bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Nuclear Respiratory Factor 1 (NRF-1) Controls the Activity Dependent Transcription of the GABA-A Receptor Beta 1 Subunit Gene in Neurons

Zhuting Li[*1,2], Meaghan Cogswell[*1], Kathryn Hixson[1], Amy R. Brooks-Kayal[3], and Shelley J. Russek[#1,4]

[1]Laboratory of Translational Epilepsy, Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, MA 02118

[2]Department of Biomedical Engineering, College of Engineering, Boston, MA 02215

[3]Department of Pediatrics, Division of Neurology, University of Colorado School of Medicine, Aurora, CO, 80045 USA; Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, Aurora, CO 80045

[4]Department of Biology, Boston, MA 02215

[#]To whom correspondence should be addressed: Shelley J. Russek, Departments of Pharmacology and Biology, Boston University School of Medicine, 72 East Concord St., Boston, MA, 02118, USA. Tel.: (617) 638-4319, E-mail: srussek@bu.edu

[*] The work of these individuals was equally important to the reported findings.

Running Title: NRF-1 regulates *GABRB1* transcription in neurons

Keywords: GABA-A receptor; *GABRB1*; NRF-1; Cortical Neurons

Funding: This work was supported by grants from the National Institutes of Health [NIH/NINDS R01 NS4236301 to SJR and ABK, T32 GM00854 to ZL, MC, and KH].

Abbreviations: ChIP, Chromatin-immunoprecipitation; DIV, days *in vitro*; EMSA, Electrophoretic mobility shift assay; GABAR, GABA type A receptors; *GABRB1*, GABA receptor subtype A β1 subunit gene; HRP, horseradish peroxidase; Inr, Initiator element; NRF-1, Nuclear Respiratory Factor 1; PhF1, Polycomb-like protein; TSS, Transcriptional start site; VP16, herpes simplex virus virion protein 16.

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

34 **ABSTRACT**

35 While the exact role of β1 subunit-containing GABA-A receptors (GABARs) in brain function is
36 not well understood, altered expression of the β1 subunit gene (*GABRB1*) is associated with neurological
37 and neuropsychiatric disorders. In particular, down-regulation of β1 subunit levels is observed in brains of
38 patients with epilepsy, autism, bipolar disorder, and schizophrenia. A pathophysiological feature of these
39 disease states is imbalance in energy metabolism and mitochondrial dysfunction. The transcription factor,
40 nuclear respiratory factor 1 (NRF-1), has been shown to be a key mediator of genes involved in oxidative
41 phosphorylation and mitochondrial biogenesis. Using a variety of molecular approaches (including
42 mobility shift, promoter/reporter assays, and overexpression of dominant negative NRF-1), we now report
43 that NRF-1 regulates transcription of *GABRB1* and that its core promoter contains a conserved canonical
44 NRF-1 element responsible for sequence specific binding and transcriptional activation. Our identification
45 of *GABRB1* as a new target for NRF-1 in neurons suggests that genes coding for inhibitory
46 neurotransmission may be coupled to cellular metabolism. This is especially meaningful as binding of
47 NRF-1 to its element is sensitive to the kind of epigenetic changes that occur in multiple disorders
48 associated with altered brain inhibition.

49

50 **INTRODUCTION**

51 The type A γ-aminobutyric acid receptor (GABAR) is a ligand-gated Cl^- ion channel that
52 mediates inhibitory neurotransmission in the adult mammalian central nervous system. The majority of
53 GABARs are composed of two α and two β subunits, and either a γ2 or δ subunit (Barrera *et al.* 2008,
54 Patel *et al.* 2014, Farrar *et al.* 1999). For each receptor, there is the binding of two molecules of GABA,
55 one molecule at each α and β subunit interface (Connolly & Wafford 2004, Olsen & Sieghart 2009,
56 Rabow *et al.* 1995). In the mature neuron, activation of GABARs leads to hyperpolarization. Depending
57 on its subunit composition, GABARs may contain binding sites for barbiturates, benzodiazepines,
58 ethanol, and/or neuroactive steroids. There are nineteen different subunit genes to date, grouped into eight
59 classes (i.e. α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3) that contribute to the diversity and differential assembly of
60 receptor subtypes. The β subunits, which contain the domains that interact with mediators of receptor
61 trafficking and endocytosis (for reviews see (Vithlani *et al.* 2011, Jacob *et al.* 2008)), play an important
62 role in the expression of GABARs at the cell surface.

63 The human *GABRB1* gene, located on chromosome 4, is part of a GABAR gene cluster that
64 contains the genes that encode the α2, α4, and γ1 subunits. A dysregulation of GABAR-mediated
65 neurotransmission has been implicated in various neurological disorders (Hines *et al.* 2012) that show

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

66 altered levels of GABAR subunits, including β1. Through linkage studies, *GABRB1* has been associated
67 with alcohol dependence (Parsian & Zhang 1999, Sun *et al.* 1999, Zinn-Justin & Abel 1999, Song *et al.*
68 2003); and more recently, specific mutations in mouse *Gabrb1* have been shown to produce increased
69 alcohol consumption that is linked to increased tonic inhibition (Anstee *et al.* 2013). Interestingly, single
70 nucleotide polymorphisms in *GABRB1* are also associated with altered brain responses in human
71 adolescents susceptible to addictive behaviors (Duka *et al.* 2017).
72 *GABRB1* expression is also reduced in the lateral cerebella of subjects with bipolar disorder,
73 major depression, and schizophrenia compared to healthy subjects (Fatemi *et al.* 2013). Particularly in
74 schizophrenia, a significant association of *GABRB1* has been identified by genome-wide association
75 studies that were coupled to a protein-interaction-network-based analysis (Yu *et al.* 2014). As *GABRB1*
76 and *GABRA4* lie within the same GABAR gene cluster and their promoters are head-to-head, it is
77 interesting to note that the association of *GABRA4* with autism risk increases with a *GABRB1* interaction
78 (Ma *et al.* 2005, Collins *et al.* 2006), suggesting that these genes may be coordinately regulated. Further
79 support for an association of *GABRB1* with autism is evidenced by a decrease in β1 subunit levels in the
80 brains of autistic subjects (Fatemi *et al.* 2009, Fatemi *et al.* 2010). In addition, the levels of both β1 and
81 β2 subunit mRNAs are reduced in a Fragile X mental retardation mouse model, where the gene Fragile X
82 mental retardation 1 (fmr1) was removed (D'Hulst *et al.* 2006). Finally, down-regulation of β1 subunit
83 mRNAs and protein are observed in the rat pilocarpine model of epilepsy (Brooks-Kayal *et al.* 1998).
84 Yet, despite its prevalent association with brain disorders, there is still little known about the function
85 and/or regulation of β1 in neurons.
86 The TATA-less *GABRB1*/*Gabrb1* promoter (*GABRB1-p* (human)/*Gabrb1-p* (rodent)) contains
87 multiple transcriptional start sites that lie within a CpG island (Russek *et al.* 2000, Saha *et al.* 2013). In
88 unraveling the molecular determinants of GABAR β1 subunit gene regulation, our laboratory
89 demonstrated that the minimal *GABRB1-p* lies within the first 500 bp of the 5' flanking region. Within
90 this region, there is a conserved initiator element (Inr) that mediates down-regulation in response to
91 chronic GABA exposure, implicating an autologous mechanism of transcriptional control.
92 Nuclear respiratory factor 1 (NRF-1) is a transcription factor that functions primarily as a positive
93 regulator of nuclear genes involved in mitochondrial biogenesis and oxidative phosphorylation, such as
94 Tfam, which moves into the mitochondria and regulates mitochondrial DNA transcription (Scarpulla
95 2006, Scarpulla 2008). However, it has also been shown that the binding of NRF-1 to a co-factor, such as
96 SIRT7, can influence its polarity (from activator to repressor) (Mohrin et al., 2015). In addition, binding
97 of NRF-1 to DNA is regulated by the methylation state of its regulatory element (Domcke et al., 2015),

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

98 suggesting that its role in neuronal gene expression will be sensitive to the epigenetic changes that occur
99 in neurological and neuropsychiatric disorders.
100 It is well known that increased neuronal activity results in a parallel change in cellular
101 metabolism, as orchestrated by the synthesis of NRF-1 and its control over mitochondrial biogenesis.
102 Moreover, it has been reported that NRF-1 is a transcriptional activator of glutamate receptor subunit
103 genes under conditions of depolarizing stimulation in neurons (Dhar & Wong-Riley 2009) suggesting that
104 in addition to its role in cellular metabolism, via regulation of the mitochondrial genome, NRF-1
105 coordinates activities in the nucleus to couple neuronal excitability with energy demands of synaptic
106 neurotransmission.
107 Here, we ask whether NRF-1 may control the transcription of GABAR subunit genes (*GABRs*),
108 and in particular the human β1 subunit gene (*GABRB1*), a gene that has been associated with neuronal
109 developmental disorders, the pathophysiology of epilepsy, and alcohol dependence. In this study, we have
110 uncovered a functional regulatory element within *GABRB1* that demonstrates sequence specificity and is
111 responsible for the majority of *GABRB1* promoter-reporter activity, as well as a role for NRF-1 in the
112 activity dependent transcription of endogenous *Gabrb1* in rat primary cortical neurons.
113
114 **METHODS**
115 *Cell Culture and Drug treatment*— The use of animals for our culture studies was under the guidance and
116 protocol approval of the Boston University Institutional Care and Use Committee (IACUC).
117 Primary neocortical neurons were isolated from embryonic day 18 Sprague-Dawley rat embryos
118 (Charles River Laboratories). Isolated embryonic brains and the subsequently dissected cortices were
119 maintained in ice-cold modified calcium-magnesium free Hank's Balanced Salt Solution (HBSS) (4.2mM
120 sodium bicarbonate, 1 mM sodium pyruvate, and 20 mM HEPES, 3mg/ml BSA) buffering between pH
121 range 7.25 – 7.3. Tissues were then separated from HBSS dissection solution and trypsinized (0.05%
122 trypsin-EDTA) for 10 minutes in 37°C and 5% CO_2. The trypsin reaction was stopped with serum
123 inactivation using plating medium (Neural Basal Medium, 10% FBS, 10 U/ml penicillin/streptomycin,
124 2mM L-glutamine). Tissues were triturated with a 1000 mL micropipette and diluted to a concentration of
125 $0.5x10^6$ cells/mL in plating media for plating. Cells were allowed to adhere onto Poly-L-lysine coated
126 culturing surface for 1 h prior to changing to serum-free feeding medium (2% B-27, 2mM glutamine, 10
127 U/ml penicillin/streptomycin supplemented neurobasal medium). Neuronal cultures were maintained at
128 37°C in a 5% CO_2 incubator. Primary cortical neurons (DIV7-8) were treated with either Vehicle or 20
129 mM KCl for 6 hours before harvesting for analysis.

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

130 *Expression Constructs*— pCDNA 3.1 hygro hNRF-1 VP16 was generously provided by Dr. Tod
131 Gulick (Ramachandran *et al.* 2008) (Sanford-Burnham Medical Research Institute, Orlando FL). pcDNA
132 3.1 hygro hNRF-1 VP16 encodes a constitutively active form of NRF-1, consisting of the full-length
133 human NRF-1 and the herpes simplex virus VP16 transactivation domain. The pcDNA3.0-NRF-1 DN
134 expresses amino acid residues 1-304 of human NRF-1, which encodes the DNA-binding, dimerization
135 and nuclear localization domains of NRF-1, but lacks the transactivation domain (amino acids 305-503).
136 With the exception of a single conservative mutation at amino acid residue 293 (A→T), the 304 amino
137 acid residues of NRF-1 are conserved between human and rat. The construct was created using PCR with
138 the forward primer sequence 5'-CGGGGTACCACCATGGAGGAACACGGAGTGACCCAAAC-3',
139 containing the underlined Kpn1 restriction site and the kozak sequence on the 5' end, and the reverse
140 primer 5'GCTCTAGATCACTGTGATGGTACAAGATGAGCTATACTATGTGTGGCTGTGGC-3',
141 containing stop codon and Xba1 restriction site. PCR products were digested with restriction enzymes
142 Kpn1 and Xba1, and ligated into pcDNA3.0 vector (Invitrogen).
143 *Electrophoretic mobility shift (EMSA) and supershift assays*—Briefly, 30 bp DNA probes containing
144 the putative NRF-1 binding sequence were incubated with 25μg of neocortical nuclear extracts for
145 electrophoresis under non-denaturing conditions. Following electrophoresis, the protein-DNA complexes
146 were detected by autoradiography. The DNA probes were created from annealing synthesized
147 oligonucleotides (www.idtdna.com) and 5' end labeling using [γ-^{32}P] ATP (PerkinElmer) in a T4
148 polynucleotide kinase (NEB) reaction. Nuclear extracts were prepared from DIV7 primary neocortical
149 neurons grown on 10-cm plates in the presence of protease inhibitor cocktail. Protein-DNA binding
150 specificity was determined by adding poly (dI-dC) (Roche) or/and 100-fold excess unlabeled DNA probe
151 prior to the addition of labeled probe during the room temperature binding reaction. To generate a
152 supershift complex, NRF-1 antibody (AbCam ab34682) was added to the reaction mixture for 15 min.
153 The binding reactions were loaded onto a 5% polyacrylamide gel in 0.5X TBE buffer and run at 200V for
154 2 h at 4°C. The positive control probe consisted of a functional NRF-1 sequence (Evans & Scarpulla
155 1990) found in the Rat cytochrome c (rCycs) gene (Evans & Scarpulla 1990). Probe and competitor
156 oligonucleotide sequences were: *GABRB1* NRF1, 5'-agcgcgc**TCTGCGCATGCGCA**ggtccattc-3' and 5'-
157 gaatggacc**TGCGCATGCGCAGA**gcgcgct-3'. *GABRB1* NRF1 mutant, 5'-
158 agcgcgc**TCTGC**c**CATG**g**GCA**ggtccattc -3' and 5'-gaatggacc**TGC**c**CATG**g**GCAGA**gcgcgct-3'. *rCycs*
159 NRF1, 5'-ctgcta**GCCCGCATGCGC**gcgcacctta-3'and 5'-taaggtgcgc**GCGCATGCGGGC**tagcag-3'.
160 *Reporter Plasmids and Promoter Mutagenesis*—The *GABRB1p*-Luc (pGL2-*GABRB1*) promoter
161 construct containing the 5' flanking region of the human β1 subunit gene was previously cloned by our
162 laboratory and contains 436 bp upstream of the initiator sequence and 105 bp downstream (Russek et al.

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

163 2000). The promoter containing a mutated NRF-1 element (**TCTGC**c**CATG**g**GCA**) within
164 the *GABRB1p-Luc* was created by PCR-driven overlap extension. Using wild-type *GABRB1p*-Luc as PCR
165 template, two PCR fragments were amplified using the GL1 primer (Promega) and the antisense mutant
166 NRF-1 oligonucleotide from EMSA, and sense mutant NRF-1 oligonucleotide and the GL2 primer
167 (Promega), resulting in fragments with 30 bp overlapping sequences that contain the mutant NRF-1
168 element. A second PCR step using GL1, GL2 primers and both initial PCR products produced the
169 mutant *GABRB1* promoter insert.
170 *Luciferase Assay/Reporter Assay with Magnetofection*™— Magnetofection of DNA into primary
171 neuron cultures was achieved with the NeuroMag transfection reagent according to the manufacturer's
172 protocol. Here, 2 ml of resuspended E18 primary cortical neurons at 0.5 X 10^6 cells/ml were plated in
173 each well of a 6-well plate. On DIV7, neurons were transfected with 1µg of expression construct, 2µg of
174 promoter reporter construct, and 3µl of NeuroMag transfection reagent (1:1 DNA to reagent ratio). 24 h
175 after transfection, neurons were actively lysed by scraping. Cell lysates were cleared of precipitates by
176 centrifugation and then assayed for luciferase activity using a luciferase assay system (Promega).
177 Luciferase activity was normalized to total protein as determined using a protein assay kit (Thermo
178 Scientific Pierce). All transfections were performed in sister dishes from three or more plating sessions to
179 produce true N's.
180 *Chromatin Immunoprecipitation (ChIP)*— ChIP was performed according to the Magna ChIP A
181 protocol (Millipore). Briefly, primary neurons in 100 mm dishes were fixed with a final concentration of
182 1% formaldehyde in culturing media. The remaining unreacted formaldehyde was quenched with
183 Glycine. Genomic DNA and protein complexes were extracted from cells using nuclear lysis buffers
184 supplemented with protease and phosphatase inhibitors. The lysates containing DNA-protein complexes
185 were sonicated (nine times, 5 minutes each at a 30s on/off interval) in an ice-cold water bath with a
186 Bioruptor (Diagenode) in order to generate fragments predominantly in the range of 200 – 500 bp in size.
187 The sheared chromatin was immunoprecipitated with either anti-NRF-1 antibody (Abcam ab34682 ChIP
188 grade antibody) or normal rabbit IgG overnight at 4 ^{O}C with constant rotation. The antibody/transcription
189 factor bound chromatin was separated from unbound chromatin using Protein A conjugated magnetic
190 beads and magnetic pull-down. The isolated complexes were washed with a series of salt buffer solutions
191 prior to eluting. DNA fragments were separated from complexes using Proteinase K and heating, and
192 recovered through column purification. The co-precipitated DNA fragments were identified by
193 quantitative PCR (qPCR) using specific primers and TaqMan probes that flank putative responsive
194 elements in gene promoters using the FastStart Universal Probe Master (Roche) PCR reagent. PCR
195 cycling was performed using the ABI7900HT Fast Real-Time PCR system. The *Gabrb1* promoter

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

196 fragment (114 bp) was amplified using: forward primer 5'- TGTTTGCAAGGCACAAGGTGTC-3',
197 reverse primer 5'- TCTGCGAAGATTCAAGGAATGCAACT, TaqMan® MGB probe 5'-
198 GCGCATGCGCAGGTCCATTCGGGAAT-3'.
199 *Western Blot Analysis*—Total cellular proteins were extracted from primary neuronal cultures
200 after KCl treatment with standard procedures and the use of RIPA lysis buffer (Tris, pH 7.4, 10 mM;
201 Nonidet P-40 1%; NaCl 150 mM; SDS 0.1%; protease inhibitor mixture (Roche Applied Science) 1X;
202 EDTA 1mM; sodium orthovanadate 1 mM; sodium deoxycholate 0.1%; phenylmethylsulfonyl fluoride 1
203 mM). 30 µg of whole cell extracts were separated by SDS-PAGE under reducing conditions on either
204 10% or 4-20% Tris-glycine gel according to mass/size. The electrophoresed samples were transferred to
205 nitrocellulose membranes. Western blot analysis was performed using antibodies against NRF-1 (AbCam
206 ab34682, 1:2000 in 1X TBS-T). Membranes were incubated with peroxidase-conjugated goat anti-rabbit
207 secondary antibody (Santa Cruz Biotechnology, 1:5000) in TBS-T and visualized using the ECL
208 enhanced chemiluminescence reagent (GE Healthcare Life Sciences). Data are presented as mean ±
209 SEM. Significance was set at $p < 0.05$, as determined using the paired Student's t-test (two-tailed).
210 *RNA extraction and qRT-PCR*—Total RNA was isolated from cultured primary neocortical using the
211 RNeasy Micro Kit (Qiagen). For each reaction, 20ng of total RNA was reverse-transcribed to cDNA and
212 PCR amplified in a single reaction mixture using the TaqMan® One-Step RT-PCR Master Mix Reagents
213 Kit (Applied Biosystems). Incubation and thermal cycling conditions were performed using the
214 ABI7900HT in a 384-well PCR plate format (AppliedBiosystems). The RT reaction was held at 48°C for
215 30 min, followed by 95°C for 10 min to activate the polymerase. The PCR reaction conditions were: 15
216 sec denaturation at 95°C and coupled annealing and extension for 1 min at 60°C for 40 cycles. Co-
217 detection of rat peptidylprolyl isomerase A (cyclophilin A) gene served as an internal control for
218 normalization. Cyclophilin A expression has been shown to be stable in response to neuronal stimulation
219 in culture (Santos & Duarte, 2008), which is consistent with our previous studies. Relative gene
220 expression was quantified using $2^{\wedge}(-\Delta\Delta C_T)$ and a standard curve was generated based on the
221 amplification of total RNA extracted from untreated cultured neurons. The qRT-PCR primers and probes
222 for rat mRNAs were: *NRF-1*, 57 bp amplicon (Assay ID: Rn01455958_m1, Thermo Fisher Scientific);
223 *Gabrb1*, 81 bp amplicon (Assay ID: Rn00564146_m1, Thermo Fisher Scientific); *Ppia*, 60 bp amplicon,
224 forward primer: 5'- TGCAGACATGGTCAACCCC-3', reverse primer: 5'- CCCAAGGGCTCGCCA-3',
225 TaqMan probe with TAMARA quencher: 5'- CCGTGTTCTTCGACATCACGGCTG-3'.
226

227 **RESULTS**

228 *Neuronal depolarization increases NRF-1 and GABAR β1 subunit gene transcription*

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

229 To determine whether *Gabrb1* is activity dependent, primary cortical neurons were treated with KCl.
230 Both NRF-1 protein and NRF-1 mRNA levels have been previously shown to increase with KCl-
231 stimulated depolarization (Dhar & Wong-Riley 2009). We asked whether under conditions where NRF-1
232 levels increase in response to neuronal activity, is it accompanied by increased levels of *Gabrb1*
233 transcripts. As shown in Figure 1A and 1B, there is a 2-fold increase in NRF-1 mRNA levels (1.982 ±
234 0.445, n=5, **p < 0.01) upon KCl stimulation for 6 hours that is accompanied by a ~30% increase in the
235 levels of NRF-1 protein (fold change: 1.285 ± 0.330, n=6, *p < 0.05) when compared to vehicle control.
236 In parallel to changes in NRF-1, we now report a ~40% increase in levels of *Gabrb1* transcripts (fold
237 change: 1.424 ± 0.324, n=5, *p < 0.05).
238
239 *Identification of a Conserved NRF1 Element in the GABRB1 Promoter*
240 Our laboratory previously defined the 5'-regulatory region of the human β1 subunit gene *GABRB1*,
241 identifying transcriptional start sites (TSSs) within a 10 bp functional initiator element (Inr) that mediates
242 the response of the gene to chronic GABA exposure (Russek et al. 2000, Saha et al. 2013). Now we report
243 that directly upstream of this Inr is a canonical NRF-1 element spanning -11/+1 relative to the major TSS
244 for the rat homologue *Gabrb1* in neocortical neurons. As shown in Figure 2, the location of the NRF-1
245 element within the promoter region is conserved across multiple species. Given the ubiquitous expression
246 of NRF-1, its conservation across species, and its established role in cellular respiration and mitochondrial
247 biogenesis, the sequence comparison presented in Figure 2 strongly suggests that the NRF-1 element is
248 functionally relevant to β1 subunit expression in the mammalian brain.
249
250 *NRF-1 Recognizes the Cis-Element in the human GABRB1 Promoter*
251 To determine the specific binding site within *GABRB1-p* that binds to NRF-1, we performed an
252 electrophoretic mobility shift assay (EMSA) with a ^{32}P-labeled probe specific to its NRF-1 consensus
253 element in a binding reaction with nuclear protein extracts from E18 primary cortical neurons. To validate
254 the specificity of the NRF-1 antibody for EMSA analysis, nuclear extracts were incubated with a positive
255 control probe (Dhar *et al.* 2008) containing the NRF-1 binding site of the rat cytochrome c promoter. As
256 shown in lane 2 of Figure 3A, the control radiolabeled probe (rat Cyt C) displays specific DNA
257 recognition from nuclear extracts of cortical neurons that is confirmed by supershift with the addition of
258 an NRF-1 specific antibody (Fig. 3A, lane 4). Next, specific binding to the putative NRF-1 consensus site
259 in *GABRB1* was confirmed using the same nuclear extracts, with sequence specificity defined by
260 competition with an unlabeled double stranded oligonucleotide that was identical to the probe sequence
261 (competitor) (Fig. 3A, lanes 6 and 7). Addition of an unlabeled competitor mutant probe, containing

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

262 substitutions within the GC core, failed to compete for complex formation (Fig. 3A, lane 8). Presence of
263 endogenous NRF-1 at the *GABRB1* NRF1 consensus site was further confirmed by supershift analysis
264 using the NRF-1 specific antibody (Fig. 3A, lane 9). Finally, a radiolabeled probe containing the sequence
265 of the mutant NRF-1 site in *GABRB1* shows little or no complex formation (Fig. 3A, lanes 10-12).
266 To determine whether the endogenous β1 promoter in neurons is occupied by NRF-1, ChIP was
267 performed using genomic DNA derived from E18 rat primary cortical cultures (DIV7) that was
268 precipitated with NRF-1 antibodies. Precipitated fragments were detected using PCR primers that
269 specifically amplify DNA encompassing the putative NRF-1 binding site in rat *Gabrb1*. As can be seen in
270 Figure 3B, there is a 5-fold increase (5.045 ± 0.981, n=4, *$p < 0.05$) in PCR detection of the NRF-1 site in
271 *Gabrb1* when precipitated using an NRF-1 Ab, as compared to rabbit IgG. Moreover, NRF-1 is also
272 present at the core promoter of *GABRB1* in human embryonic stem cells (H1-hESC) as detected in ChIP-
273 sequencing (ChIP-seq) datasets of the ENCODE project (https://www.encodeproject.org) using our
274 bioinformatic analysis algorithm (in the Strand NGS pipeline, Model-based Analysis for ChIP-Seq
275 (MACS, version 2.0, Zhang et al., 2008)) with a p-value cutoff set to 1.0E-05, quality threshold >=30,
276 99% match to the sequence, and all duplicates removed (Fig. 3C). We also found coincident peak
277 detection using ENCODE datasets from NRF-1 ChIP-seq with genomic DNA from immortalized cell
278 lines (K562, HepG2, CH12.LX, GM 12878, and HeLa-S3; data not shown). Note that the detected peak
279 in H1-hESC is identical to that predicted by Figure 2 and within the wildtype oligonucleotide sequence
280 that bound nuclear extracts from rat primary neurons (Fig. 3).
281
282 *Overexpression of NRF1 Induces GABRB1 Promoter Activity in Transfected Primary Cortical Neurons*
283 To evaluate whether there is a functional consequence to NRF-1 binding to its consensus site in
284 *GABRB1-p*, primary cortical neurons were transfected with the *GABRB1p-luciferase* construct containing
285 the 541 bp 5' flanking region upstream of the human β1 subunit gene (Russek et al. 2000). We chose this
286 approach to study functional relevance of the NRF-1 site to *GABRB1* transcription in neurons because
287 NRF-1's influence on the genome is difficult to detect by siRNA knockdown due to its robust expression
288 at baseline and protein stability (Baar *et al.* 2003, Scarpulla 2006, Ramachandran et al. 2008).
289 As the expression of the NRF1:VP16 fusion protein has been shown to induce the promoter
290 activity of NRF-1 responsive genes in cell lines (Ramachandran et al. 2008, Gonen & Assaraf 2010), we
291 transfected primary cortical neurons with NRF1:VP16 along with the *GABRB1p-luciferase* reporter and
292 found a marked increase (~70%, fold change: 1.671 ± 0.404, n=5, *$p < 0.05$) above baseline (when
293 compared to co-transfection with empty vector control, 1.00 ± 0.225, n=5) (Fig. 4A). Mutations were
294 introduced into *GABRB1-p* using site-directed mutagenesis (based on the loss of specific binding of NRF-

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

295 1 as identified in EMSA (see Fig. 3, lane 8)). As can be seen in Figure 4A for *mGABRB1-p*, with and
296 without NRF1:VP16 overexpression, mutation of the NRF-1 regulatory element in *GABRB1-p* reduces
297 basal activity to around 30% (fold change: 0.314 ± 0.067, n=5, *p < 0.05) of wild type. Overexpression of
298 NRF-1:VP16 has no effect on *mGABRB1-p* (0.358 ± 0.057, n=5, ns) showing that increased *GABRB1*
299 promoter activity directed by NRF-1 is sequence specific; and, moreover, that NRF-1 may be an
300 important positive regulator of β1 subunit expression in developing neurons, especially interesting
301 because β1 is found in the germinal zones and associated with pre-migrating neurons (Ma and Barker,
302 1995). Furthermore, increased mitochondrial biogenesis has also been associated with neuronal
303 differentiation (Vayssiere *et al.* 1992, Cheng *et al.* 2010).
304
305 *Inhibition of NRF-1 Function in Neurons*
306 To evaluate the specific effect of endogenous NRF-1 on *GABRB1* transcription, a dominant negative form
307 of NRF-1 was utilized that contains the DNA binding domain but lacks the NRF-1 trans-activation
308 domain (Gugneja *et al.* 1996). Co-expression of this dominant negative NRF-1 represses *GABRB1*
309 promoter activity by 45% (fold change: 0.549 ± 0.164, n=6, *p < 0.05, Fig. 4B) compared to empty-
310 vector control (1.000 ± 0.235, n=6). Most importantly, overexpression of dominant negative NRF-1
311 blocks the activity dependent increase of endogenous *Gabrb1* mRNA levels in response to KCl treatment
312 (Fig. 5).
313
314 When taken together with the fact that the NRF-1 element in the β1 subunit gene is completely conserved
315 across species (Fig. 2), and that there is a mutation-induced loss of binding (Fig. 3) and function (Fig. 4),
316 our results strongly suggest that NRF-1 is an essential feature of β1 subunit expression in neurons and that
317 it couples transcription to the activity pattern of individual cells.
318
319 **DISCUSSION**
320 We now report that the GABAR β1 subunit gene (*GABRB1*/*Gabrb1*) is regulated by NRF-1, a
321 crucial transcription factor involved in oxidative phosphorylation and mitochondrial biogenesis. While it
322 is believed that NRF-1 coordinates synaptic activity and energy metabolism by regulating excitatory
323 neurotransmission via genes that code for subunits of the N-methyl-D-aspartate (NMDA) receptor (Dhar
324 & Wong-Riley 2009, Dhar *et al.* 2009, Dhar & Wong-Riley 2011), it is clear that the this regulatory
325 program is more complex than originally expected given our observation that elements of inhibitory
326 neurotransmission may be coordinately regulated with excitation. This possibility is especially important

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

327 as a variety of brain disorders present with a decrease in GABAR β1 subunit levels, including epilepsy
328 where there is also aberrant hyperactivity.
329 Previously, our laboratory mapped the 5' flanking region of the human β1 subunit promoter.
330 Within this TATA-less promoter, we identified the major transcriptional start site (TSS) and described an
331 initiator element (Inr) that senses the presence of prolonged GABA to mediate the autologous
332 downregulation of β1 subunit expression (Russek et al. 2000). Our recent studies have discovered that
333 such decreases in β1 subunit RNA levels may reflect a change in the chromatin state as mediated by
334 PhF1b, a polycomb-like protein (Saha et al. 2013). In our present work, we have found a conserved
335 canonical NRF-1 binding element (Fig. 2) that interacts with NRF-1 *in vitro* as verified by mobility shift
336 assays (Fig. 3). Interestingly, our results in primary rat neurons are consistent with a peak of NRF-1
337 binding over the core promoter of *GABRB1* in human embryonic stem cells, as displayed in Figure 3C,
338 using our bioinformatic analysis of ENCODE project datasets (Wang *et al.* 2012, Wang *et al.* 2013,
339 Gerstein *et al.* 2012) with the Strand NGS pipeline (MACS V2 peak detection). We identified the same
340 peak of binding in additional NRF-1 ChIP-seq ENCODE datasets from immortalized cell lines.
341 Interestingly, we did not detect any additional NRF-1 peaks on the genes that code for other β subunit
342 genes, suggesting that NRF-1 regulation may be unique to β1.
343 It is thought that NRF-1 binds as a homodimer to the consensus binding sequence
344 (T/C)GCGCA(C/T)GCGC(A/G), making contact with DNA at the guanine nucleotides (Virbasius *et al.*
345 1993). This model is supported by the results of our mutational studies which show that a single mutation
346 of G>C eliminates the ability of a cold double stranded oligonucleotide to compete for complex formation
347 as assayed by mobility shift. The location of the NRF-1 element in *GABRB1* centers at -12 relative to the
348 major TTS in neocortical neurons. The GC-rich NRF-1 binding motif is often associated with TATA-less
349 promoters and found within 100 bp DNA regions around transcriptional start sites in the human genome
350 (Virbasius et al. 1993, Xi *et al.* 2007). The proximity of the NRF-1 element in *GABRB1* to the Inr that
351 binds polycomb-like proteins associated with chromatin remodeling and DNA methylation (Vire *et al.*
352 2006) may underlie its major role in controlling basal levels of β1 subunit mRNAs in neurons. Whether
353 *GABRB1* is epigenetically regulated *in vivo* remains to be determined and could be a feature of why its
354 transcription decreases in disease, especially since NRF-1 binding is blocked by DNA methylation
355 (Gebhard *et al.* 2010).
356 Using the sensitivity of the luciferase reporter system, we have overexpressed NRF-1:VP16 in
357 living neurons and shown that it indeed regulates the *GABRB1p-luciferase* reporter construct and that
358 such regulation is lost upon mutation of the *GABRB1* NRF-1 regulatory element (Fig. 4A) and upon
359 competition for endogenous NRF-1 binding to the promoter by overexpression of a dominant negative

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

360 NRF-1 expression construct (Fig. 4B). We have also shown that the same mutation in the NRF-1
361 regulatory element of *GABRB1p* removes binding of endogenous NRF-1 to neuronal extracts in a
362 mobility shift assay, as seen in Figure 3. Finally, and perhaps most importantly, we have shown that
363 overexpression of dominant negative NRF-1 protein blocks the activity dependent increase in endogenous
364 *Gabrb1* mRNA levels identifying a key molecular determinant of β1 subunit gene expression within cells
365 (Fig. 5).
366 Our results are consistent with previous studies from the Russek laboratory, using the same wild
367 type *GABRB1p-luciferase* reporter construct, where promoter truncation and/or deletion that removes the
368 Inr and at the same time disrupts the element for NRF-1 results in a 75-90% decrease in luciferase gene
369 transcription (Russek et al. 2000) (Fig. 4). Given that GABAR blockade by bicuculine has also been
370 shown to drive NRF-1 dependent transcription (Delgado & Owens 2012) and that bicuculine reverses
371 GABA-induced downregulation of β1 mRNA levels (Russek et al. 2000), presumably through PhF1b
372 binding to the Inr, our new results suggest that the NRF-1 responsive element and Inr may act
373 synergistically to regulate β1 subunit levels as neurons adapt to changes in their activity state.
374 The direct regulation of NRF-1 in *GABRB1*/*Gabrb1* gene expression in the brain may also have
375 implications in the initiation of sleep. The use of fragrant dioxane derivatives that show a 6-fold
376 preference for β1-containing GABARs (Sergeeva *et al.* 2010) suggest that the β1 subunit is required for
377 the modulation of wakefulness that is mediated by the histaminergic neurons of the posterior
378 hypothalamus (tubermamillary nucleus-TMN) (Yanovsky *et al.* 2012). Given that energy metabolism is
379 sensitive to restoration during the sleep cycle and that NRF-1 levels rise with sleep deprivation (Nikonova
380 *et al.* 2010), it is interesting that β1-containing GABARs are the major source of inhibitory control over
381 sleep.
382 Although differential expression of α subunits in relationship to brain disorders has clearly been
383 associated with their region-specific control over changes in tonic and phasic inhibition, it is only recently
384 that the importance of differential β subunit expression to GABAR function has been noted. This
385 selective property of GABAR function ascribed to the assembly of particular β subunits, however, has
386 been limited to β2 and β3, with β1 present in only a limited population of receptors in the brain. However,
387 of all β subunits, β1 has been most associated with both neurological and neuropsychiatric disorders. The
388 reason for this functional relationship remains to be described and is an active area of investigation in our
389 laboratories.
390

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

391 **Acknowledgments**: We thank Dr. Tod Gulick (Sanford-Burnham Medical Research Institute, Orlando,
392 FL) for his helpful discussion and providing us with expression constructs that were invaluable for our
393 experiments.
394
395 **Disclosures**: The authors declare no competing conflict of financial interests.
396

REFERENCES

Anstee, Q. M., Knapp, S., Maguire, E. P. et al. (2013) Mutations in the Gabrb1 gene promote alcohol consumption through increased tonic inhibition. *Nature communications,* **4,** 2816.

Baar, K., Song, Z., Semenkovich, C. F., Jones, T. E., Han, D. H., Nolte, L. A., Ojuka, E. O., Chen, M. and Holloszy, J. O. (2003) Skeletal muscle overexpression of nuclear respiratory factor 1 increases glucose transport capacity. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* **17,** 1666-1673.

Barrera, N. P., Betts, J., You, H., Henderson, R. M., Martin, I. L., Dunn, S. M. and Edwardson, J. M. (2008) Atomic force microscopy reveals the stoichiometry and subunit arrangement of the alpha4beta3delta GABA(A) receptor. *Molecular pharmacology,* **73,** 960-967.

Brooks-Kayal, A. R., Shumate, M. D., Jin, H., Rikhter, T. Y. and Coulter, D. A. (1998) Selective changes in single cell GABA(A) receptor subunit expression and function in temporal lobe epilepsy. *Nature medicine,* **4,** 1166-1172.

Cheng, A., Hou, Y. and Mattson, M. P. (2010) Mitochondria and neuroplasticity. *ASN neuro,* **2,** e00045.

Cheng, A., Wan, R., Yang, J. L., Kamimura, N., Son, T. G., Ouyang, X., Luo, Y., Okun, E. and Mattson, M. P. (2012) Involvement of PGC-1alpha in the formation and maintenance of neuronal dendritic spines. *Nature communications,* **3,** 1250.

Collins, A. L., Ma, D., Whitehead, P. L. et al. (2006) Investigation of autism and GABA receptor subunit genes in multiple ethnic groups. *Neurogenetics,* **7,** 167-174.

Connolly, C. N. and Wafford, K. A. (2004) The Cys-loop superfamily of ligand-gated ion channels: the impact of receptor structure on function. *Biochemical Society transactions,* **32,** 529-534.

D'Hulst, C., De Geest, N., Reeve, S. P., Van Dam, D., De Deyn, P. P., Hassan, B. A. and Kooy, R. F. (2006) Decreased expression of the GABAA receptor in fragile X syndrome. *Brain research,* **1121,** 238-245.

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Delgado, J. Y. and Owens, G. C. (2012) The cytochrome c gene proximal enhancer drives activity-dependent reporter gene expression in hippocampal neurons. *Frontiers in molecular neuroscience,* **5,** 31.

Dhar, S. S., Liang, H. L. and Wong-Riley, M. T. (2009) Nuclear respiratory factor 1 co-regulates AMPA glutamate receptor subunit 2 and cytochrome c oxidase: tight coupling of glutamatergic transmission and energy metabolism in neurons. *Journal of neurochemistry,* **108,** 1595-1606.

Dhar, S. S., Ongwijitwat, S. and Wong-Riley, M. T. (2008) Nuclear respiratory factor 1 regulates all ten nuclear-encoded subunits of cytochrome c oxidase in neurons. *J Biol Chem,* **283,** 3120-3129.

Dhar, S. S. and Wong-Riley, M. T. (2009) Coupling of energy metabolism and synaptic transmission at the transcriptional level: role of nuclear respiratory factor 1 in regulating both cytochrome c oxidase and NMDA glutamate receptor subunit genes. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* **29,** 483-492.

Dhar, S. S. and Wong-Riley, M. T. (2011) The kinesin superfamily protein KIF17 is regulated by the same transcription factor (NRF-1) as its cargo NR2B in neurons. *Biochimica et biophysica acta,* **1813,** 403-411.

Evans, M. J. and Scarpulla, R. C. (1990) NRF-1: a trans-activator of nuclear-encoded respiratory genes in animal cells. *Genes & development,* **4,** 1023-1034.

Farrar, S. J., Whiting, P. J., Bonnert, T. P. and McKernan, R. M. (1999) Stoichiometry of a ligand-gated ion channel determined by fluorescence energy transfer. *J Biol Chem,* **274,** 10100-10104.

Fatemi, S. H., Folsom, T. D., Rooney, R. J. and Thuras, P. D. (2013) mRNA and protein expression for novel GABAA receptors theta and rho2 are altered in schizophrenia and mood disorders; relevance to FMRP-mGluR5 signaling pathway. *Translational psychiatry,* **3,** e271.

Fatemi, S. H., Reutiman, T. J., Folsom, T. D., Rooney, R. J., Patel, D. H. and Thuras, P. D. (2010) mRNA and protein levels for GABAAalpha4, alpha5, beta1 and GABABR1 receptors are altered in brains from subjects with autism. *Journal of autism and developmental disorders,* **40,** 743-750.

Fatemi, S. H., Reutiman, T. J., Folsom, T. D. and Thuras, P. D. (2009) GABA(A) receptor downregulation in brains of subjects with autism. *Journal of autism and developmental disorders,* **39,** 223-230.

Fazio, I. K., Bolger, T. A. and Gill, G. (2001) Conserved regions of the Drosophila erect wing protein contribute both positively and negatively to transcriptional activity. *J Biol Chem,* **276,** 18710-18716.

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Gebhard, C., Benner, C., Ehrich, M. et al. (2010) General transcription factor binding at CpG islands in normal cells correlates with resistance to de novo DNA methylation in cancer cells. *Cancer research,* **70,** 1398-1407.

Gerstein, M. B., Kundaje, A., Hariharan, M. et al. (2012) Architecture of the human regulatory network derived from ENCODE data. *Nature,* **489,** 91-100.

Ghosh, A., Carnahan, J. and Greenberg, M. E. (1994) Requirement for BDNF in activity-dependent survival of cortical neurons. *Science,* **263,** 1618-1623.

Gonen, N. and Assaraf, Y. G. (2010) The obligatory intestinal folate transporter PCFT (SLC46A1) is regulated by nuclear respiratory factor 1. *J Biol Chem,* **285,** 33602-33613.

Gugneja, S., Virbasius, C. M. and Scarpulla, R. C. (1996) Nuclear respiratory factors 1 and 2 utilize similar glutamine-containing clusters of hydrophobic residues to activate transcription. *Molecular and cellular biology,* **16,** 5708-5716.

Han, Y., Lin, Y., Xie, N. et al. (2011) Impaired mitochondrial biogenesis in hippocampi of rats with chronic seizures. *Neuroscience,* **194,** 234-240.

Hines, R. M., Davies, P. A., Moss, S. J. and Maguire, J. (2012) Functional regulation of GABAA receptors in nervous system pathologies. *Current opinion in neurobiology,* **22,** 552-558.

Jacob, T. C., Moss, S. J. and Jurd, R. (2008) GABA(A) receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nature reviews. Neuroscience,* **9,** 331-343.

Li, Zhuting (2015) Activity-dependent gene regulation in neurons: energy coupling and a novel biosensor [dissertation]. [Boston (MA)]: Boston University.

Lund, I. V., Hu, Y., Raol, Y. H., Benham, R. S., Faris, R., Russek, S. J. and Brooks-Kayal, A. R. (2008) BDNF selectively regulates GABAA receptor transcription by activation of the JAK/STAT pathway. *Science signaling,* **1,** ra9.

Ma, D. Q., Whitehead, P. L., Menold, M. M. et al. (2005) Identification of significant association and gene-gene interaction of GABA receptor subunit genes in autism. *American journal of human genetics,* **77,** 377-388.

Ma, W., Barker, J. L., (1995) Complementary expressions of transcripts encoding Gad(67) and Gaba(A) receptor alpha-4, beta-1, and gamma-1 subunits in the proliferative zone of the embryonic rat central nervous system. *J. Neurosci.* **15**, 2547–2560.

Mo, J., Kim, C. H., Lee, D., Sun, W., Lee, H. W. and Kim, H. (2015) Early growth response 1 (Egr-1) directly regulates GABAA receptor alpha2, alpha4, and theta subunits in the hippocampus. *Journal of neurochemistry,* **133,** 489-500.

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Mohrin, M., Shin, J., Liu, Y., Brown, K., Luo, H., Xi, Y., Haynes, C.M. and Chen, D. (2015) A mitochondrial UPR-mediated metabolic checkpoint regulates hematopoietic stem cell aging. *Science*, **347**, 1374-1377.

Nikonova, E. V., Naidoo, N., Zhang, L., Romer, M., Cater, J. R., Scharf, M. T., Galante, R. J. and Pack, A. I. (2010) Changes in components of energy regulation in mouse cortex with increases in wakefulness. *Sleep,* **33,** 889-900.

Olsen, R. W. and Sieghart, W. (2009) GABA A receptors: subtypes provide diversity of function and pharmacology. *Neuropharmacology,* **56,** 141-148.

Parsian, A. and Zhang, Z. H. (1999) Human chromosomes 11p15 and 4p12 and alcohol dependence: possible association with the GABRB1 gene. *American journal of medical genetics,* **88,** 533-538.

Patel, B., Mortensen, M. and Smart, T. G. (2014) Stoichiometry of delta subunit containing GABA(A) receptors. *British journal of pharmacology,* **171,** 985-994.

Quadrato, G., Elnaggar, M. Y., Duman, C., Sabino, A., Forsberg, K. and Di Giovanni, S. (2014) Modulation of GABAA receptor signaling increases neurogenesis and suppresses anxiety through NFATc4. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* **34,** 8630-8645.

Rabow, L. E., Russek, S. J. and Farb, D. H. (1995) From ion currents to genomic analysis: recent advances in GABAA receptor research. *Synapse,* **21,** 189-274.

Ramachandran, B., Yu, G. and Gulick, T. (2008) Nuclear respiratory factor 1 controls myocyte enhancer factor 2A transcription to provide a mechanism for coordinate expression of respiratory chain subunits. *J Biol Chem,* **283,** 11935-11946.

Roberts, D. S., Hu, Y., Lund, I. V., Brooks-Kayal, A. R. and Russek, S. J. (2006) Brain-derived neurotrophic factor (BDNF)-induced synthesis of early growth response factor 3 (Egr3) controls the levels of type A GABA receptor alpha 4 subunits in hippocampal neurons. *J Biol Chem,* **281,** 29431-29435.

Russek, S. J., Bandyopadhyay, S. and Farb, D. H. (2000) An initiator element mediates autologous downregulation of the human type A gamma -aminobutyric acid receptor beta 1 subunit gene. *Proceedings of the National Academy of Sciences of the United States of America,* **97,** 8600-8605.

Saha, S., Hu, Y., Martin, S. C., Bandyopadhyay, S., Russek, S. J. and Farb, D. H. (2013) Polycomblike protein PHF1b: a transcriptional sensor for GABA receptor activity. *BMC pharmacology & toxicology,* **14,** 37.

Scarpulla, R. C. (2006) Nuclear control of respiratory gene expression in mammalian cells. *Journal of cellular biochemistry,* **97,** 673-683.

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Scarpulla, R. C. (2008) Nuclear control of respiratory chain expression by nuclear respiratory factors and PGC-1-related coactivator. *Annals of the New York Academy of Sciences,* **1147,** 321-334.

Scharfman, H. E. (2002) Epilepsy as an example of neural plasticity. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry,* **8,** 154-173.

Sergeeva, O. A., Kletke, O., Kragler, A. et al. (2010) Fragrant dioxane derivatives identify beta1-subunit-containing GABAA receptors. *J Biol Chem,* **285,** 23985-23993.

Song, J., Koller, D. L., Foroud, T. et al. (2003) Association of GABA(A) receptors and alcohol dependence and the effects of genetic imprinting. *American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics,* **117B,** 39-45.

Sun, F., Cheng, R., Flanders, W. D., Yang, Q. and Khoury, M. J. (1999) Whole genome association studies for genes affecting alcohol dependence. *Genetic epidemiology,* **17 Suppl 1,** S337-342.

Vayssiere, J. L., Cordeau-Lossouarn, L., Larcher, J. C., Basseville, M., Gros, F. and Croizat, B. (1992) Participation of the mitochondrial genome in the differentiation of neuroblastoma cells. *In vitro cellular & developmental biology : journal of the Tissue Culture Association,* **28A,** 763-772.

Virbasius, C. A., Virbasius, J. V. and Scarpulla, R. C. (1993) NRF-1, an activator involved in nuclear-mitochondrial interactions, utilizes a new DNA-binding domain conserved in a family of developmental regulators. *Genes & development,* **7,** 2431-2445.

Vire, E., Brenner, C., Deplus, R. et al. (2006) The Polycomb group protein EZH2 directly controls DNA methylation. *Nature,* **439,** 871-874.

Vithlani, M., Terunuma, M. and Moss, S. J. (2011) The dynamic modulation of GABA(A) receptor trafficking and its role in regulating the plasticity of inhibitory synapses. *Physiological reviews,* **91,** 1009-1022.

Wang, J., Zhuang, J., Iyer, S. et al. (2012) Sequence features and chromatin structure around the genomic regions bound by 119 human transcription factors. *Genome research,* **22,** 1798-1812.

Wang, J., Zhuang, J., Iyer, S. et al. (2013) Factorbook.org: a Wiki-based database for transcription factor-binding data generated by the ENCODE consortium. *Nucleic acids research,* **41,** D171-176.

Xi, H., Yu, Y., Fu, Y., Foley, J., Halees, A. and Weng, Z. (2007) Analysis of overrepresented motifs in human core promoters reveals dual regulatory roles of YY1. *Genome research,* **17,** 798-806.

Yanovsky, Y., Schubring, S., Fleischer, W. et al. (2012) GABAA receptors involved in sleep and anaesthesia: beta1- versus beta3-containing assemblies. *Pflugers Archiv : European journal of physiology,* **463,** 187-199.

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Yu, H., Bi, W., Liu, C., Zhao, Y., Zhang, J. F., Zhang, D. and Yue, W. (2014) Protein-interaction-network-based analysis for genome-wide association analysis of schizophrenia in Han Chinese population. *Journal of psychiatric research,* **50,** 73-78.

Yu, L. and Yang, S. J. (2010) AMP-activated protein kinase mediates activity-dependent regulation of peroxisome proliferator-activated receptor gamma coactivator-1alpha and nuclear respiratory factor 1 expression in rat visual cortical neurons. *Neuroscience,* **169,** 23-38.

Zinn-Justin, A. and Abel, L. (1999) Genome search for alcohol dependence using the weighted pairwise correlation linkage method: interesting findings on chromosome 4. *Genetic epidemiology,* **17 Suppl 1,** S421-426.

FIGURE LEGENDS

FIGURE 1. **Activity-dependent regulation of *NRF-1* and *Gabrb1* in primary cortical neurons.** Primary cortical neurons (DIV7-8) were treated with either Vehicle or 20 mM KCl for 6 hours. (A) Total protein was extracted from neurons and probed for the presence of NRF-1 and β-actin. A representative western blot is shown (*left*) for comparison. NRF-1 levels were quantified by densitometry and normalized to levels of β-actin. Levels of NRF-1 are expressed relative to vehicle (*right*) ($n = 6$, paired Students t-test, $p<.05$ as significant. (B) Levels of mRNAs were quantified by TaqMan qRT-PCR. Transcripts specific to *NRF-1* and *Gabrb1* were normalized to Cyclophilin A. Messenger RNA levels are expressed relative to vehicle treated neurons plated in a 6 well dish. Data represent the average ± SEM of $n = 6$ independent neuronal cultures with neurons extracted from different animals and plated on different days. *, $p < 0.05$, **, $p < 0.01$, Student's t-test. This dataset was originally published in the thesis of Li, Zhuting (2015) Activity-dependent gene regulation in neurons: energy coupling and a novel biosensor [dissertation]. [Boston (MA)]: Boston University.

FIGURE 2. Sequence alignment of the 5' promoter regions of β1 subunit genes. The β1 subunit promoters in mammals contain a conserved NRF-1 element, indicated in bold type upstream of the major initiator element (Inr) specific to each gene, underlined for reference. Sequences were aligned using ClustalW, where conserved nucleotides are as indicated "*". Modified from the figure originally published in the thesis of Li, Zhuting (2015) Activity-dependent gene regulation in neurons: energy coupling and a novel biosensor [dissertation]. [Boston (MA)]: Boston University.

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

FIGURE 3. ***In vitro and in vivo*** **binding of NRF-1 to the putative NRF-1 site in** ***GABRB1***. (A) ^{32}P-labeled probes encompassing the NRF-1 binding site were incubated with 20 μg of DIV7 primary rat cortical nuclear extracts. 100-fold excess of unlabeled probe was added to the binding reaction to assess specificity. NRF-1 Abs were pre-incubated with nuclear extracts and radiolabeled probe to test for "supershift" and protein identification. (Left Panel) The NRF-1 element in the rat cytochrome c (Cyt C) promoter displays NRF-1 specific binding (lane 2) and "supershift" (lane 4). (Right Panel) The proposed NRF-1 element in the human *GABRB1* promoter displays a probe specific shift (lane 6) (note that excess probe was run off of the gel to provide room for the detection of the shifted probe), competition of complex formation with cold competitor (lane 7), lack of competition with mutant cold competitor (lane 8), and supershift upon addition of NRF-1 specific Ab (lane 9). In contrast, binding to radiolabeled probe for NRF-1 mutant *GABRB1* shows markedly reduced signal strength (lanes 11 and 12). "*" indicates specific interaction between labeled probe and nuclear extract, "←" indicates location of supershift. (B) Chromatin Immunoprecipitation (ChIP) assays were performed using sonicated genomic DNA from DIV7 primary rat cortical neurons and either ChIP grade NRF-1 polyclonal antibody (Abcam, ab34682) that recognizes the full length protein or rabbit IgG (Vector Laboratories, I-1000). Co-precipitated *Gabrb1* gene promoter fragments were detected with specific qPCR primers and probe. Data represent the average ± SEM of n = 4 independent primary cultures and co-precipitations. *, $p < 0.05$, student t-test. (C) Representative ChIP-seq track from the Strand NGS software platform for *GABRB1* in H1-hESC cells after peak detection (MACS version 2.0). Read density profile plots of forward reads (blue) and reverse reads (red) aligned to the UCSC transcript model are depicted; each brown box represents a single 27-bp sequencing read. The NRF-1 motif sequence is shown in black text above its position. Relative position of the Inr in *GABRB1* is shown for reference. Chr: chromosome. A and B datasets were originally published in the thesis of Li, Zhuting (2015) Activity-dependent gene regulation in neurons: energy coupling and a novel biosensor [dissertation]. [Boston (MA)]: Boston University.

FIGURE 4. Evidence for the regulation of the GABAR β1 promoter by NRF-1. (A) Primary cortical neurons were co-transfected with 2 μg of wild type *GABRB1p* (*wtGABRB1p*) or the NRF-1 binding site mutant (*mGABRB1p*) and 1 μg of empty vector pcDNA3 or the NRF-1:VP16 fusion construct. Cells were assayed for luciferase activity 24 hours after transfection. Data represent the average ± SEM (n = 5 independent transfections) of luciferase activity relative to wild type *GABRB1p* in the absence of NRF-1:VP16. "*"and "ns" represent presence or absence respectively of significance based on $p < 0.05$ according to Student's t-test. (B) Primary cortical neurons were co-transfected with either pcDNA3 or the

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

dominant negative variant of NRF-1 (NRF-1 DN) and *GABRB1p* reporter (2 µg). Twenty-four hours after transfection, cells were assayed for luciferase activity. Data represent the average ± SEM of n = 6 independent transfections, normalized to *wtGABRB1p* and pcDNA3. *, $p < 0.05$, Student's t-test. This dataset was originally published in the thesis of Li, Zhuting (2015) Activity-dependent gene regulation in neurons: energy coupling and a novel biosensor [dissertation]. [Boston (MA)]: Boston University.

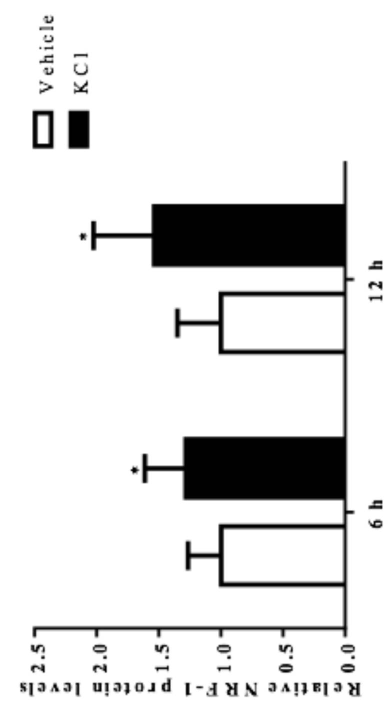
FIGURE 5. Overexpression of dominant negative NRF-1 attenuates the increase in β1 subunit mRNA levels in response to neuronal stimulation of primary cortical neurons. Primary cortical neurons were transfected with empty vector (pcDNA3) or dominant negative NRF-1 (NRF-1 DN) (using Nucleofection™) and plated in 6-well plates. DIV7 cells were treated with either vehicle or 20 mM KCl for 6 hrs. Total mRNA was isolated from cells and quantified by TaqMan qRT-PCR. *Gabrb1* mRNA expression was normalized to Cyclophilin A mRNA levels and is presented relative to its levels in pcDNA3 transfected neurons that were treated with vehicle (expressed as 1). Data represent the average ± SEM of n = 3 independent neuronal cultures. *, $p < 0.05$,**, $p < 0.01$, One-way ANOVA with Tukeys post hoc analysis.

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

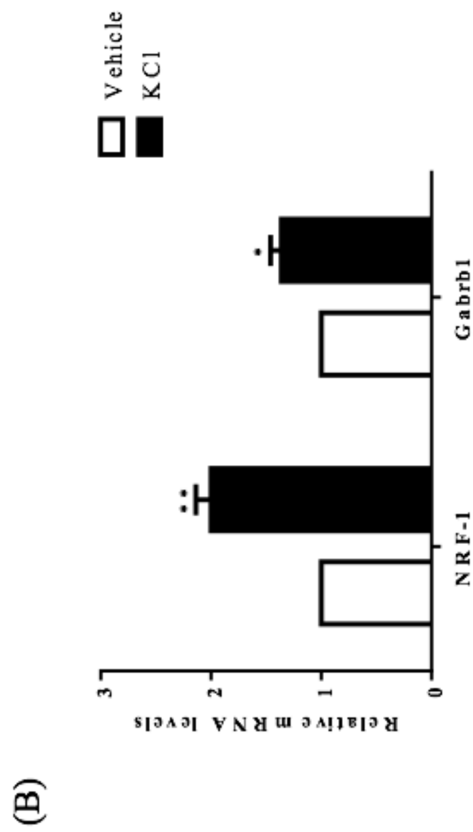
Figure 1.

(A)

Veh KCl Veh KCl
NRF-1
β-actin
6 h 12 h



(B)



bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Figure 2.

```
Species
Human      AACAGCAGCT TGTCTCTGAC TACCCGGAGG ACATGGAGCA CCCCAAATAG
Rat        GACAGCAGGT TGTATTCGAC TACCCAGAGA ACGCGGAGCA CCTCAGACAG
Mouse      GACAGCAGGT TGTATTCGAC TACCCAGAGA ACGCGGAGCA CCTCAGACAG
Chimpanzee AACAGCAGCT TGTCTCTGAC TACCCGGAGG ACATGGAGCA CCCCAAATAG
Marmoset   AACAGCAGCT TGTCTCTGAC TACCCGGAGG ACACGGAGAA CCCCAAAAAG
Cat        AAGAGCAGGA TATCTCCGAC TACCCAGAGG ACGTGCAGCA CCACAAACAG
Cow        AACAGCAGGA TATCTTTTAC TATC-GGAGA ACGCGGAGCA CCACAAACAG
            * *****   * * *   ** ** *  ***  **  * ** * ** ** * **
Species
Human      GAACTTTAGA GGGATTGAAA TCTGTTGCCT GTTCCACTAG GAATATTGTT
Rat        GAACTTTAAA GGGATTGAAA TCTGTTGCCT GTTCCACTAG GAATATTGTT
Mouse      GAACTTTAAA GGGATTGAAA TCTGTTGCCT GTTCCACTAG GAATATTGTT
Chimpanzee GAACTTTAGA GGGATTGAAA TCTGTTGCCT GTTCCACTAG GAATATTGTT
Marmoset   GAACTTTAGA AGGATTGAAA TCTGTTGCCT GTTCCACTAG GAATACTGTT
Cat        GAACTTTAAA GGGATTGAAA TCTGTTGCCT GTTCCACTAG GAATATTGTT
Cow        GAACTTTAAA GGAACTGAAA TCTGTTGCCT GTTCCACTAG GAATATTGTT
           ******** *  *** ***** ********** ********** ***** ****
Species                                NRF-1 Consensus: yG CGCAYGCGCr
Human      TGCAAGGCAC AAGGTGTCTT TTGGTAGTGA GCGCGCTCTG CGCATGCGCA
Rat        TGCAAGGCAC AAGGTGTCTT TTGGTAGTGA GTACACTCTG CGCATGCGCA
Mouse      TGCAAGGCAC AAGGTGTCTT TTGGTAGTGA GTACCCTCTG CGCATGCGCA
Marmoset   TGCAAGGCAC AAGGTGTCTT TTGGTAGTGA GCGCCCTCTG CGCATGCGCA
Chimpanzee TGCAAGGCAC AAGGTGTCTT TTGGTAGTGA GCGCGCTCTG CGCATGCGCA
Cat        TGCAAGGCAC AAGGTGTCTT TTGGTAGTGA GCACCCTCTG CGCATGCGCA
Cow        TGCAAGGCAC AAGGTGTCTT TTGGTAGTGA GCACCCTCTG CGCATGCGCA
           ********** ********** ********** *  * ***** **********
                            NRF-1 binding site mutant: yG CcCAYGgGCr

Species   Inr:YYANWYY
Human      GGTCCATTCG GGAATTACTG CCCAGCAGCC GACTAAGTTG CATTCCTTGA
Rat        GGTCCATTCG GGAATTACTG CCCAGCCGCC GACTAAGTTG CATTCCTTGA
Mouse      GGTCCATTCG GGAATTACTG CCCAGCCGCC GACTAAGTTG CATTCCTTGA
Chimpanzee GGTCCATTCG GGAATTACTG CCCAGCAGCC GACTAAGTTG CATTCCTTGA
Marmoset   GGTCCATTCG GGAATTACTG CCCAGCAGCC GACTAAGTTG CATTCCTTGA
Cat        GGTCCATTCG GGAATTACTG CCCTGCCGCC GACTAAGTTG CATTCCTTGA
Cow        GGTCCATTCG GGAATTACTG CCCTGCCGCC GACTAAGTTG CATTCCTTGA
           ********** ********** *** ** *** ********** **********
Species                                           Met
Human      ATCTTCGCAG AAAAGACAAT TCTTTTAATC AGAGTTAGTA ATG
Rat        ATCTTCGCAG AAAAGACAAT TCTTT-CATC AGAGTTAGTA ATG
Mouse      ATCTTCGCAG AAAAGACAAT TCTTT-CATC AGAGTTAGGA ATG
Chimpanzee ATCTTCGCAG AAAAGACAAT TCTTTTAATC AGAGTTAGTA ATG
Marmoset   ATCTTCGCAG AAAAGACAAT TCTTT-AATC AGAGTTAGTA ATG
Cat        ATCTTCGCAG AAAAGACAAT TCTTT-AATC AGAGTTAGTA ATG
Cow        ATCTTCGCAG AAAAGACAAT TCTTT-AATC AGAGTTAGTA ATG
           ********** ********** *****  *** ******** * ***
```

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

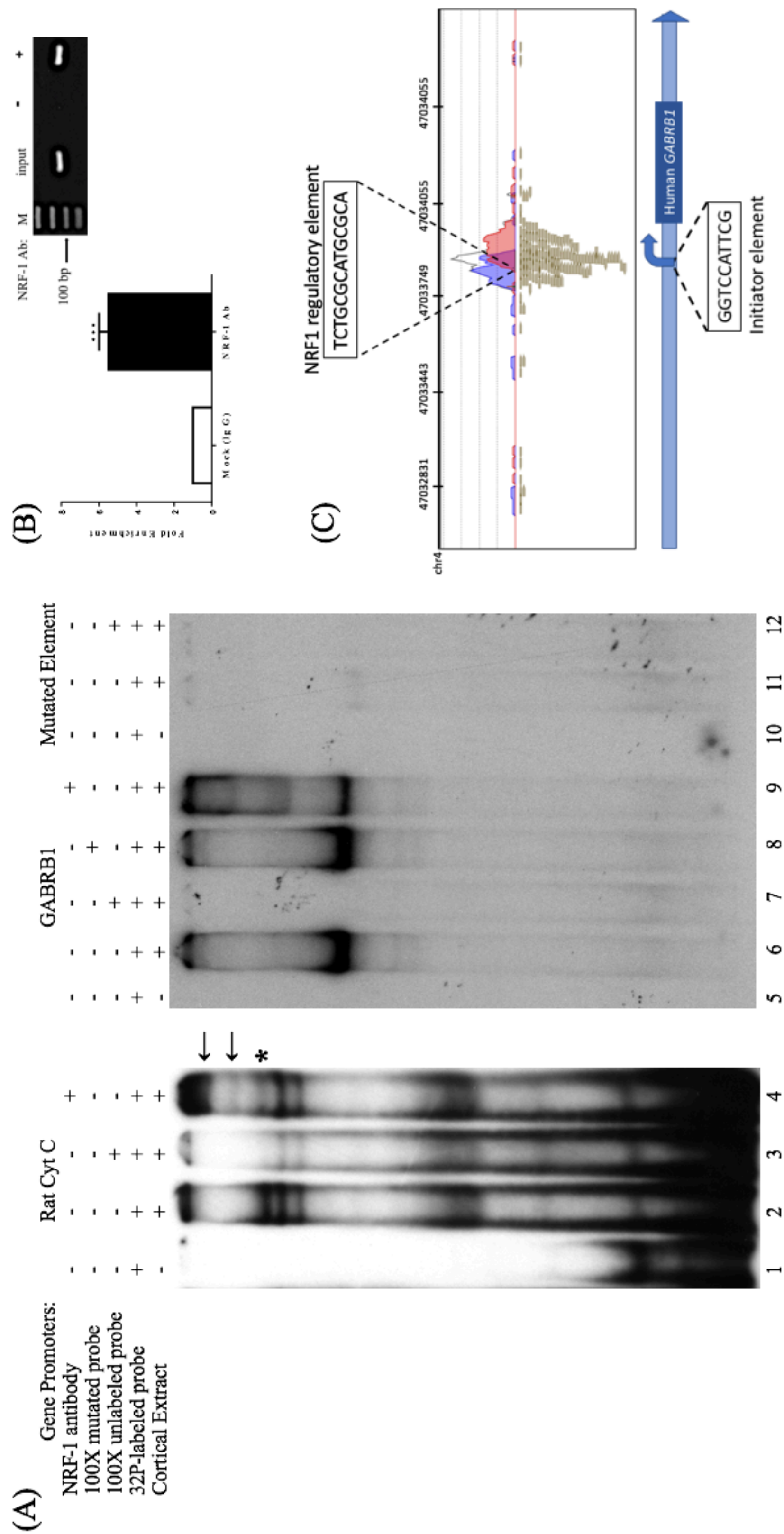
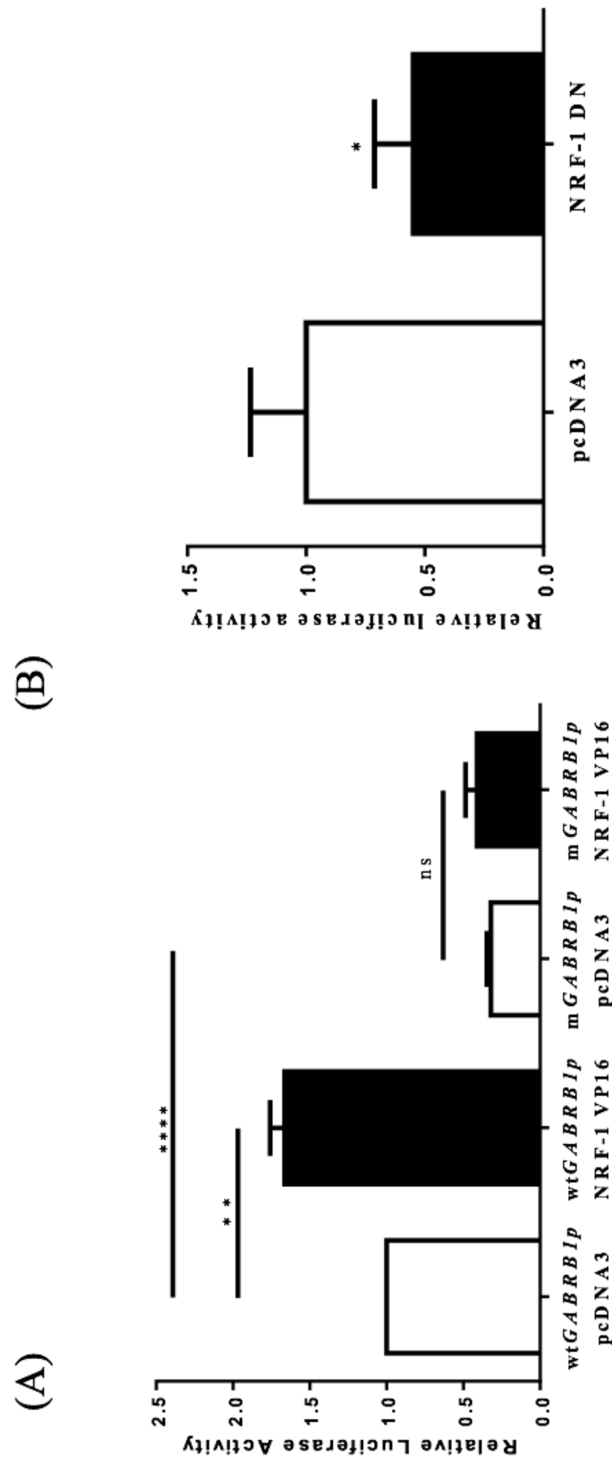


Figure 3.

bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Figure 4.



bioRxiv preprint doi: https://doi.org/10.1101/321257; this version posted May 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Figure 5.