Western blot analysis of pS6, S6, pS6K1, S6K1, pAKT, AKT and GAPDH from soluble protein extracts (n=3/3). (Extended Data Fig. 4-1, 4-2)

Figure 5. Metabolite analysis of P16 brain extracts. Relative abundance of A) amino acids, and B) biogenic amines were measured from NPRL2 nKO (white bars) and littermate (black bars) controls using LC-MS/MS. (n=7/10, respectively). Error bars represent the mean $\pm$ SEM. (Extended Data Fig. 5-1, 5-2) *a*, $p<0.05$; *b*, $p<0.01$; *c*, $p<0.001$.

Figure 6. NPRL2 contributes to the regulation of sodium channel expression. A) Quantitative RT-PCR analysis of sodium voltage-gated channel alpha subunits (*Scn1A*, *Scn2A*, *Scn3A*, and *Scn5A*) and sodium voltage-gated channel beta subunits (*Scn1B*, *Scn2B*, *Scn3B* and *Scn4B*) in control and NPRL2 nKO brain at P16. (n=5/4, respectively) B) Western blot analysis of SCN1A and metabotropic glutamate receptor 1 (mGluR1) from membrane fractions of P16 WT and NPRL2 nKO brain (n=3/3). C) Quantitative RT-PCR analysis of *Nprl2* and *Scn1A* from *Nprl2*^{loxP/loxP} primary neurons infected with control or CRE-expressing lentivirus and treated with 10 nM rapamycin, or DMSO control, for 24 hours (n=6/6; 2-independent experiments). (Extended Data Fig. 6-1) *a*, $p<0.05$; *b*, $p<0.01$; *c*, $p<0.001$.

Extended Data Figure Legends:

Extended Data Figure 1-1. Seizure analysis of NPRL2 nKO mice. Quantification of the tonic and clonic seizures of the NPRL2 nKO mice before their death.

Extended Data Figure 4-1. Quantification of A) P-S6 signal intensity from cortical immunohistochemistry staining and B) relative signaling intensity of P-S6:S6 of western blot analysis.

Extended Data Figure 4-2. Representative image of immunohistochemical staining of P-S6, NeuN, and GFAP in the cortex of A) control or B) NPRL2 nKO at P16 (n=3 per genotype). Scale bar is 25 μ m.

564 Extended Data Figure 5-1. Quantitative gene expression of *Bax*, *Dapk2*, *Dapk3*, and *Pcna*, in control of
565 NPRL2 nKO brain. (n=5/4 per genotype, respectively).

566 Extended Data Figure 5-2. Relative abundance of A) acylcarnitines, B) sphingolipids, and C)
567 lysophosphatidylcholines. (n=7/10, respectively). Error bars represent the mean $\pm$ SEM.

568 Extended Data Figure 6-1. Regulation of potassium channel expression. A) Quantitative RT-PCR analysis
569 of potassium channels (*Kv1.1*, *Kv1.3*, *Kv1.4*, *Kv1.6*, *Kv2.2*) in control and NPRL2 nKO brain at P16. (n=5/4,
570 respectively). *b*, $p<0.01$

571

Protein Target	Vendor	Catalog #
AKT	Cell Signaling Technology	9272
DEPDC5	Santa Cruz Biotechnology, Inc.	sc-86115
GAPDH	Sigma	G9295
GFAP	Millipore	MAB5541
mGluR1	Fisher Scientific	12370
NeuN	Millipore	AB5541
NPRL2	Santa Cruz Biotechnology, Inc.	sc-376986
NPRL3	Abcam	ab121346
p70 S6 Kinase	Cell Signaling Technology	9202
Phospho-p70 S6 Kinase (T389)	Cell Signaling Technology	9205
Phospho-AKT (Ser473)	Cell Signaling Technology	9271
Phospho-rpS6 (Ser240/244)	Cell Signaling Technology	2215
rpS6	Cell Signaling Technology	2217
Scn1A	Cell Signaling Technology	18339
VGLUT2	Synaptic Systems	135404

Table 1. List of antibodies used in this study.

Gene	Forward	Reverse
<i>Bax</i>	GTCTCCGGCGAATTGGA	TCCACGTCAGCAATCATCCT
<i>Dapk2</i>	GCAGGGAGCAGAGCAGAG	CCTGGACCATAACAATCGGCG
<i>Dapk3</i>	CGTAGATGCCGTCTCAGGAT	TGGGGACACAGGAGCGTC
<i>Kv1.1</i>	CCCGCCAGCGTCCATC	CTGCAGCCCTTCTAGGACAC
<i>Kv1.3</i>	CTTCTGGTGCGGTTCTTTGC	TACCTTGTCGTTCAAGCAGC
<i>Kv1.4</i>	GGCTTAACAAGTGATCGCTGC	CCAAGGTAGACCCCGGAGAT
<i>Kv1.6</i>	GACTGCCAATTTCTGCTTGGG	AGTTATTCAGTGGGGAGGCG
<i>Kv2.2</i>	AACGTAGGGGGCCTTAACCA	TTGTAGTCATCGCACACCTCC
<i>Pcna</i>	CGAAGGCTTCGACACATACC	GGACATGCTGGTGAGGTTCA
<i>Scn1A</i>	CTGGCTGGACTTCACTGTCAT	GATGGTCTTCAGGCCTGGAA
<i>Scn1B</i>	AACACCAGCGTCGTCAAGAA	CCATCTCTGCCACAAGCCAT
<i>Scn2A</i>	ACCTTTGCGTATGTAACAGAATTT	ACAGCTTCTTCACCGACTGG
<i>Scn2B</i>	CGCCTAACGTCACAGTCTCT	GGACACTAAGAGTGGTGGGC
<i>Scn3A</i>	GGACGTGGGGTCTGAGAATG	GTGGTACCGTTACTGTTGCG
<i>Scn3B</i>	GCTCAGGAAAATGCGTCTGACTAC	TGCCTGTACATCACCTCAAGT
<i>Scn4B</i>	GTGGACAACACGGTGACTCT	CACTCCTTCTTCTCTCGGG
<i>Scn5A</i>	AAGCTAGGCAATTTGTCGGC	GTCTTCAGGCCTGGAATAACTG
<i>U36B4</i>	CGTCCTCGTTGGAGTGACA	CGGTGCGTCAGGGATTG

Table 2. List of all qRT-PCR primers used in this study.