Figure S1

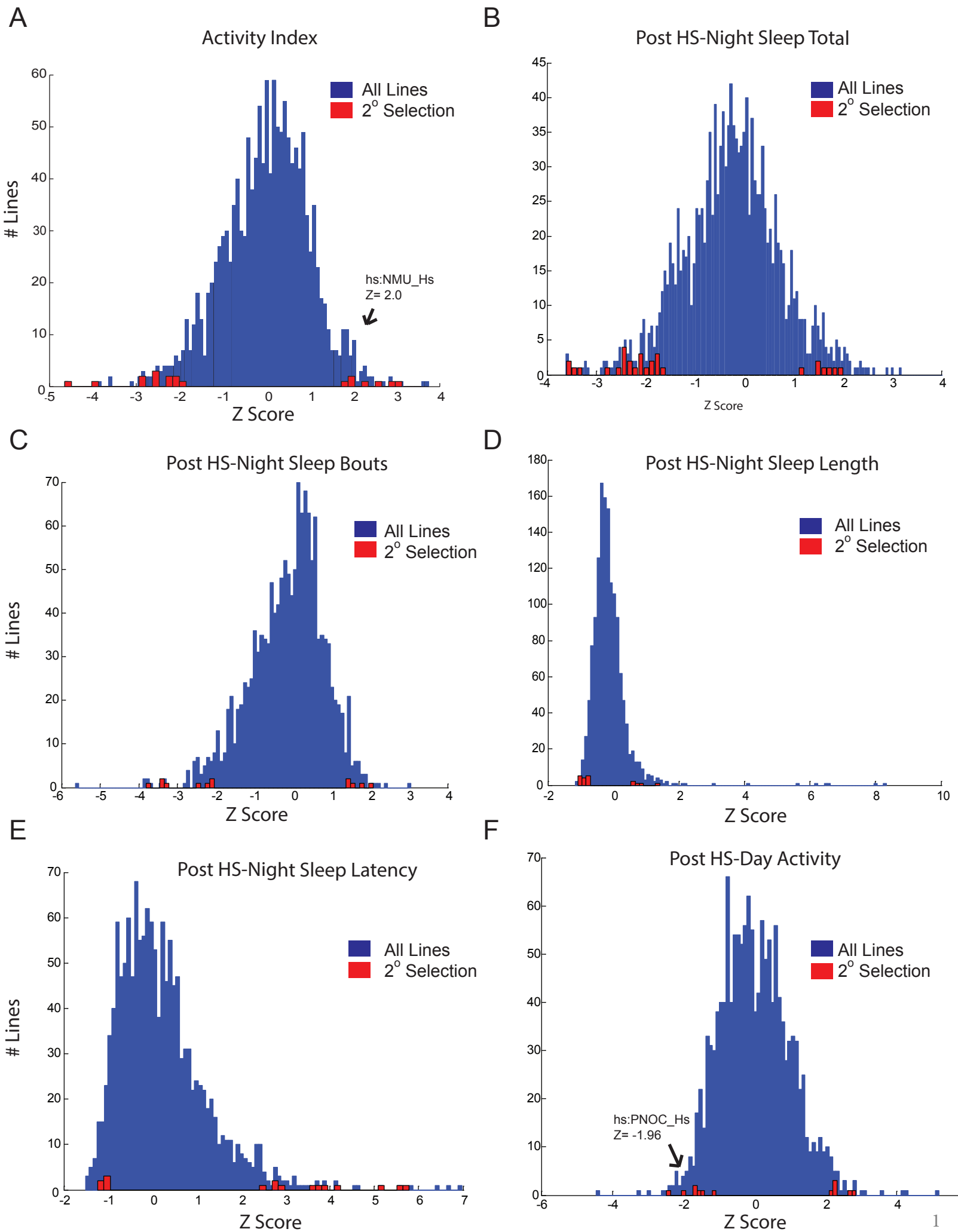
A
Activity Index
All Lines
2° Selection
Lines
hs:NMU_Hs
Z= 2.0
Z Score
B
Post HS-Night Sleep Total
All Lines
2° Selection
Z Score
C
Post HS-Night Sleep Bouts
All Lines
2° Selection
Lines
Z Score
D
Post HS-Night Sleep Length
All Lines
2° Selection
Z Score
E
Post HS-Night Sleep Latency
All Lines
2° Selection
Lines
Z Score
F
Post HS-Day Activity
All Lines
2° Selection
hs:PNOC_Hs
Z= -1.96
Z Score

Figure S2

A

```
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Nmu_Dr d4  ATGAGGAACAGCAATCAATGTGAACGCGCAACCGCTCACAGCGCGATGAGCCCGGGAAACACCTCCGCCCT----CTCGC 76

Nmu_Dr     GGTACTACTCCTCTCCTTCATACCCATCACCACAAGTGCTCCGATGCTCTTGAATCCATCTTCACTAGAGCATGAGCAGC 160
Nmu_Dr d4  GGTACTACTCCTCTCCTTCATACCCATCACCACAAGTGCTCCGATGCTCTTGAATCCATCTTCACTAGAGCATGAGCAGC 156

Nmu_Dr     TACTAACCCAGATAACTGATTTGTGTTCATTCTACCTCTCCGCAGACCCGTCCTTTAGAACATCTGACGTCCTGGAGGAC 240
Nmu_Dr d4  TACTAACCCAGATAACTGATTTGTGTTCATTCTACCTCTCCGCAGACCCGTCCTTTAGAACATCTGACGTCCTGGAGGAC 236

Nmu_Dr     CTGTGTTTCCTAATGCTGGGATCACTGCAAAAATCGAAGGAGATCACAGCTCGAGAGACTAGCAAAAGGTTTTTATTTCA 320
Nmu_Dr d4  CTGTGTTTCCTAATGCTGGGATCACTGCAAAAATCGAAGGAGATCACAGCTCGAGAGACTAGCAAAAGGTTTTTATTTCA 316

Nmu_Dr     TTACACTAAACCAAACGGGGCAGGATTGTCTGATGGGACGTCTACTGTGTTGCACCCTCTTCTGGAGCTCATACCCCAGC 400
Nmu_Dr d4  TTACACTAAACCAAACGGGGCAGGATTGTCTGATGGGACGTCTACTGTGTTGCACCCTCTTCTGGAGCTCATACCCCAGC 396

Nmu_Dr     TTGCCAGAAGAAGAAGCAGGAGAATGAAATTAAATGAGAACCTTCAAGGTCCGGGACGCATCCAGAGCAGAGGATACTTC 480
Nmu_Dr d4  TTGCCAGAAGAAGAAGCAGGAGAATGAAATTAAATGAGAACCTTCAAGGTCCGGGACGCATCCAGAGCAGAGGATACTTC 476

Nmu_Dr     CTTTATCGGCCAAGAAATGGAAGAAGATCTGATGAGTATGTGTAA                                    525
Nmu_Dr d4  CTTTATCGGCCAAGAAATGGAAGAAGATCTGATGAGTATGTGTAA                                    521
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B

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Nmu_Hs     MLRTESCRPRSPAGQVAAA--SPLLLLLLLLAWCAGACRGAPIL-PQGLQPEQQLQLWNEIDDTCSSFLSIDSQPQASNALEELCFMIM  86
Nmu_Mm     MSRAAGHRPGLSAGQLAAATASPLLSLLLLLACCADACKGVPIS-PQRLQPEQELQLWNEIHEACASFLSIDSRPQASVALRELCRIVM  88
Nmu_Dr     MRNSNQCERATAHSAMSPGNTSALMLAVLLLSFIPITTSAPMLLNPSSLEHEQ---LLTQITDLCSFYLSADPSFRTSDVLEDLCFLML  86
Nmu_Dr d4  MRNSNQCERATAHSAMSPGNTSALSRYYSSPSYPSPQVLRCS                                              42

Nmu_Hs     GMLPKPQEQDEKDNTKRFLFHYSKTQKLGKSNVVSSVVHPLLQLVPHLHERRMKRFRVDEEFQSPFASQSRGYFLFRPRNGRRSAGFI  174
Nmu_Mm     EISQKPQEQSEKDNTKRFLFHYSKTQKLGNSNVVSSVVHPLLQLVPQLHERRMKRFKA--EYQSPSVGQSKGYFLFRPRNGKRSTSFI  174
Nmu_Dr     GSLQKSKEITARETSKRFLFHYTKPNGAGLSDGTSTVLHPLLELIPQLARRRSRRMKLNENLQGPGRIQSRGYFLYRPRNGRRSDEYV  174
Nmu_Dr d4                                                                                        42
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C

```
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Nmur1a_Dr i11  ATGGAGATTGAAGACTTCTGCCTTGACCAAGACGAGTATCTGGAGAAATACCTCGGGCCAAGACGATCCCCAGTGTTCCTGCCTGTAT 88

Nmur1a_Dr      GCCTGACCTACCTCCTGATCTTCCTGGTGGGAGCGGTGGGAAACATCCTTACCTGCATTGTCATTGCTAAAAACAAAGTCATGCGGAC 176
Nmur1a_Dr i11  GCCTGACCTACCTCCTGATCTTCCTGGTGGGAGCGGTGGGAAACATCCTTACCTGCATTGTCATTGCTAAAAACAAAGTCATGCGGAC 176

Nmur1a_Dr      GCCGACCAACTTCTACCTGTTCAGCCTGGCCATTTCAGATCTTCTAGTGCTTCTCCTGGGAATGCCTTTGGAGCTTTATGAAATGTGG 264
Nmur1a_Dr i11  GCCGACCAACTTCTACCTGTTCAGCCTGGCCATTTCAGATCTTCTAGTGCTTCTCCTGGGAATGCCTTTGGAGCTTTATGAAATGTGG 264

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Nmur1a_Dr i11  GATATTGAGCGTCTGGAGCATTTCCGTGCTTTGCGCCATTCCCAACACGATCCTCCACGGTATATTTACTCTCCCGCCTCCTAAAGGG 528

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Nmur1a_Dr i11  TCCCGAGAAAGCGCTCTCTGAGCATGACCAGAGTCACCGTTCGCAGCACCGTCAGTGACGTCCCGCCATGCAACGGCACTGTGACTAT 1144

Nmur1a_Dr      TGAAGGAGACGATTATGACGTGGATGAAGGTCAAGAAAATAAGACATGTCCCTAA                              1188
Nmur1a_Dr i11  TGAAGGAGACGATTATGACGTGGATGAAGGTCAAGAAAATAAGACATGTCCCTAA                              1199
```

Figure S2

D


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Nmur1b_Dr CAAAACAGAACAGCAAACCTATCAGACCTCTGTCTATCCCGCAGTGCCTACCTGGAGAAATACCTTGGACCCTGTCGTTCACCTTTC 174
Nmur1b_Dr d5 CAAAACAGAACAGCAAACCTATCAGACCTCTGTCTATCCCGCAGTGCCTACCTGGAGAAATACCTTGGACCCTGTCGTTCACCTTTC 169
Nmur1b_Dr TTTCTGCCTATATGTGTCACCTACCTGCTCATCTTTGTAGTAGGTGTGGTGGGCAACATCCTAACTTGTATTGTCATTACCCGTCAT 261
Nmur1b_Dr d5 TTTCTGCCTATATGTGTCACCTACCTGCTCATCTTTGTAGTAGGTGTGGTGGGCAACATCCTAACTTGTATTGTCATTACCCGTCAT 256
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Nmur1b_Dr d5 CGCATCATGCGCACAACGACAAACTACTACCTGTTCAGCCTGGCCATTTCTGACCTCCTTGTGCTGTTGCTTGGCCTCCCGCTGGAG 343
Nmur1b_Dr CTTTATGAGCTCTGGAGCAACTATCCTTTTCTGTTTGGAATTTCTGGCTGCTACTTCAAAACCTGTCTCTTTGAGACAGTCTGCTTT 435
Nmur1b_Dr d5 CTTTATGAGCTCTGGAGCAACTATCCTTTTCTGTTTGGAATTTCTGGCTGCTACTTCAAAACCTGTCTCTTTGAGACAGTCTGCTTT 430
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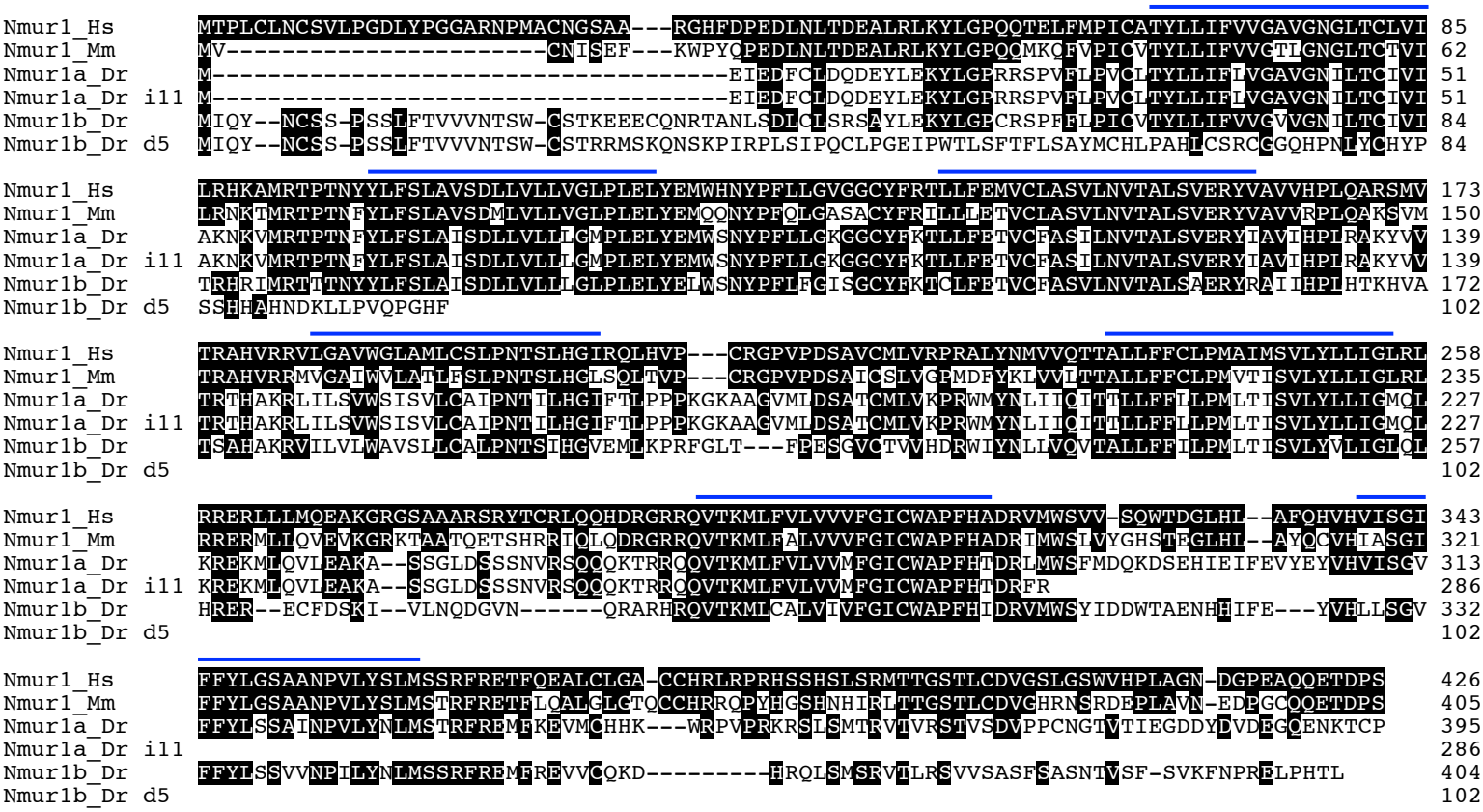
E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Nmur1a_Dr M----------EIEDFCLDQDEYLEKYLGPRRSPVFLPVCLTYLLIFLVGAVGNILTCIVI 51
Nmur1a_Dr i11 M----------EIEDFCLDQDEYLEKYLGPRRSPVFLPVCLTYLLIFLVGAVGNILTCIVI 51
Nmur1b_Dr MIQY--NCSS-PSSLFTVVVNTSW-CSTKEEECQNRTANLSDLCLSRSAYLEKYLGPCRSPFFLPICVTYLLIFVVGVVGNILTCIVI 84
Nmur1b_Dr d5 MIQY--NCSS-PSSLFTVVVNTSW-CSTRRMSKQNSKPIRPLSIPQCLPGEIPWTLSFTFLSAYMCHLPAHLCSRCGGQHPNLYCHYP 84
Nmur1_Hs LRHKAMRTPTNYYLFSLAVSDLLVLLVGLPLELYEMWHNYPFLLGVGGCYFRTLLFEMVCLASVLNVTALSVERYVAVVHPLQARSMV 173
Nmur1_Mm LRNKTMRTPTNFYLFSLAVSDMLVLLVGLPLELYEMQQNYPFQLGASACYFRILLLETVCLASVLNVTALSVERYVAVVRPLQAKSVM 150
Nmur1a_Dr AKNKVMRTPTNFYLFSLAISDLLVLLLGMPLELYEMWSNYPFLLGKGGCYFKTLLFETVCFASILNVTALSVERYIAVIHPIRAKYVV 139
Nmur1a_Dr i11 AKNKVMRTPTNFYLFSLAISDLLVLLLGMPLELYEMWSNYPFLLGKGGCYFKTLLFETVCFASILNVTALSVERYIAVIHPIRAKYVV 139
Nmur1b_Dr TRHRIMRTTTNYYLFSLAISDLLVLLLGLPLELYELWSNYPFLFGISGCYFKTCLFETVCFASVLNVTALSAERYRAIIHPLHTKHVA 172
Nmur1b_Dr d5 SSHHAHNDKLLPVQPGHF 102
Nmur1_Hs TRAHVRRVLGAVWGLAMLCSLPNTSLHGIRQLHVP---CRGPVPDSAVCMLVRPRALYNMVVQTTALLFFCLPMAIMSVLYLLIGLRL 258
Nmur1_Mm TRAHVRRMVGAIWVLATLFSLPNTSLHGLSQLTVP---CRGPVPDSAICSLVGPMDFYKLVVLTTALLFFCLPMVTISVLYLLIGLRL 235
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Nmur1b_Dr d5 102
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Nmur1_Mm RRERMLLQVEVKGRKTAATQETSHRRIQLQDRGRRQVTKMLFALVVVFGICWAPFHADRIMWSLVYGHSTEGLHL--AYQCVHIASGI 321
Nmur1a_Dr KREKMLQVLEAKA--SSGLDSSSNVRSQQQKTRRQQVTKMLFVLVVMFGICWAPFHTDRLMWSFMDQKDSEHIEIFEVYEYVHVISCV 313
Nmur1a_Dr i11 KREKMLQVLEAKA--SSGLDSSSNVRSQQQKTRRQQVTKMLFVLVVMFGICWAPFHTDRFR 286
Nmur1b_Dr HRER--ECFDSKI--VLNQDGVN------QRARHRQVTKMLCALVIVFGICWAPFHIDRVMWSYIDDWTAENHHIFE---YVHLLSGV 332
Nmur1b_Dr d5 102
Nmur1_Hs FFYLGSAANPVLYSLMSSRFRETFQEALCLGA-CCHRLRPRHSSHSLSRMTTGSTLCDVGSLGSWVHPLAGN-DGPEAQQETDPS 426
Nmur1_Mm FFYLGSAANPVLYSLMSTRFRETFLQALGLGTQCCHRRQPYHGSHNHIRLTTGSTLCDVGHRNSRDEPLAVN-EDPGCQQETDPS 405
Nmur1a_Dr FFYLSSAINPVLYNLMSTRFREMFKEVMCHHK---WRPVPRKRSLSMTRVTVRSTVSDVPPCNGTVTIEGDDYDVDEGCENKTCP 395
Nmur1a_Dr i11 286
Nmur1b_Dr FFYLSSVVNPILYNLMSSRFREMFREVVCQKD---------HRQLSMSRVTLRSVVSASFSASNTVSF-SVKFNPRELPHTL 404
Nmur1b_Dr d5 102

Figure S2

F

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Nmur2_Dr d17  ATGATGGCCCTGATCTGCCTTTGCGAGTCTGTGAATACATCAGACCAGGATTATGAGTTCTGCAACAGCAGTACGTTCAACTTCACTGG 89

Nmur2_Dr      AAATGACAGTGCACACTGTTACCTTCAGACCATTGATGAAGTTCTGTTTAAGCTTCTGGGCCCAAGACGATCTCCGTTCTTCTTCCCTG 178
Nmur2_Dr d17  AAATGACAGTGCACACTGTTACCTTCAGACCATTGATGAAGTTCTGTTTAAGCTTCTGGGCCCAAGACGATCTCCGTTCTTCTTCCCTG 178

Nmur2_Dr      TAACCTGTACTTACATCCTCATCTTCATGACCGGGGTCTTAGGAAACCTCCTGACCTGCGCTGTAATCACTAAAGATCGAAAAATGCAA 267
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Nmur2_Dr      ATCGCTGGGGTTTGGGCTATGTCTCTGCTCTGCGCCGTCCCGAACACCTCCCTCCATGGCCTGCAGTATCAGTATCTGCCGGAGAGGGT 623
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Nmur2_Dr d17  TCAGGAATCGGCTACCTGCAACCTGCTCAAGCCCAAATGGATGTACAACTTGGTGATCCAGATCACAACTGTGCTCTTCTACTTTGTTC 695

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Nmur2_Dr d17  CCATGATGATGATCAGCGTGCTGTATCTGATGATCGGTCTGACGCTTGGCAGAGGGCAGAAGCAGAAAAAGGACAAGCTGGGAAGCAAT 784

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Nmur2_Dr d17  TGATTTCAAACGGACTGGA                                                                      1159
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G

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Nmur2_Dr      MMALICLCESVNTSDQDYEFCNSSTFNFTGNDSAHCYLQTIDEVLFKLLGPRRSPFFFPVTCTYILIFMTGVLGNLITCAVITKDRKMQ  89
Nmur2_Dr d17  MMALICLCESVNTSDQDYEFCNSSTFNFTGNDSAHCYLQTIDEVLFKLLGPRRSPFFFPVTCTYILIFMTGVLGNLITCAVITKDRKMQ  89

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Nmur2_Mm      TPTNYYLFSLAVSDLLVLLLGMPLEVYELWHNYPFLFGPVGCYFKTALFETVCFASILSVTTVSIERYVAIVHPFRAKLESTRRRALRI 158
Nmur2_Dr      TPTNLYLFSLAISDLLVLLFGMPLEIYELWQNYPFPFGESICCFKIFLFETVCFASVLNVTVLSVERYIAVIHPLKTRYAITNKHARRV 178
Nmur2_Dr d17  TPTNLYLRSSSATLRDASGNLRTLAKLPFSLRREHLLL                                                     127

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Nmur2_Mm      LSLVWSFSVVFSLPNTSIHGIKFQQFPNGSSVPGSATCTVTKPIWVYNFIIQATSFLFYILPMTLISVLYYLMGLRLKRDESLEADKV- 246
Nmur2_Dr      IAGVWAMSLLCAVPNTSIHGLQYQYLPE--RVQESATCNLLKPKWMYNLVIQITTVLFYFVPMMMISVLYLMIGLTLIGRGQKQKKDKLG 265
Nmur2_Dr d17                                                                                            127

Nmur2_Hs      --------NANIQRPCRKSVNKMLFVLVLVFAICWAPFHIDRLFFSFVEEWSESLAAVFNLVHVVSGVFFYLSSAVNPIIYNLLSRRFQ 335
Nmur2_Mm      --------TVNIHRPSRKSVTKMLFVLVLVFAICWTPFHVDRLFFSFVDEWTESLAAVFNLIHVVSGVFFYLSSAVNPIIYNLLSRRFR 327
Nmur2_Dr      SNHSNDSWKIHLDSRRRRQVTKMHFVVVLVFAICWAPFHIDRLLWSFITSWTDHMHNIFEYVHIISGVLFYLSSAVNPIIYNLLSSRFR 354
Nmur2_Dr d17                                                                                            127

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Nmur2_Mm      AAFRNVVSPSCK-WCHPQHRPQGPPAQKVIFLTECHLVELTEDAGPQFPCQSSIHNTQLTTVPCVEEVP                      395
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Nmur2_Dr d17                                                                                            127
```

H

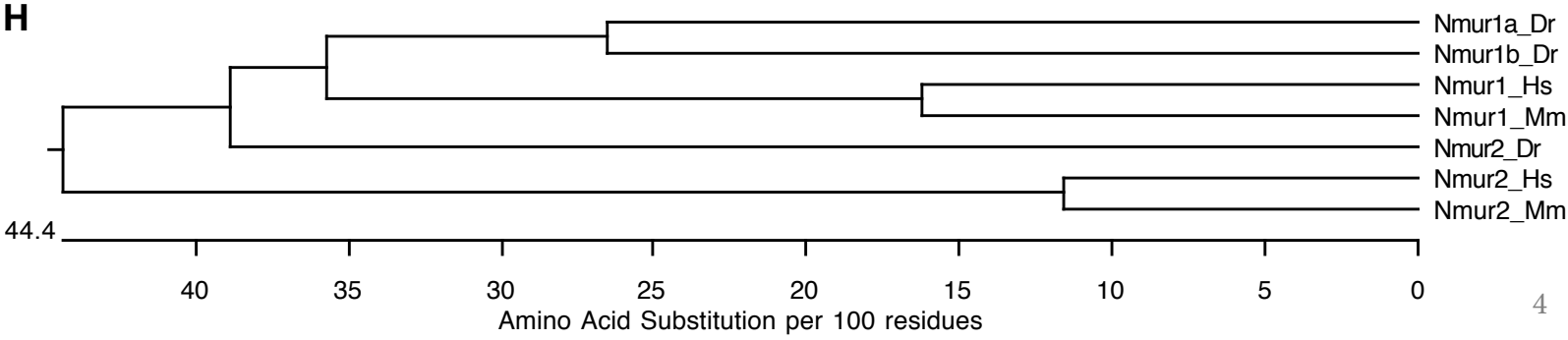


Figure S3

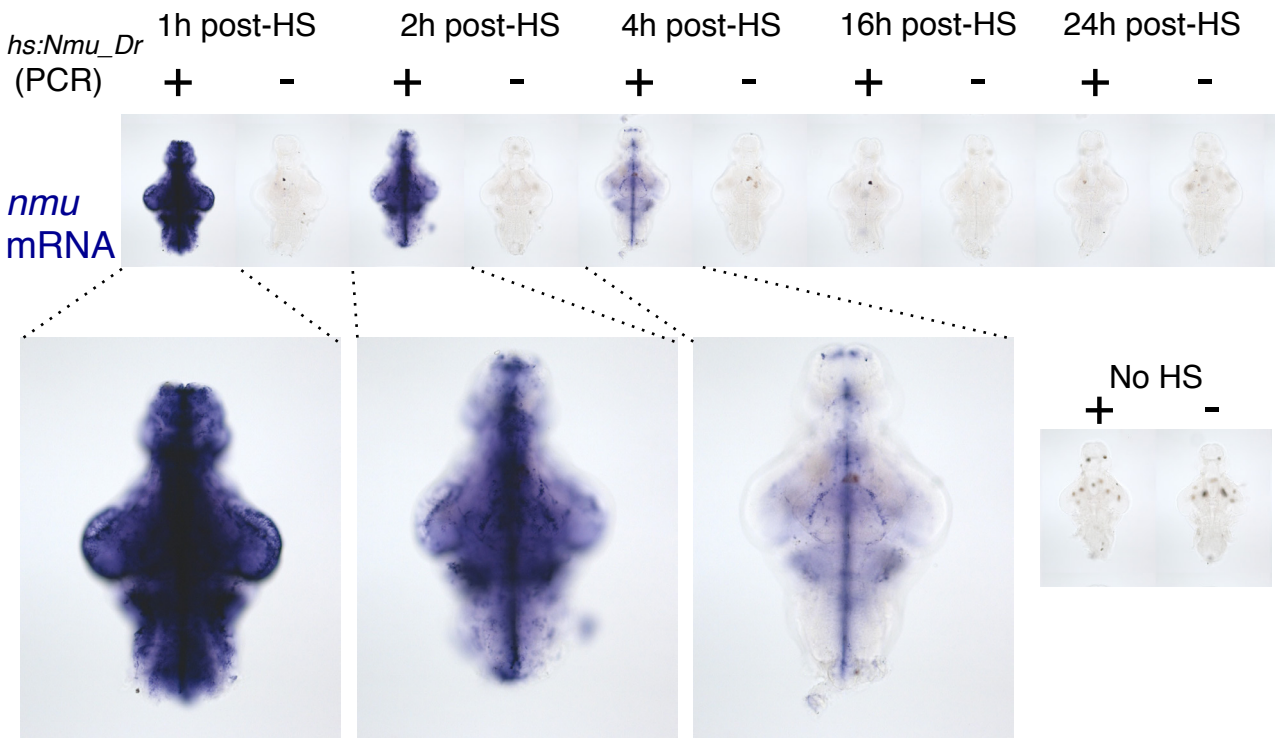

hs:Nmu_Dr
(PCR)
1h post-HS
2h post-HS
4h post-HS
16h post-HS
24h post-HS
+ - + - + - + - + -
nmu
mRNA
No HS
+ -

Figure S4

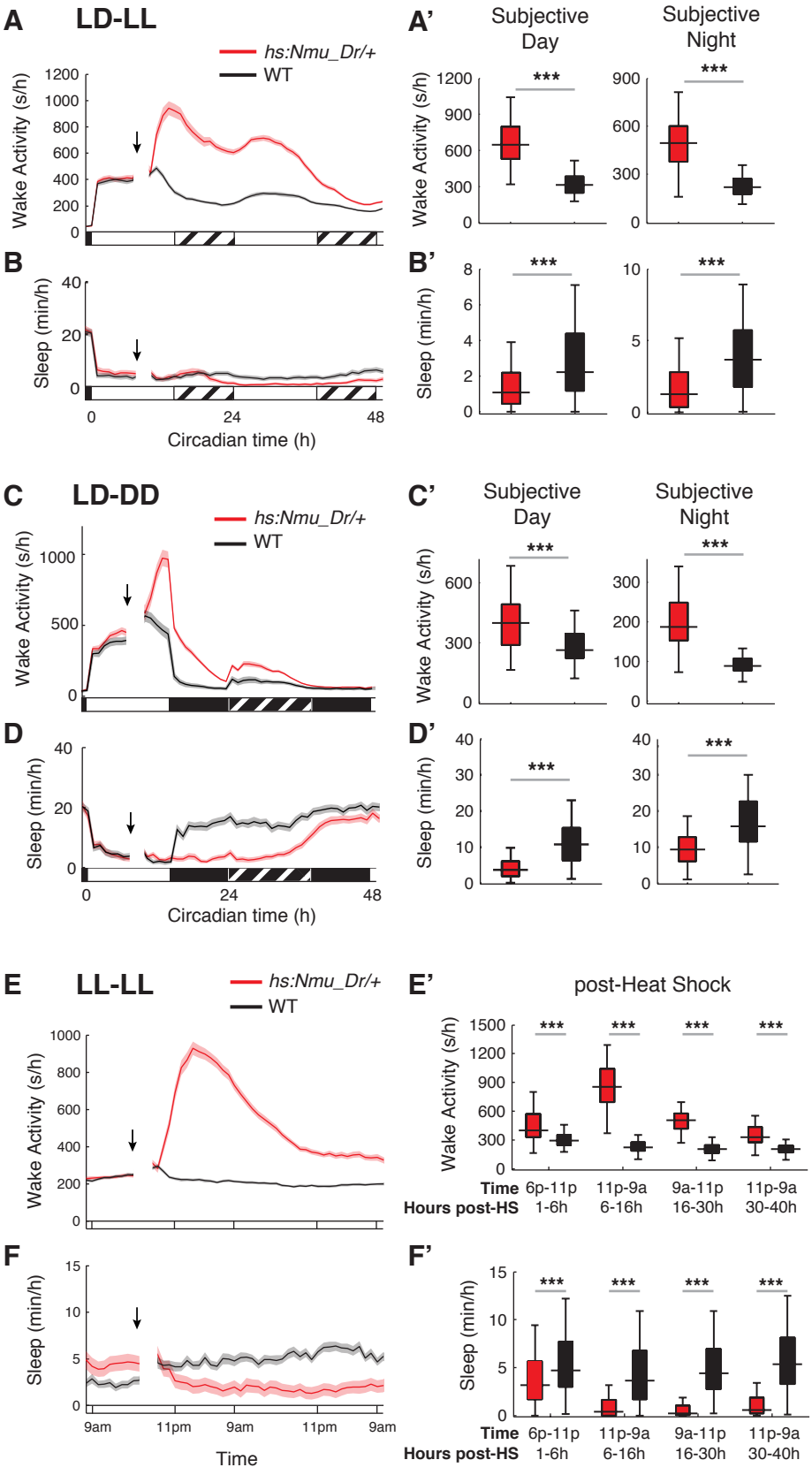

A
LD-LL
hs:Nmu_Dr/+
WT
Wake Activity (s/h)
A'
Subjective Day
Subjective Night
B
Sleep (min/h)
B'
Circadian time (h)
C
LD-DD
C'
D
D'
E
LL-LL
E'
post-Heat Shock
Time
Hours post-HS
6p-11p 1-6h
11p-9a 6-16h
9a-11p 16-30h
11p-9a 30-40h
F
F'
9am
11pm
***

Figure S5

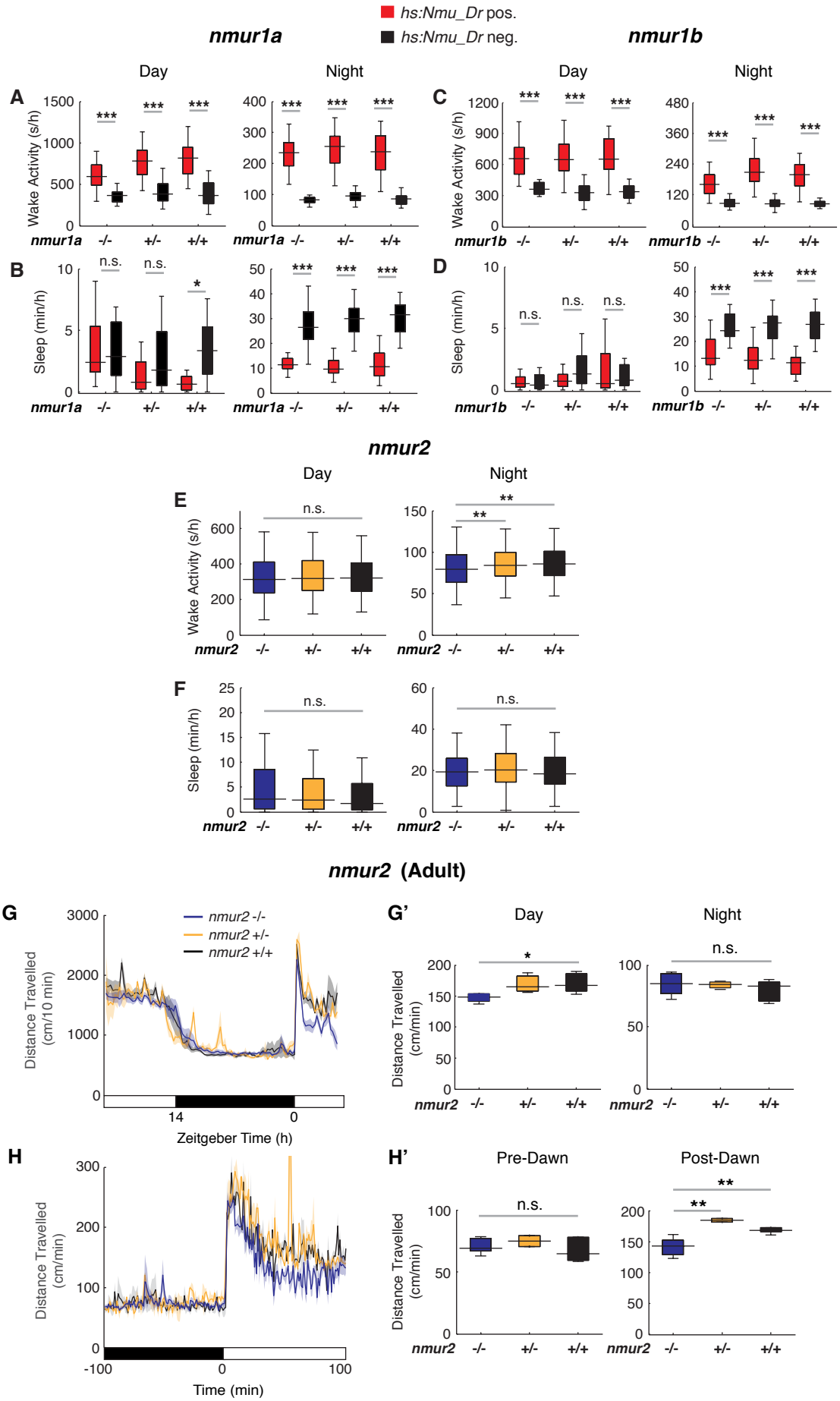
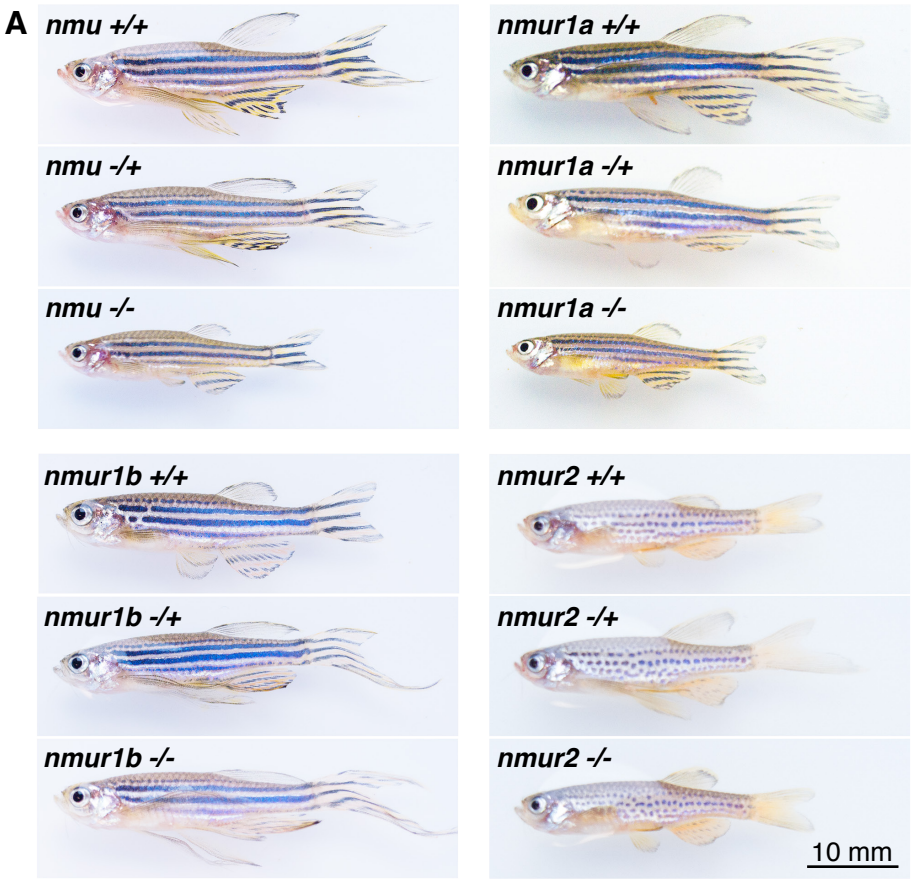

hs:Nmu_Dr pos.
hs:Nmu_Dr neg.
nmur1a
nmur1b
nmur2
Day
Night
A
B
C
D
E
F
Wake Activity (s/h)
Sleep (min/h)
nmur1a -/- +/- +/+
nmur1b -/- +/- +/+
nmur2 -/- +/- +/+
n.s.
nmur2 (Adult)
G
G'
H
H'
nmur2 -/-
nmur2 +/-
nmur2 +/+
Distance Travelled (cm/10 min)
Zeitgeber Time (h)
Distance Travelled (cm/min)
Time (min)
Pre-Dawn
Post-Dawn

Figure S6


A
nmu +/+
nmur1a +/+
nmu -/+
nmur1a -/+
nmu -/-
nmur1a -/-
nmur1b +/+
nmur2 +/+
nmur1b -/+
nmur2 -/+
nmur1b -/-
nmur2 -/-
10 mm

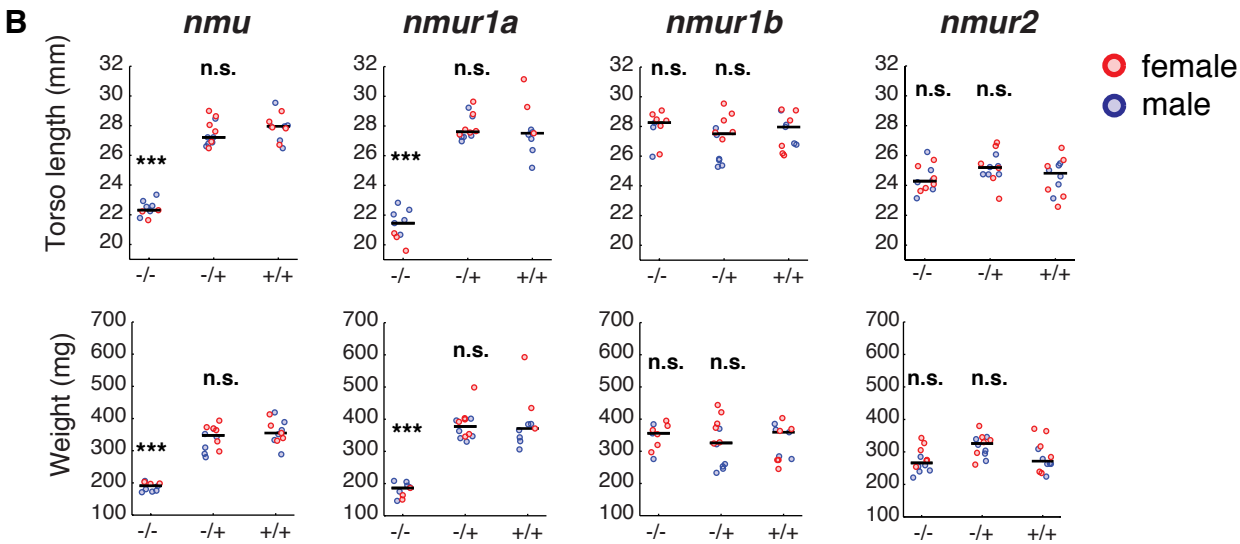

B
nmu
nmur1a
nmur1b
nmur2
female
male
Torso length (mm)
Weight (mg)
n.s.
***
-/-
-/+
+/+

Table S1. Primary Screen

Experiments Performed	1432
Unique ORFs	1286
Unique Genes	1126
Duplicate ORFs	146
Duplicate Genes	306

Controls	768
empty hs vector	660
hs:Hcrt	72
hs:EGFP	13

Table S2. Primary Screen Raw Data

Sleep (min/h)	**Screen Data** Mean +/- SD	**Control Data** Mean +/- SD
Pre-HS		
Night	34.0 +/- 5.3	34.9 +/- 5.3
Day	18.1 +/- 5.6	18.3 +/- 6.1
Post-HS		
Day 1	15.7 +/- 4.7	16.0 +/- 4.9
Night	21.9 +/- 4.5	23.0 +/- 4.5
Day 2	14.0 +/- 4.5	14.7 +/- 4.9
Sleep Bouts (#/h)		
Pre-HS		
Night	7.8 +/- 1.0	7.8 +/- 1.02
Day	4.71 +/- 1.1	4.8 +/- 1.1
Post-HS		
Day 1	3.9 +/- 0.9	3.9 +/- 1.7
Night	7.6 +/- 1.2	7.7 +/- 1.2
Day 2	5.7 +/- 1.4	6.1 +/- 1.5
Sleep Bout Length (min/bout)		
Pre-HS		
Night	4.9 +/- 1.6	5.2 +/- 1.9
Day	4.0 +/- 1.2	4.0 +/- 1.5
Post-HS		
Day 1	5.0 +/- 5.2	4.9 +/- 2.8
Night	3.0 +/- 0.9	3.1 +/- 1.4
Day 2	2.4 +/- 1.2	2.7 +/- 6.0
Sleep Latency (min)		
Pre-HS		
Night	26.2 +/- 10.0	25.4 +/- 10.6
Day	54.6 +/- 32.6	56.4 +/- 33.2
Post-HS		
Day 1	N/A	N/A
Night	60.8 +/- 25.0	55.2 +/- 24.4
Day 2	101.6 +/- 43.6	99.0 +/- 52.0
Average Activity (s/h)		
Pre-HS		
Night	48.4 +/- 12.5	43.9 +/- 11.4
Day	240.6 +/- 49.9	218 +/- 47.9
Post-HS		
Day 1	257.0 +/- 49.1	230.8 +/- 47.0
Night	66.2 +/- 15.1	59.1 +/- 12.9
Day 2	213.5 +/- 43.3	183.7 +/- 42.8
Wake Activity (s/h)		
Pre-HS		
Night	108 +/- 18	102 +/- 18
Day	342 +/- 54	312 +/- 54
Post-HS		
Day 1	348 +/-54	312 +/- 48
Night	102 +/- 18	90 +/- 18
Day 2	270 +/- 42	240 +/- 42

Table S3. Secondary Screen

Experiments Conducted	N
HS-Constructs Made into Stable Lines	**60**
Stable Repeats Primary Phenotype	10
Stable Differs from Primary	2
% Stables w/Phenotype	**20%**
#Lines Tested/Construct	
1 line	22
2 lines	24
3 lines	14
Total Stable Experiments	**232**
Constructs Tested 1x	9
2x	13
≥3x	38

Supplemental Figure Legends

Figure S1. Screen-wide frequency distributions of sleep/wake behavioral parameters (Related to Figure 1). (A-F) Histograms of behavioral indices from the primary screen, normalized as standard deviations from the mean (Z-score). Blue indicates the whole collection of experiments and red indicates the secondary selection of 60 Secretome genes that were chosen based on that parameter result. Most of the outliers with effects as strong as Nmu that were not chosen had failed to reproduce in repeated transient injection tests. See Figure 1, Table S2, and Supplemental Experimental Procedures for additional information about how the parameters and indices were calculated.

Figure S2. Sequences of wild type and mutant Nmu, Nmur1 and Nmur2 (Related to Figure 2). Nucleotide sequences of open reading frames of zebrafish wild type and mutant *nmu* (A, ENSDARG00000043299), *nmur1a* (C, ENSDARG00000060884), *nmur1b* (D, ENSDARG00000003944) and *nmur2* (F, ENSDARG00000022570) are shown. Zebrafish orthologs were identified by reciprocal Blast searches of mammalian and zebrafish genomes. Red boxes indicate binding sites of ZFNs (C, D) and TALENs (A, F) used to generate mutants. Alignments of amino acid sequences of human, mouse, wild type zebrafish and mutant zebrafish Nmu (B; human, ENSG00000109255; mouse, ENSMUSG00000029236), Nmur1 (E; human, ENSG00000171596; mouse, ENSMUSG00000026237) and Nmur2 (G; human, ENSG00000132911; mouse, ENSMUSG00000037393) are shown. Amino acids shaded in black are identical to human. Blue lines indicate mature human Nmu peptide (B) and predicted human Nmur transmembrane domains (E, G). Asterisks in (B) indicate conserved residues that are critical for biological activity in mammals. H. Phylogenetic tree of human, mouse and zebrafish Nmu receptors.

Figure S3. Time course of Nmu overexpression (Related to Figure 3). Larvae from a *hs:Nmu_Dr* /+ to WT mating were heat shocked for 1 hour at 37^{o}C at 5 dpf and fixed at the indicated times after heat shock. ISH was then performed using an *nmu*-specific riboprobe on dissected larval brains. Transgenic and WT larvae were subsequently identified by PCR. Ubiquitous *nmu* overexpression was observed at 1 hour post-heat shock, with reduced levels at 2 and 4 hours post-heat shock. No ectopic transcript was observed at 16 and 24 hours post-heat shock. No ectopic *nmu* expression was observed in non-heat shocked transgenic brains (No HS, insert). Chromogenic development was stopped at the same time for all samples prior to the visualization of endogenous *nmu* transcript. While this data shows that ectopic *nmu* transcript levels are ubiquitously elevated for a few hours after heat shock, the perdurance of Nmu peptide is not known.

Figure S4. Nmu overexpression phenotypes persist in the absence of circadian cues (Related to Figure 3). Nmu overexpression increases wake activity (A, A') and decreases sleep (B, B') in larvae entrained to LD and switched to constant light (LD-LL) conditions. Hatched boxes in the x-axis indicate subjective night. Nmu overexpression increases wake activity (C, C') and decreases sleep (D, D') in larvae entrained to LD and switched to constant dark conditions (LD-DD). Hatched boxes in x-axis indicate subjective day. (E, F) Larvae raised in constant light from birth (LL-LL) do not exhibit a circadian wake activity or sleep rhythms, and Nmu-induced phenotypes persist in the absence of entrained or external circadian cues. Number of subjects: (A, B) *hs:Nmu*/+ (n=98), WT (n=92). (C, D) *hs:Nmu*/+ (n=90), WT (n=98). (E, F) *hs:Nmu*/+ (n=92), WT (n=98). ***p<0.001, Mann-Whitney U-test.

Figure S5. Larval *nmu receptor* mutants lack sleep/wake phenotypes and Nmu-induced hyperactivity and insomnia do not require *nmur1a* or *nmur1b* (Related to Figure 5). Larvae with homozygous and heterozygous mutations of *nmur1a* (A, B) or *nmur1b* (C, D) respond to Nmu overexpression, indicating that these receptors are not required for *hs:Nmu*-induced phenotypes. For *hs:Nmu*/+ neg. larvae, all comparisons between *nmur1a*-/-, *nmur1a*+/- and *nmur1a*+/+ siblings, and between *nmur1b*-/-, *nmur1b*+/- and *nmur1b*+/+ siblings, were not significantly different. *nmur2*-/- larvae exhibit slightly less wake activity at night compared to *nmur2*+/- and *nmur2*+/+ siblings (E), but there is no significant difference in wake activity during the day (E) or in sleep (F) among the three genotypes. Two month old *nmur2*-/- adults exhibit reduced activity compared to their *nmur2*+/- and *nmur2*+/+ siblings during the day (G, G') and following night to day transitions (H, H'). Data in adult line graphs are averaged in 10 (G) or 1 (H) minute bins. Adult animals were derived from a *nmur2*+/- to *nmur2*+/- mating. Number of subjects for *nmur1a* mutant experiment: *hs:Nmu*/+; *nmur1a*-/- (n=22), *hs:Nmu*/+; *nmur1a*+/- (n=9), *hs:Nmu*/+; *nmur1a*+/+ (n=22), *nmur1a*-/- (n=23), *nmur1a*+/- (n=54), *nmur1a*+/+ (n=18). Number of subjects for *nmur1b* mutant experiment: *hs:Nmu*/+; *nmur1b*-/- (n=29), *hs:Nmu*/+; *nmur1b*+/- (n=43), *hs:Nmu*/+; *nmur1b*+/+ (n=25), *nmur1b*-/- (n=22), *nmur1b*+/- (n=39), *nmur1b*+/+ (n=17). Number of subjects for *nmur2* mutant larval experiment: *nmur2*-/- (n=106), *nmur2*+/- (n=233), *nmur2*+/+ (n=108). Number of subjects for *nmur2* mutant adult experiment: *nmur2*-/- (n=8), *nmur2*+/- (n=3), *nmur2*+/+ (n=5). *p<0.05, **p<0.01, ***p<0.001, n.s. p>0.05, Kruskal-Wallis test followed by the Steel-Dwass test for pairwise multiple comparisons.

Figure S6. *nmu*-/- and *nmur1a*-/- but not *nmur1b*-/- or *nmur2*-/- adult zebrafish exhibit defects in size and weight (Related to Figure 5). (A) Representative images of male 20-22 week old adults from each genotype tested. For each mutation, fish from heterozygous mutant matings

were raised to adulthood, measured and then genotyped to identify homozygous mutant, heterozygous mutant and WT individuals. All samples are displayed at the same scale (scale bar = 10 mm). (B) Size, as measured from rostral tip to end of torso (fin length not included), and weight of individual fish. Red=female, blue=male. Black horizontal bars indicate median value for each genotype. ***$p<0.001$, n.s. $p>0.05$, Kruskal-Wallis test followed by the Steel-Dwass test for pairwise multiple comparisons.

Table S1. Primary screen summary data (Related to Figure 1). This table shows a summary of the experiments performed in the primary screen. Fewer unique genes were tested than unique ORFs because some genes contained splice variants that encode for more than one ORF. Duplicate ORFs and duplicate genes refer to the number of unique ORFs and unique genes that were tested more than once. Empty hs vector denotes negative control consisting of the Gateway destination vector that was not recombined to insert an ORF 3' to the heat shock promoter. *hs:Hcrt* denotes positive control containing the zebrafish Hcrt ORF. *hs:EGFP* denotes negative control containing the EGFP ORF. Most experiments used the empty hs vector as a negative control.

Table S2. Primary screen raw data (Related to Figure 1). This table shows mean±standard deviation values of all behavioral parameters quantified in the primary screen during day and night periods before and after heat shock. Data for overexpressed ORFs (Screen Data) and negative controls (Control Data) are shown. Most overexpressed ORFs did not induce phenotypes, so Screen Data and Control Data values are similar.

Table S3. Secondary screen summary data (Related to Figure 1). This table shows summary statistics for the 60 human ORFs for which stable transgenic lines were generated. Of the 60

ORFs, 10 produced overexpression phenotypes similar to those observed in the primary screen. Two ORFs produced overexpression phenotypes different from those observed in the primary screen. Two or three independent stable transgenic lines were generated for 24 or 14 of the ORFs, respectively. Most stable transgenic lines were tested for overexpression phenotypes in 3 or more independent behavioral experiments.

Movie S1. *nmu* expression in a 5 dpf larval zebrafish brain (Related to Figure 2). At 5 dpf, *nmu* is expressed in several ventral hypothalamic cell clusters, a dorsal hypothalamic bilateral cluster, and a few cells in the lateral-caudal brainstem. The movie progresses from ventral to dorsal focal planes imaged at 5 μm intervals. The large dark spots in the most dorsal images are pigment cells.

Movie S2. *nmur2* expression in a 5 dpf larval zebrafish brain (Related to Figure 2). At 5 dpf, *nmur2* is broadly expressed in discrete regions of the brain, including cell clusters in the brainstem, hypothalamus and forebrain. The movie progresses from ventral to dorsal focal planes imaged at 5 μm intervals. The large dark spots in the most dorsal images are pigment cells.

Supplemental Experimental Procedures

Animal use

Zebrafish were raised on a 14 hour:10 hour light:dark cycle at 28.5°C, with lights on at 9 a.m. and off at 11 p.m. WT, transgenic, and mutant stocks come from a background of TL x AB WT strains. All experiments with zebrafish followed standard protocols (Westerfield, 2000) in accordance with the Harvard University and California Institute of Technology Institutional Animal Care and Use Committee guidelines.

Generation of Secretome library and microinjection assay

The LOCATE database (http://locate.imb.uq.edu.au) identifies 4418 human proteins that are predicted or known to be secreted. ORFs that encode 1632 of these proteins were present in the hORFeome 3.1 collection in Gateway entry vectors (Lamesch et al., 2007). We constructed a Gateway destination vector containing a heat shock-inducible promoter (Halloran et al., 2000) 5' to attR1 and an SV40 polyadenylation signal 3' to attR2, and the entire cassette was flanked by Tol2 transposase arms. LR clonase (Invitrogen) was used to transfer each ORF into the destination vector. Plasmid preps were obtained using a Qiagen Biorobot 8000, purified using multiscreen PCR μ96 filter plates (LSKMPCR10, Millipore), dried using a speedvac, and dissolved in 10 μL water. Plasmid concentrations were measured using the Quant-iT kit (Invitrogen) and adjusted to 100 ng/μL. The purification step allowed injection of twice as much DNA compared to non-purified samples without increased toxicity. One nL of an injection mix (50 ng/μL plasmid, 150 ng/μL *tol2 transposase* mRNA and 0.05% phenol red in PBS (P0290, Sigma-Aldrich)) was injected into the yolk of TLAB embryos at the one-cell stage. Each plasmid was injected into 50 embryos, and up to 32 larvae were tested in the behavioral assay. Injected animals were screened at 24 hpf and immediately prior to the behavioral assay to remove any larvae that exhibited

abnormal morphology or locomotor behaviors. Plasmids for which fewer than 24 healthy larvae were available to test were reinjected at 25 ng/μL. For behavioral testing, individual larvae were placed into each of 80 wells of a 96-well plate (7701-1651, Whatman) containing 650 μL embryo water (0.3 g/L Instant Ocean, 1 mg/L methylene blue, pH 7.0). Two plasmids were tested in each plate on 32 animals per plasmid. At 4 dpf, larvae injected with each plasmid were loaded in alternating columns, and control larvae injected with the destination vector lacking an ORF or containing EGFP were loaded in columns 1 and 10.

After the primary screen, post-HS day and night sleep, wake activity, and sleep bout number, length and latency were calculated for each clone tested as described (Rihel et al., 2010): sleep is defined as one minute of inactivity; sleep bouts are the number of uninterrupted runs of sleep; sleep length is the average time a sleep bout lasts; sleep latency is the time from lights out to first sleep bout; and wake activity is the amount of locomotor activity during active bouts. The activity index was determined as:

$$\text{Activity Index} = \frac{(\text{PostNight}_n - \text{PreNight}_n) + 0.5(\text{PostD1}_n + \text{PostD2}_n) - \text{PreDay}_n)}{1/N \sum(\text{PostNight}_c - \text{PreNight}_c + 0.5(\text{PostD1}_c + \text{PostD2}_c) - \text{PreDay}_c)}$$

Z-scores were determined by subtracting the mean of all tested clones from the measure of each clone, divided by the standard deviation of all tested clones.

Secondary screening in stable transgenic lines

Sixty clones that produced the same phenotype upon retesting in the injection overexpression assay were used to generate stable transgenic lines using Tol2 transposase or ISce1 meganuclease. Stable lines were identified by heat shocking the progeny of potential founders at 24 hpf, fixing the embryos with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) 1 hour after

heat shock, and performing ISH using a probe specific for the overexpressed ORF. Lines that produced strong and ubiquitous gene overexpression were used for behavioral assays. When available, up to three independent stable lines were behaviorally tested for each ORF (Table S3) in the locomotor activity assay.

Locomotor activity assay

At 4 dpf, individual larvae were placed into each 650 μL well of a 96-well plate (7701-1651, Whatman). For the primary screen, only 80 wells of the plate were used due to software and hardware limitations. For subsequent experiments, all 96 wells were used. In experiments using stable transgenic or mutant lines, larvae were blindly assigned a position in the plate, and were genotyped after the behavioral experiment was completed. Except in the primary screen or as noted, plates were filled E3 embryo medium and sealed with an optical adhesive film (4311971, Applied Biosystems) to prevent evaporation. Occasionally, the sealing process introduces air bubbles in some wells, which occlude tracking of larvae and are discarded from analysis. Locomotor activity was monitored using an automated videotracking system (Viewpoint Life Sciences) with a one-third inch monochrome camera (primary screen: LTC0385, Bosch; subsequent experiments: Dragonfly 2, Point Grey) fitted with a fixed-angle megapixel lens (M5018-MP, Computar) and infrared filter. Larvae were heat shocked at 37˚C for one hour starting around 4 p.m. or 5 p.m. on 5 or 6 dpf. The movement of each larva was captured at 15 Hz and recorded using the quantization mode with 1-minute integration time bins. In the primary screen, data from two cameras were collected in alternating minutes by one computer; in subsequent experiments, one computer collected data from two cameras simultaneously. The 96-well plate and camera were housed inside a custom-modified, opaque Zebrabox (Viewpoint Life Sciences) that was continuously illuminated with infrared LEDs, and illuminated with white LEDs

from 9 a.m. to 11 p.m., except as noted in constant light or constant dark experiments. The 96-well plate was housed in a chamber filled with recirculating water to maintain a constant temperature of 28.5°C. The parameters used for detection with the Bosch (primary screen) / Point Grey (subsequent experiments) cameras were the following: detection threshold, 40/14; burst, 25/29; freeze, 3/4. Data were processed using custom PERL and Matlab (The Mathworks, Inc) scripts.

For adult behavioral assays, individual two month (*nmur2* mutant) or three month (*nmu* mutant) old zebrafish were randomly placed into a 7 cm x 12 cm by X 8.5 cm (WxLxH) open topped, transparent plastic chamber, with three small holes (5 mm, partially covered with parafilm for smaller adults) to allow water exchange. The chambers (8 per tracking session) were placed into a semi-transparent, plastic 46 cm x 54 cm chamber, which was supplied with aquarium fish water (water height in chamber: 4.5 cm), pumped from a 45 L reservoir with an aquarium pump (Maxi-Jet, MJ500) at a flow rate of 1.3 L/min and heated to 28°C with an aquarium heater (HT100; Tetra). The chamber was continuously illuminated from below with two panels of infrared lights (60 Degree, 54 LED Video Camera Red Infrared Illuminator Lamp, SourcingMap) with the detector covered, and illuminated from above with white light (180 lux at water surface) from 9 a.m. to 11 p.m. The chambers were monitored with a ceiling mounted (143 cm from chamber to lens) Dinion one-third inch Monochrome camera (LTC0385; Bosch) fitted with a 13-36 mm, 1:2:8, 2/3'' lens (Computar). The entire setup was housed in an isolated darkroom. Fish were continuously tracked for two to four days at 15 Hz using an automated videotracking system (Viewpoint Life Sciences) in tracking mode, with a background threshold of 40, inactive/small movement cutoff of 1.3 cm/sec, and small/large movement cutoff of 8 cm/sec. Each track was visually inspected at 1 minute resolution for any artifactual movements (e.g. from stray particles or air bubbles) and then analyzed using custom Matlab scripts (The Mathworks, Inc). Fish were

derived from a *nmu*-/- to *nmu*+/- mating and a *nmur2*+/- to *nmur2*+/- mating. Mixed gender siblings were raised in groups of 30-40 per tank and were genotyped by fin clip after the behavioral assay.

Light pulse-evoked arousal assay

Larval zebrafish were housed and monitored as described for the locomotor assay, except activity was stored in 1-second integration time bins and data were subsequently smoothed with a 10-second running average. One-minute light pulse trials were presented at 2 hour intervals starting 2 hours after the start of the dark phase (i.e. 1 a.m.). The full dataset consisted of 870 trials each for *hs:Nmu*/+ and WT sibling genotypes, which were randomly subdivided to generate n=29 samples per genotype, with each sample representing a 30-trial average. The baseline for each sample was measured as the median activity level during the 30 minutes prior to stimulus onset. The peak amplitude (A_1) during first "stimulus-on" response epoch was defined as the peak raw activity level during the stimulus period minus the sample baseline. The peak amplitude (A_2) of the "post-stimulus" response epoch was determined in two steps. First, an alpha function was fit to the "post-stimulus" response, as given in Equation 1:

$$\text{Eq. 1: } y(t) = \text{baseline} + t * e^{-(t/\tau)}$$

with time t, and free decay parameter τ_{decay}. Second, the A_2 was calculated as peak value of the alpha curve fit minus baseline. Next, the exponential decay function that was fit to the "stimulus-on" or "post-stimulus" epochs of the response curve is given in Equation 2:

$$\text{Eq. 2: } y(t) = \text{baseline} + A * e^{-(t/\tau)}$$

with time t, peak amplitude A_1 or A_2, and free decay parameter τ_1 or τ_2, for the "stimulus-on" and post-stimulus response periods, respectively. Total activity for "stimulus-on" was calculated as the integral of raw activity ($\int \text{Activity}_1$) data during the 6 minutes after stimulus onset. Total activity

for "post-stimulus" response was calculated as the integral of raw activity ($\int Activity_2$) during subsequent time points through τ_2.

Generation of zebrafish heat-shock inducible transgenic lines

We used reciprocal BLAST to identify zebrafish orthologs for each human ORF that produced a locomotor activity phenotype in stable transgenic lines. To clone the zebrafish ORFs, we performed RT-PCR (Superscript III Reverse Transcriptase, Life Technologies) using mRNA isolated from 5 dpf zebrafish larvae (Trizol, Life Technologies). In cases where the 5' or 3' end of an ORF was not annotated in Ensembl or was ambiguous, 5' and 3' RACE (First Choice RLM-RACE Kit, Life Technologies) was performed to identify the entire ORF. Each ORF was cloned into a vector containing a heat-shock inducible promoter (Halloran et al., 2000) and ISce1 meganuclease sites, and each vector was injected with ISce1 (New England Biolabs) into zebrafish embryos at the one-cell stage to generate stable lines, which were identified as described above.

Generation of zebrafish mutants

nmu and *nmur2* mutant zebrafish were generated using TAL effector nucleases (TALENs), as described (Chen et al., 2013; Reyon et al., 2012). *nmur1a* and *nmur1b* mutant zebrafish were generated using zinc finger nucleases (ZFNs), as described (Chen et al., 2013). Plasmids were obtained from Addgene. *nmu* mutants were genotyped using the primers 5'-TGACCGACAGAGAGCATGAG-3', 5'-GGAGTAGTACCGCGAGCATC-3' and 5'-CGATTAAAACAGTAAAAACGCAGA-3', which generate 172 bp and 106 bp bands for the WT allele and a 168 bp band for the mutant allele. *nmur1a* mutants were genotyped using the primers 5'-AGACACCCTGTATTTTCTCCTCA-3', 5'-GTAGAGGACGGGGTTTATGG-3' and 5'-CACATCGGAGCTAGCGAAAC-3', which generate a 203 bp band for the WT allele, and 214 bp

and 96 bp bands for the mutant allele. *nmur1b* mutants were genotyped using the primers 5'-TCAATGATACAGTACAACTGCTCCTC-3', 5'-AGGGTCCAAGGTATTTCTCCA-3' and 5'-ATGGTGCTCCACCAAAGAA-3', which generate 163 bp and 101 bp bands for the WT allele and a 158 bp band for the mutant allele. *nmur2* mutants were genotyped using the primers 5'-ATGACCGGGGTCTTAGGAAA-3' and 5'-TGACGTTTAACACGGAAGCA-3', which generate a 244 bp band for the WT allele and a 227 bp band for the mutant allele. The gr^{s357} mutant (Ziv et al., 2013) and was a kind gift from Herwig Baier. These animals were genotyped using the primers 5'-GGAAGAACTGaCCTGCCTGT-3' and 5'-TCTCAGTTTATCCACATTTATGCAG-3'. DrdI digests the WT PCR product into 114 and 14 bp bands.

in situ hybridization and imaging

Samples were fixed in 4% PFA in PBS for 12-16 hours at room temperature. *in situ* hybridizations (ISH) were performed using digoxygenin (DIG) labeled antisense riboprobes (DIG RNA Labeling Kit, Roche) or 2,4-dinitrophenol (DNP) labeled antisense riboprobes (DNP-11-UTP, Perkin Elmer) as previously described (Thisse and Thisse, 2008). Double-fluorescent ISH was performed using DIG- and DNP- labeled riboprobes and the TSA Plus Fluorescein and Cyanine 3 Systems (Perkin Elmer). PCR products generated from larval zebrafish cDNA were used as templates for riboprobe synthesis using the following primers. *nmu*: 5'-CATGAGGAACAGCAATCAATG-3' and 5'-TAATACGACTCACTATAGGGACACATACTCATCAGATCTTCTTCC-3' (524 bp). *nmur1a*: 5'-GGCATTAAACCTCACCGAGA-3' and 5'-TAATACGACTCACTATAGGGCACGTTTCGTCAAGAAATCAAA-3' (1620 bp). *nmur1b*: 5'-GTGAACACGTCATGGTGCTC-3' and 5'-TAATACGACTCACTATAGGGGCGTTGGTATTCAGAAACTGC-3' (1206 bp). *nmur2*: 5'-

CTCCTGACCTGCGCTGTAAT-3' and 5'-TAATACGACTCACTATAGGGGAGGAGCTGAACTTGACTTGC-3' (1486 bp). Plasmids containing *crh* (genbank clone CK352624, 849 bp) and *cfos* (genbank clone CA787334, 870 bp) expressed sequence tags were also used for riboprobe synthesis.

Samples were coverslip mounted in 80% glycerol in PBS and imaged using a compound microscope (Axioimager with EC Plan-Neofluar 10x/0.30 NA Air or Plan-Apochromat 20x/0.8 NA Air objective, Carl Zeiss MicroImaging, Inc.) for chromogenic ISH samples, or for double fluorescent ISH samples, a confocal microscope (LSM 780 with a Plan-Apochromat 10x/0.45 NA Air or LD C-Apochromat 40x/1.1 NA Water objective, Carl Zeiss MicroImaging, Inc.). Fluorescein and Cyanine were imaged in separate channels with a 488 nm or 561 nm laser, respectively. Confocal images are displayed as the maximum intensity z-projection of a stack of optical sections of approximately 1 airy unit (A.U.) thickness and 0.5 A.U. spacing.

Drug treatment

A 20 mM stock of 5-Chloro-*N*-(cyclopropylmethyl)-2-methyl-*N*-propyl-*N*'-(2,4,6-trichlorophenyl)-4,6-pyrimidinediamine hydrochloride (NBI 27914, Cat#1591, Tocris) was freshly prepared in dimethyl sulfoxide (DMSO) just prior to drug treatment. Immediately after the 37°C heat shock treatment from noon to 1 p.m. on 5 dpf, drug and vehicle were loaded into alternating columns of an open (i.e. not sealed) 96-well plate to make a final working concentration of 5 μM NBI 27914 in 0.05% DMSO, or 0.05% DMSO (vehicle only) in E3 embryo medium. Separate experiments were performed with the same procedure as above at 2 μM or 0.5 μM NBI 27914 in 0.05% DMSO and included within-experiment, vehicle only controls. For the dose-response graph, post-HS Day waking activity data across separate experiments (i.e. different drug

concentrations and controls) were normalized to within-experiment, vehicle-only controls before being pooled for analysis.

Weight and size measurements of adult zebrafish

Samples were generated from heterozygous x heterozygous mutant crosses for each mutation. To match environmental conditions across genotypes, sibling fish were raised in genotype-intermixed tanks (6 per 1.8 L tank) at 28.5°C until measurement at 20-22 weeks post fertilization. Thus, phenotypes for each mutation were compared across age- and housing-matched siblings. Fish were briefly anesthetized with tricaine, photographed, then blotted and weighed. Torso length was measured as the linear distance between the rostral tip to the end of the trunk, not including the tail fin. Each fish was then genotyped by fin clip and PCR as described above. Up to 6 each of males and females were measured for each genotype.

Supplemental References

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