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Supplemental information

NOVA1 acts on *Impact* to regulate hypothalamic function and translation in inhibitory neurons

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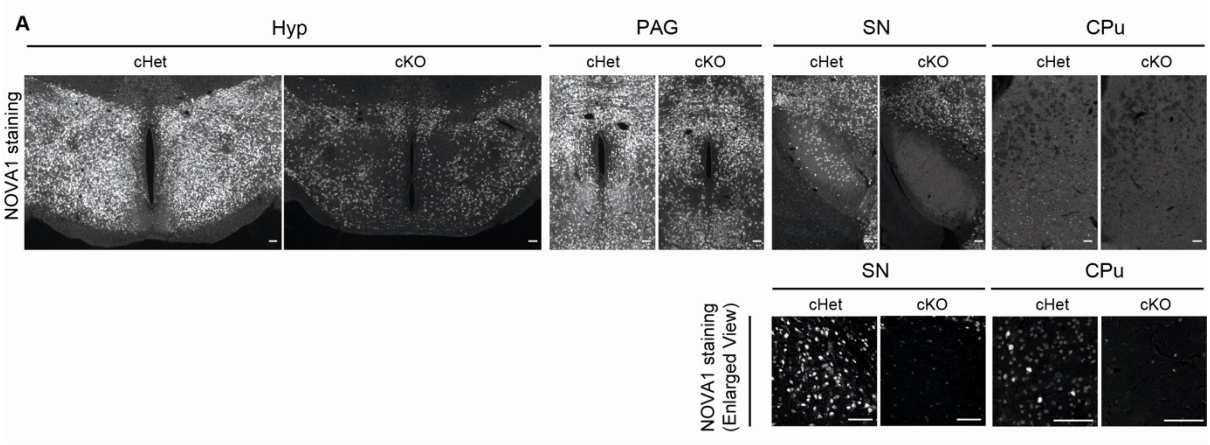
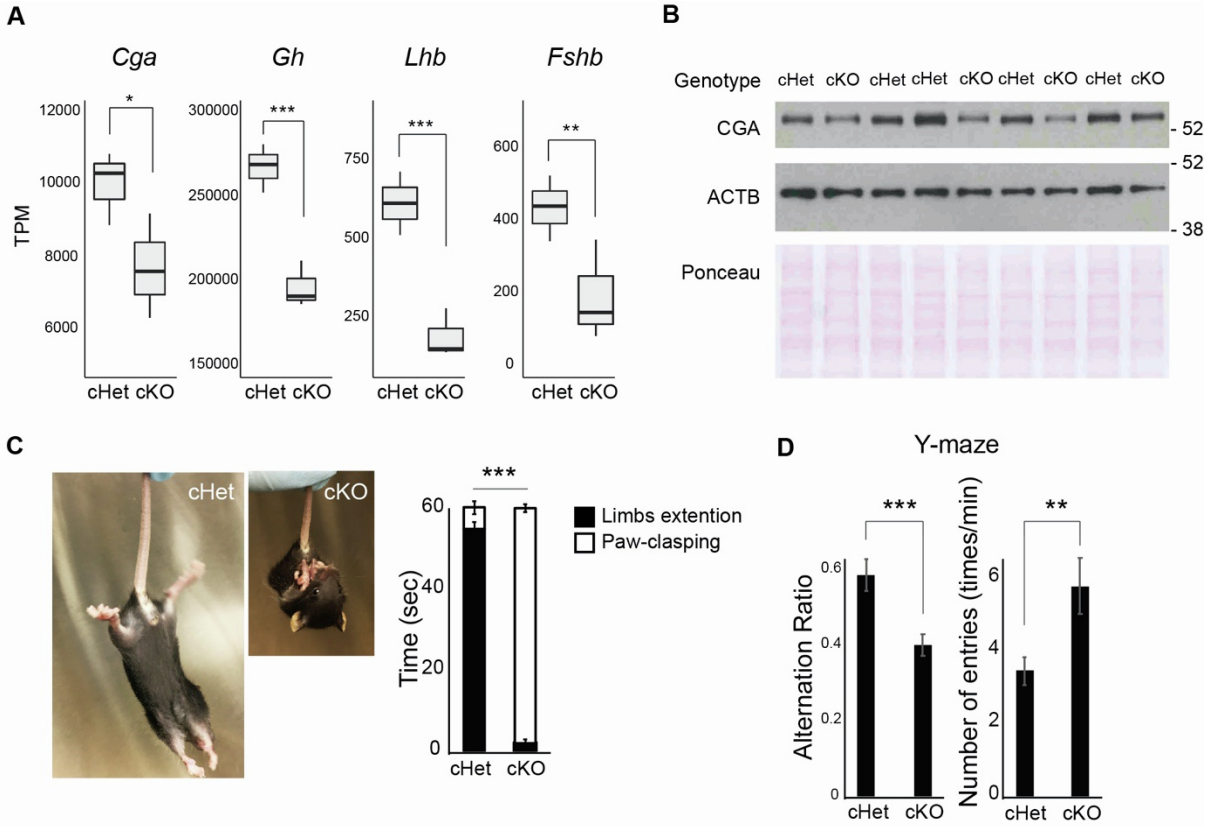
Supplemental Figure 1



Supplemental Figure 1. Immunostaining for NOVA1 in each brain region, related to Figure 1.

A. Magnified view of Figure 1D: P21 cHet (*Gad2*^{cre}-*Nova1*$^{f/w}$) and cKO (*Gad2*^{cre}-*Nova1*$^{f/f}$) mouse brain immunostained with NOVA1 antibody. Areas with significant NOVA1 depletion are presented. Hyp = Hypothalamus, SN = Substantia nigra, PAG = Periaqueductal gray, CPu = Caudate putamen. Upper panel: 40x magnification, Lower panel: 200x magnification. Scale bar = 100μm.

Supplemental Figure 2



Supplemental Figure 2. Expression of endocrine hormones, behavioral deficits, and learning capabilities in cKO mice, related to Figure 2.

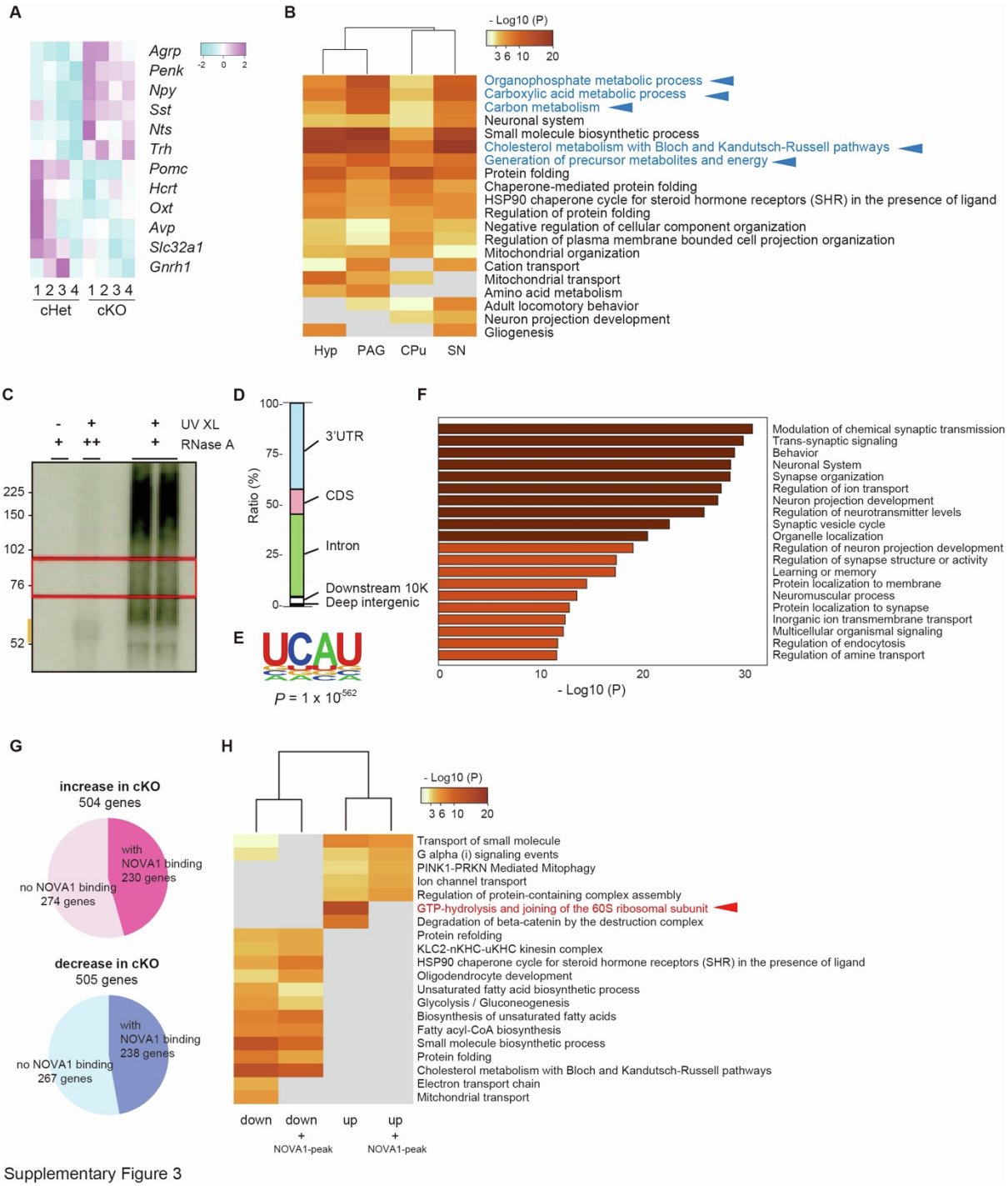
A. Box-and-whisker plots of significantly altered transcripts encoding pituitary hormones from RNA seq data of the pituitary gland of 3-week-old mice. Median values are indicated as horizonal lines with 25th – 75th percentiles and whiskers (minimum and maximum values). (*n*=4 per group, *Wilcoxon* test, *P<0.05, **P<0.01, ***P<0.005). Cga: chorionic gonadotropin, alpha subunit; Gh: growth hormone; Lhb: luteinizing hormone, beta subunit: Fshb: follicle stimulating hormone, beta subunit.

B. Western blot analysis of pituitary gland lysates from 3-week-old mice against anti-Cga antibody (cHet *n*=5, cKO *n*=4). Western blot analysis of beta-Actin and ponceau S stainings are displayed as loading control.

C. Paw-clasping phenotype. cKO mouse curls its limbs into bat-like posture when lifted. (Right) Quantified time spent in the paw-clasping state per minute (mean ± SEM, cHet *n*=12, cKO *n*=8, unpaired *t*-test, ***P<0.005).

D. Y-maze test. 8- to 12-week-old mice were allowed to freely explore a Y-shaped maze for 8 minutes. The number of entries into the arms and the number of triads were recorded to calculate the percentage of alternation. Alternations are consecutive entries into each arm of the Y-maze without any repeats (mean ± SEM, cHet *n*=7, cKO *n*=8, unpaired *t*-test, **P<0.01, ***P<0.005).

Supplemental Figure 3



Supplementary Figure 3

Supplemental Figure 3. Gene expression analysis in the hypothalamus of cKO mice, related to Figure 3.

A. Heatmap of neuropeptide/ hormone genes significantly affected in the hypothalamus of cKO mice (*n*=4 per group).

B. Gene ontology analysis of genes significantly downregulated in cKO relative to

control (cHet) in P21 hypothalamus (Hyp), substantia nigra (SN), periaqueductal gray (PAG) and caudate putamen (CPu) RNAseq data. Terms involved in metabolism are shown in blue.

C. HITS-CLIP analysis of NOVA1 in P21 mouse diencephalon/mesencephalon. Anatomically removed mice diencephalon/mesencephalon were used as samples. Protein gel autoradiograph of ^{32}P-labeled complex of NOVA1-RNA: The yellow line indicates NOVA1 molecular size, and the red square indicates the portion that was cut out and sequenced.

D. Bar graph of NOVA1-CLIP peak distribution on transcriptome.

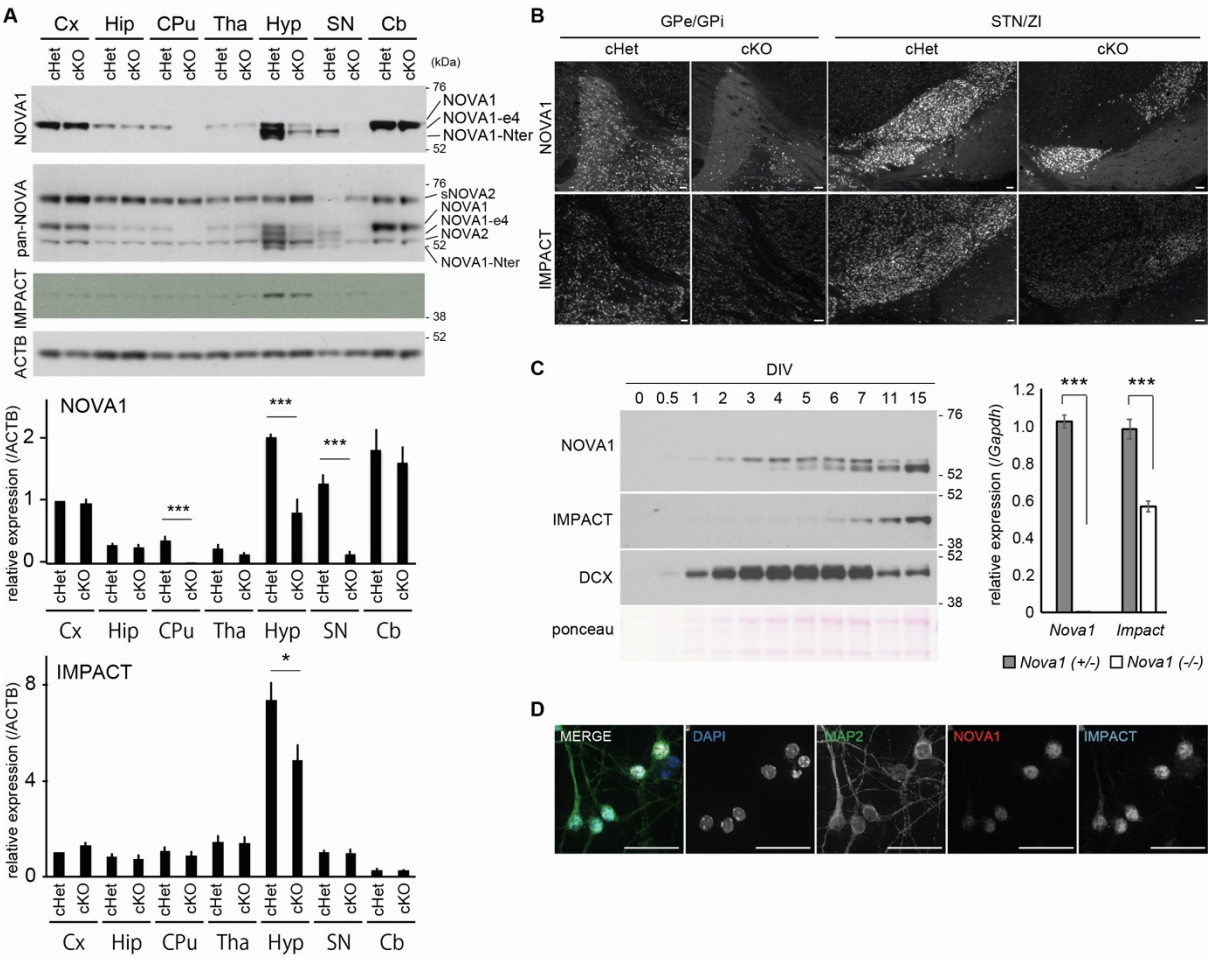
E. Motif analysis of NOVA1-CLIP peak.

F. GO analysis of genes with NOVA1 binding peak. The top 500 genes were analyzed in order of number of binding.

G. Pie charts showing the number of genes with NOVA1-CLIP peaks that were significantly altered in hypothalamus between cHet and cKO mice RNAseq data.

H. Gene ontology analysis of genes whose expression was significantly altered in cKO compared to control (cHet) in P21 hypothalamus (Hyp) RNAseq data. '+NOVA1 peak' is an analysis of those genes that were altered and had NOVA1 binding peaks on the transcripts.

Supplemental Figure 4



Supplemental Figure 4. Expression analysis of NOVA1 and IMPACT in brain and primary neuron culture, related to Figure 4.

A. Immunoblot of lysates prepared from dissected brain regions of adult mice (upper panel). NOVA1-e4: NOVA1 without exon 4. NOVA1-Nter: Nova1 isoform lacking canonical N-terminus. sNova2: superNova2 (an isoform of Nova2). Quantification of NOVA1 and IMPACT immunoblot analysis (lower panel). Each bar represents the average of three independent trials (mean ± SEM, unpaired *t*-test, *P<0.05, ***P<0.005). The immunoblot data of NOVA1 and beta-Actin are the same data as in Figure 1C.

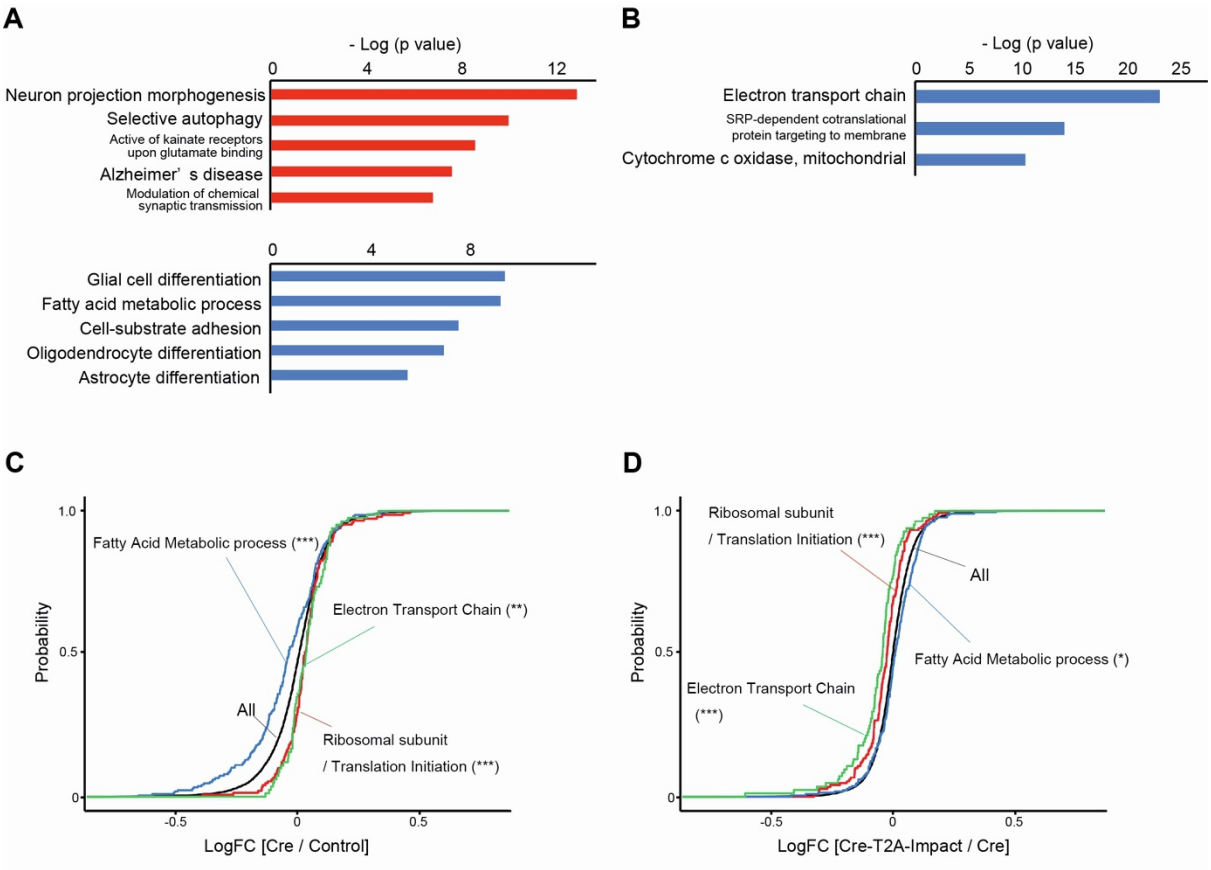
B. Immunostaining for NOVA1 and IMPACT in the brain of 12-week-old mice. Serial sections were used in each genotype. GPe: external globus pallidus. GPi: internal globus pallidus. STN: subthalamic nucleus. ZI: zona incerta. 40x magnification. Scale bar = 100μm.

C. (Left) Immunoblot of lysates prepared from primary neurons derived from WT E13.5 diencephalon/mesencephalon. Changes for NOVA1 and IMPACT expression during culture for 15 days in vitro (DIV) were monitored. Doublecortin (DCX) is used as an

immature neuron marker, and ponceau S staining as a measure of total protein per lane. (Right) Quantitative PCR analysis of *Nova1* and *Impact* at DIV15 in primary neurons prepared from wild-type and Nova1-deficient mice (mean ± SEM, n=3, unpaired *t*-test, ***P<0.005).

D. Immunostaining of NOVA1 and IMPACT on day 15 of culture. 400x magnification. Scale bar = 30μm.

Supplemental Figure 5



Supplemental Figure 5. Gene expression changes in primary neurons upon loss of *Nova1* and induction of *Impact* expression, related to Figure 5

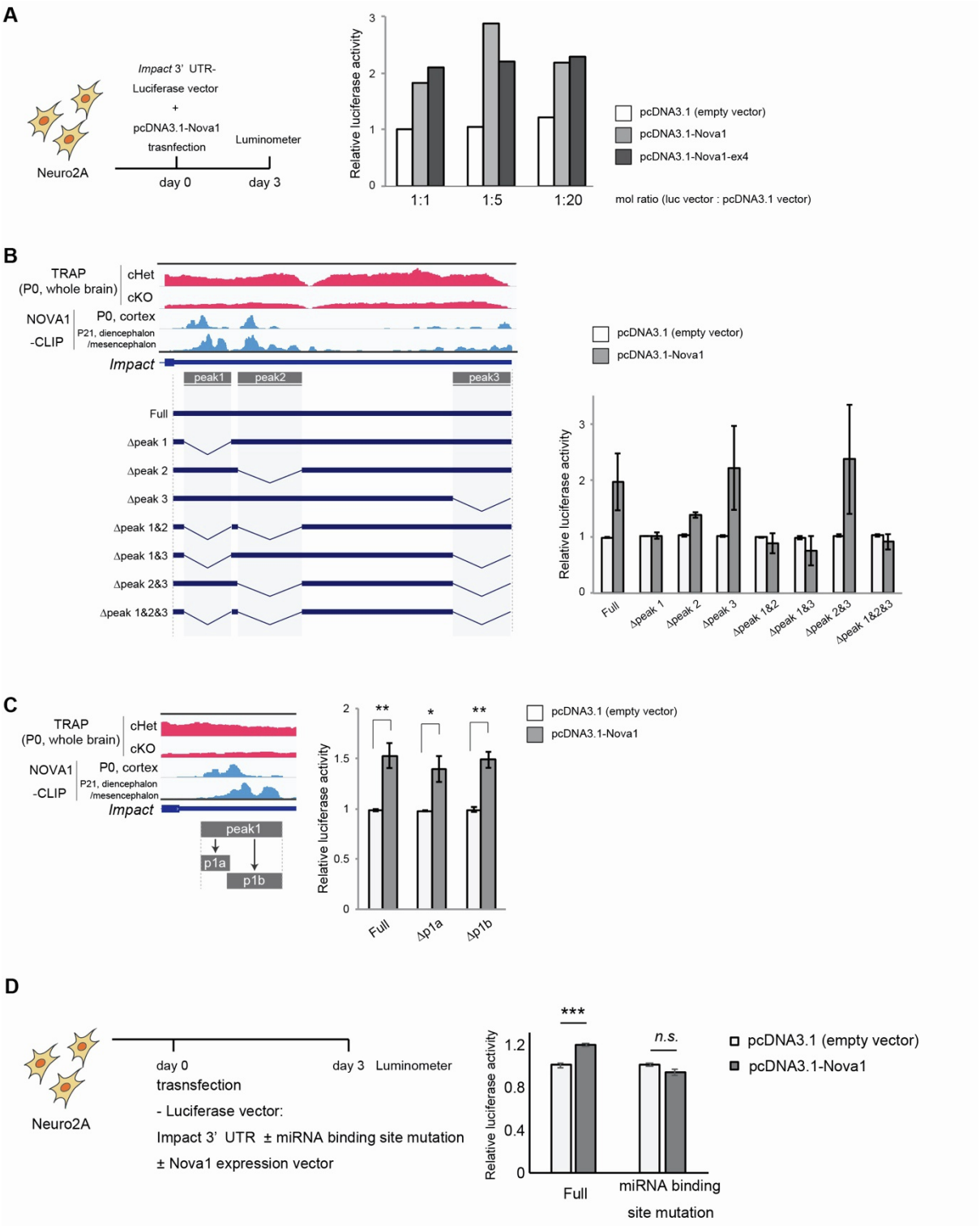
A. GO analysis of genes upregulated (top) and downregulated (bottom) when *Nova1* is deleted in primary neurons of e13.5 mice diencephalon/mesencephalon (related to Figure 5C left).

B. GO analysis of genes whose expression was downregulated in primary neurons of the mouse diencephalon/mesencephalon when Impact gene was overexpressed in *Nova1*-deficient cells (related to Figure 5C right).

C. Empirical cumulative distribution function (CDF) plot of *Nova1* loss in primary neuron culture. All expressed genes are shown as solid black lines, fatty acid metabolic pathways are shown in blue, ribosomal subunits and translation initiation are shown in red, and translation elongation and termination are shown as dotted black lines. *p*-values determined by Mann-Whitney-U test (**P<0.01, *** P<0.005).

D. CDF plot of overexpression of *Impact* in *Nova1*-deficient primary neurons. *p*-values determined by Mann-Whitney-U test (*P<0.05, *** P<0.005).

Supplemental Figure 6



Supplemental Figure 6. Luciferase reporter assays using deletion or substitution constructs on Impact 3'UTR, related to Figure 6.

A. Effect of *Nova1* on the regulation of Impact expression in Neuro2a cells. Luciferase

construct containing *Impact*-3'UTR was transfected into Neuro2a cells together with *Nova1* expression vector at indicated molar ratio. Luciferase activity was measured 3 days after transfection. Each value represents the ratio of Firefly luciferase activity over Renilla luciferase and all samples were normalized to that of the empty vector control.

B. Luciferase assay using constructs containing various deletion mutants of *Impact*-3'UTR. Using NOVA1-CLIP peak as a reference, NOVA1-binding region on *Impact*-3'UTR was divided into three regions (peak1, 2 and 3), and constructs were prepared by deleting each region, then cloned into a Luciferase vector. These constructs were transfected into Neuro2a cells together with *Nova1*-expressing vector. Each value was normalized to that of control empty vector-transfected sample (mean ± SEM, n=3).

C. Luciferase assay containing deletion mutants with a finer dissection of peak 1 into two parts (p1a and p1b). The constructs were transfected into Neuro2a cells together with *Nova1*-expressing vector. Each value was normalized to that of control empty vector transfected sample (mean ± SEM, n=3, unpaired t-test, *P<0.05, **P<0.01).

D. Luciferase assay using Impact-3'UTR with mutations in the miRNA binding sites upon Nova1 overexpression. Luciferase construct and Nova1-expression vector were transfected into Neuro2a cells, and the luciferase activity was measured 3 days after transfection. Each value represents the ratio of Firefly Luciferase over Renilla Luciferase activity. Values of the samples were normalized to that of the full-length Impact 3'UTR-transfected sample (mean ± SEM, n=3, unpaired t-test, ***P<0.005).