Reelin protein is increased in the hypothalamus of diet-induced obesity (DIO) mice and has direct actions on arcuate proopiomelanocortin (POMC) neurons

Brandon L. Roberts[1]*, Camdin Bennett[1], Louise Dalboege[2], Baylin Bennett[1,2]
Kevin Grove[2] and Paul Kievit[1]

[1]Division of Cardiometabolic Health, Oregon National Primate Research Center, Beaverton, OR 97006, USA;
[2] Obesity Research Center, Novo Nordisk, Seattle, WA 98109, USA

INTRODUCTION

The prevalence of obesity is increasing at a disquieting rate, afflicting roughly one-third of the U.S. adult population [1]. Over a quarter-million deaths per year are attributed to obesity, which has a paucity of available therapeutics [2-4].

The arcuate nucleus of the hypothalamus (ARH) is a critical brain region involved in the homeostatic regulation of energy metabolism, food intake and hedonic drive [5, 6].

Proopiomelanocortin (POMC) neurons are one of two primary neuronal phenotypes in the ARH that have been characterized extensively in regard to their role in the regulation of energy homeostasis [7]. POMC neurons are heavily involved in energy homeostasis, thermogenesis in brown adipose tissue, long-term satiety pathways, and also potentiate fast-acting satiety pathways [5, 8].

Reelin (RELN) is a large glycoprotein, which is best characterized for its role in neuronal migration and organization throughout development [9]. RELN is also implicated in several brain pathologies including autism spectrum disorder, schizophrenia, anxiety disorders and bipolar disorder [10, 11, 12]. Diet-induced obesity (DIO) reduces hippocampal RELN expression, however, its role in neuronal pathways associated with energy homeostasis has yet to be explored [13].

Full-length RELN (411 kDa) has 6 unique repeats and is cleaved into three primary fragments, the N-terminus (NT; N-R2, 180kDa), central fragment (CF; R3-6, 164kDa), and the C-terminus fragment (CT; R7-C, 80kDa) [14, 15]. Two receptors have a high-affinity for both the CF and FL forms of RELN, apolipoprotein E receptor 2 (ApoER2) and very-low density lipoprotein receptor (VLDLR) [16].

Here we aim to elucidate the actions of RELN-CF on ARH-POMC neurons and explore its role in glucose homeostasis and food intake.

RESULTS:

Diet-induced obesity (DIO) increases reelin protein in the mouse hypothalamus

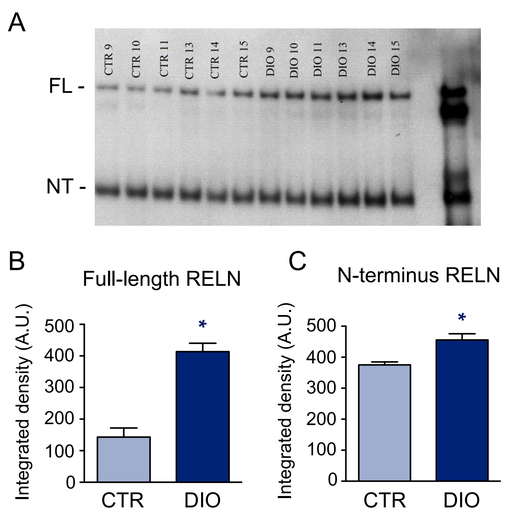


Figure 1. ***A***, Western blot showing reelin (RELN) protein of control (CTR; *n* = 6) and DIO (*n* =6) ***B***, Average integrated density (A.U.) of full-length (FL) RELN protein levels in CTRL and DIO mice. ***C***, Average integrated density (A.U.) of N-terminus (NT) RELN protein levels in CTRL and DIO mice.

Hypothalamic ApoER2 and VLDLR expression is decreased in DIO mice

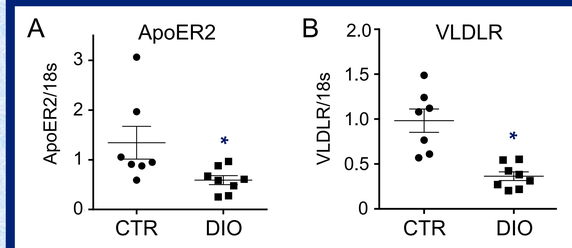


Figure 2. ***A***, Relative hypothalamic expression of ApoER2 in CTR (*n* = 7) and DIO (*n* = 8) mice. ***B***, Relative hypothalamic expression of VLDLR in CTR (*n* = 7) and DIO (*n* = 8) mice. Individual data points shown for each mouse.

Reelin CF has two distinct effects on action potential firing and membrane potential in arcuate POMC-GFP neurons

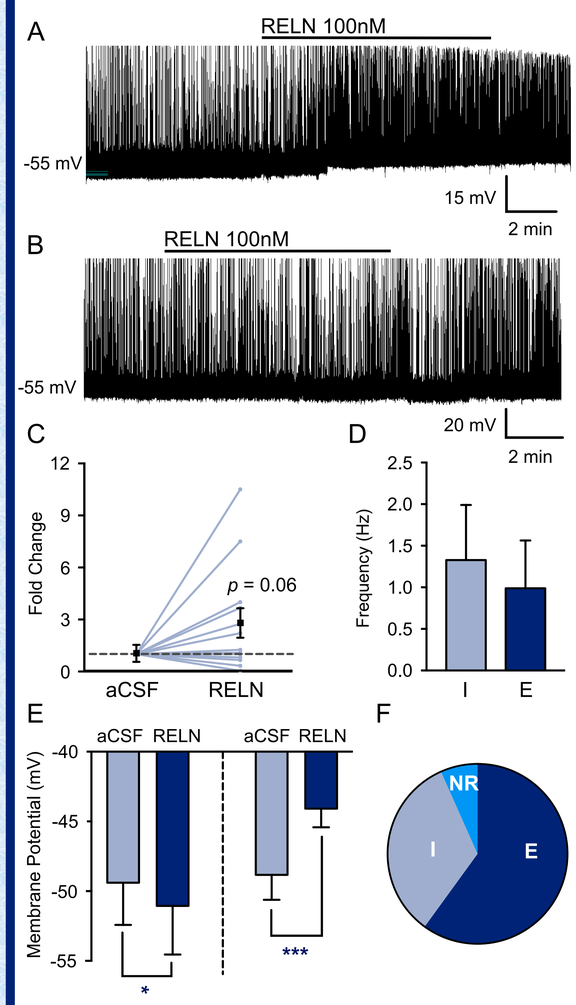


Figure 3. ***A***,***B***, Representative traces showing the firing rate and membrane potential before and after bath application of RELN (100nM) in a depolarizing (***A***) and hyperpolarizing (***B***) POMC-GFP neuron. ***C***, Normalized frequency of action potential firing from individual POMC-GFP neurons (*n* = 15) before and after RELN treatment (Bold line, cumulative mean change; pairwise analysis). ***D***, Baseline action potential firing frequency of RELN inhibited (I, *n* = 5) and excited (E, *n* = 9) neurons ***E***, Mean membrane potential in RELN inhibited (left) and excited (right) neurons ***F***, Distribution of RELN inhibited, excited and non-responsive (NR; *n* = 1) neurons.

Reelin CF has direct actions on arcuate POMC-GFP neurons

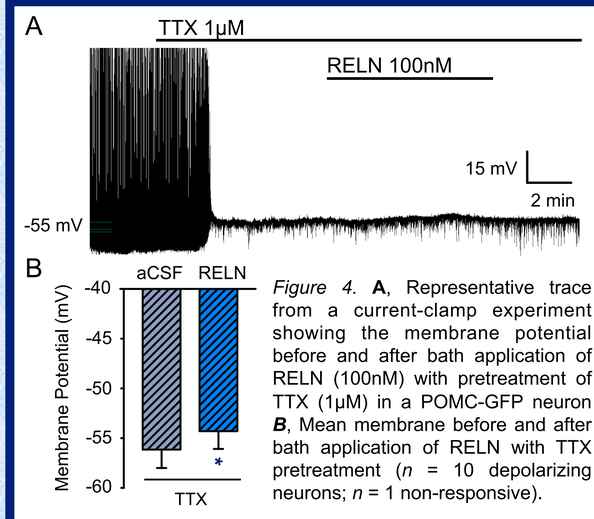


Figure 4. **A**, Representative trace from a current-clamp experiment showing the membrane potential before and after bath application of RELN (100nM) with pretreatment of TTX (1μM) in a POMC-GFP neuron ***B***, Mean membrane before and after bath application of RELN with TTX pretreatment (*n* = 10 depolarizing neurons; *n* = 1 non-responsive).

Reelin CF alters pre- and post-synaptic inputs onto arcuate POMC-GFP neurons

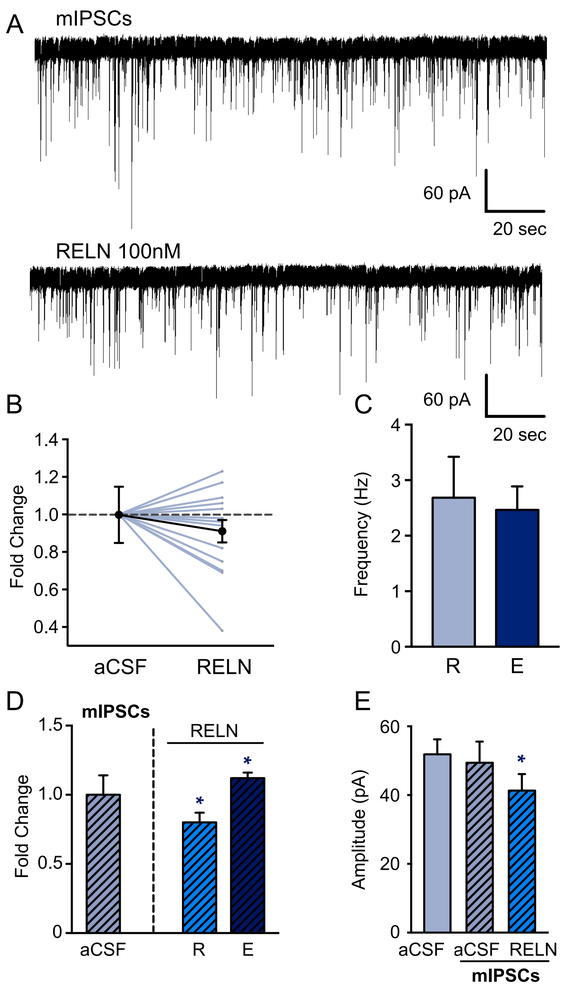


Figure 5. ***A***, Representative trace from a voltage-clamp experiment showing reduced mIPSC frequency and amplitude after bath application of RELN (100nM) in a POMC-GFP neuron. ***B***, Normalized mIPSC frequency (*n* = 14) before and after treatment of RELN (100nM) (Bold line, cumulative mean change; pairwise analysis) ***C***, Baseline mIPSC frequency (Hz) of neurons which RELN reduced (R; *n* = 5) or enhanced (E; *n* = 9) mIPSC frequency (Hz) ***D***, Fold change of mIPSC frequency in RELN (R; left) and (E; right). (*aCSF error bar represents pooled between-cell mIPSC variance from all neurons*) ***E***, Mean amplitude of mIPSCs before and after application of RELN ion (*n* = 14).

Central administration of reelin CF does not alter glucose homeostasis or food intake

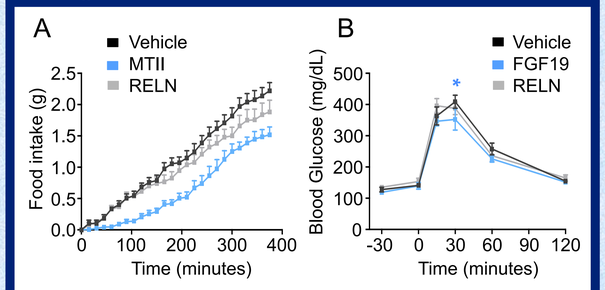
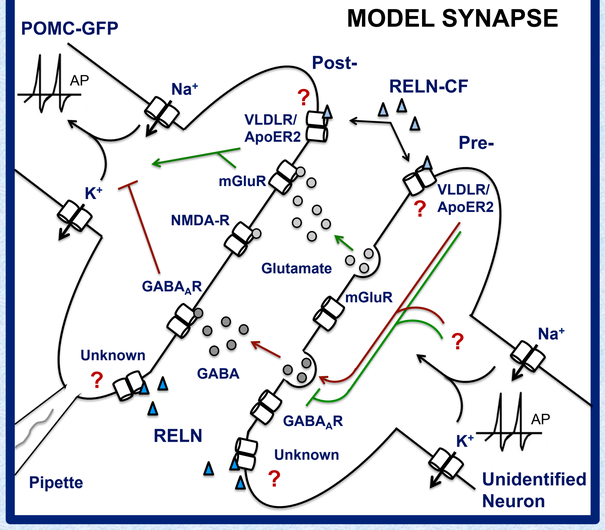


Figure 6. **A**, Cumulative food intake after third ventricle i.c.v. administration of vehicle (*n* = 10), MTII (0.5nM; *n* = 10) or RELN (0.3nM; *n* = 9) ***B***, Blood glucose time-response curve (-30 – 120 min) in response to third ventricle i.c.v. administration of vehicle (*n* = 10), FGF19 (0.14nM; *n* = 10) or RELN (0.3nM; *n* = 10).

SUMMARY

Here we show that:

- DIO increases RELN protein in the mouse hypothalamus
- ApoER2 and VLDLR expression are decreased in the hypothalamus of DIO mice
- RELN has two distinct effects on ARH-POMC neurons
- RELN acts pre- synaptically via a GABAergic mechanism and has post-synaptic actions onto POMC neurons
- RELN given i.c.v does not effect short-term food intake or glucose tolerance



METHODS

Western Blot. Hypothalamic protein samples were denatured and separated on Tris-Acetate gels. Membranes were incubated with Ms-α-reelin primary antibody (1:500) ON at 4C in 1% BSA prepared in TBST. Gels were washed four times for 5 minutes each in TBST prior to incubation with a Donkey α-Ms secondary antibody (1:10,000) in TBST containing 5% non-fat milk protein for 1h at RT. Band intensity was determined using scanning densitometry.

qPCR. Hypothalami were blocked from control and DIO mice. Trizol and RNeasy mini kit were used to isolate RNA. Reverse transcriptase reactions were prepared with a cDNA Synthesis Kit (Promega). Gene expression was measured using TaqMan probes for ApoER2, VLDLR and 18s (control). Real-time PCR was run using a BioRad CFX384.

Brain Slice Preparation. Coronal slices from POMC-GFP mice were cut to preserve the ARH. Whole cell recordings were made using an external bath solution containing: (mM) 124 NaCl, 5 KCl, 2.6 NaH_2PO_4, 10 HEPES, 2 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, 5 Dextrose and bubbled with 95% O2 / 5% CO2 30-34 C; pH=7.3, adjusted to 305-315 mOsm using sucrose. The internal current-clamp recording solution contained (mM): 125 K-Gluconate, 2 KCl, 5 HEPES, 10 EGTA, 5 MgATP, 0.25 NaGTP; the voltage-clamp internal recording solution contained: 140 CsCl, 5 $MgCl_2$, 1 BAPTA, 10 HEPES, 5 MgATP, 0.25 NaGTP, pH=7.3, 295-305 mOsm. Application of TTX, CNQX and AP-5 was used to record mIPSCs. Neurons were recorded from the lateral ARH. Only neurons not exceeding holding currents of 50 pA at V_H= -60 mV for the 10min control period (input resistance > 120 MΩ) were studied further.

Food intake. Cannulas were placed i.c.v. in the third ventricle. Animals were randomized according to body weight and food intake. Mice were dosed i.c.v. (2μl) 1h prior to lights out and food intake data collected over 24h using a BioDAQ system. Mice were fasted 4h prior to the i.c.v. injection.

Blood glucose. Food is removed 5h prior to the i.p. glucose load (2 g/kg dose volume: 10 ml/kg) at t=0 . Test compounds are administered i.c.v. at t =−30min, and blood glucose is measured at time points t =−30 (before dosing), 0 (before i.p. glucose), 15, 30, 60, and 120 min.

Mice. All mice were on a C57Bl/6J background. Mice were 8-14 weeks old at time of cell recordings. DIO animals were placed on a 60% HFD for 12-16 weeks before tissue collection.

Statistics. WB and PCR data were analyzed with a student t-test test. For recordings, within cell analysis was determined using Kolmogorov-Smirnov test and between cell analysis using a one-way ANOVA with Tukey's post-hoc analysis. For *in vivo* behavior experiments a two-way ANOVA with Bonferroni's post-hoc against vehicle was used. Error bars indicate SEM; **p* < 0.05, ****p* < 0.0001 denotes a significant change.

REFERENCES

1. **General, O.o.t.S.** (2010). Dept. of Health and Human Services: Rockville, MD, U.S.
2. WMJ (1998). **97**(9): p. 20-1, 24-5, 27-37.
3. **Allison, D.B., et al.**, JAMA, (1999). **282**(16): p. 1530-8.
4. **Mokdad, A.H., et al.** JAMA, (2004). **291**(10): p. 1238-45.
5. **Schwartz, M.W.**, Exp Biol Med (2001) **226**(11): p. 978-81.
6. **Lutter, M. and E.J. Nestler,** J Nutr, (2009).
7. **Jong-Woo Sohn, J.K.E., Kevin W. Williams.,** Trends in Neurosci (2013). **36**(9): p. 504-512.
8. **Fenselau, H., et al.,** Nat Neurosci, (2016).
9. **Tissir, F. and A.M. Goffinet,** Nat Rev Neurosci, (2003). **4**(6): p. 496-505.
10. **Baker, K.D. and A.C. Reichelt.,** Neurobiol Learn Mem, (2016). **136**: p. 127-138.
11. **Wang, Z., et al.,** Am J Med Genet B Neuropsychiatr Genet, (2014). **165B**(2): p. 192-200.
12. **Ovadia, G. and S. Shifman,** PLoS One, (2011). **6**(5): p. e19955.
13. **Reichelt, A. et al.,** Brain, Behav, and Immunity, (2015). **43**: p. 68-75.
14. **Lee, G.H.,** J Biol Chem, (2014) **289**(29): p. 20307-17.
15. **Jossin, Y., L. Gui, and A.M. Goffinet,**. J Neurosci, (2007). **27**(16): p. 4243-52
16. **D'Arcangelo G, et al.,** Neuron (1999). **24**:471–479.

SUPPORT

Thanks to Novo Nordisk for producing and supplying reelin central fragment protein. This work was supported by SRA 11-061-E and 15-088 from Novo Nordisk to PK.