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7 **TRANSIENT EXPRESSION OF NEUROPEPTIDE W IN POSTNATAL MOUSE**

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10 **HYPOTHALAMUS - A PUTATIVE REGULATOR OF ENERGY HOMEOSTASIS**

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17 T. MOTOIKE ^{a*}, A. G. SKACH ^a, J. K. GODWIN ^a, C. M. SINTON ^b, M. YAMAZAKI ^c,
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20 M. ABE ^c, R. NATSUME ^c, K. SAKIMURA ^c AND M. YANAGISAWA ^{a, d*}
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27 ^a Howard Hughes Medical Institute and Department of Molecular Genetics, University of
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31 Texas, Southwestern Medical Center, Dallas, TX 75390-8584, USA;
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34 ^b Arizona Respiratory Center, Department of Medicine, University of Arizona, Tucson, AZ
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38 85724-5030, USA;
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40

41 ^c Department of Cellular Neurobiology, Brain Research Institute, Niigata University,
42
43
44
45 Niigata 951-8585, Japan;
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47

48 ^d International Institute for Integrative Sleep Medicine (WPI-IIIS), University of Tsukuba,
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52 Tsukuba 305-8575, Japan
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* To whom correspondence should be addressed.

Masashi Yanagisawa

International Institute for Integrative Sleep Medicine (WPI-IIIS), University of Tsukuba, 1-

1-1 Tennodai, Tsukuba, 305-8575, Japan

Phone: +81-29-853-3301

E-mail: yanagisawa.masa.fu@u.tsukuba.ac.jp

And

Toshiyuki Motoike

Howard Hughes Medical Institute and Department of Molecular Genetics,

University of Texas, Southwestern Medical Center,

5323 Harry Hines Blvd., Dallas, TX 75390-8584, USA

Phone: 940-465-3596

E-mail: Toshiyuki.Motoike@UTSouthwestern.edu

Author Contributions

T.M., C.M.S., K.S., and M.Y. designed research.

T.M., A.G.S., J.K.G., M.Yam., M.A., and R.N. performed research.

T.M., C.M.S. and M.Y., analyzed data and wrote the paper.

Abbreviations

AGRP, Agouti-related protein; ARC, arcuate nucleus; CART, cocaine amphetamine-regulated transcript; DA, dopamine; DMH, dorsomedial hypothalamus; FRT, flippase recognition target; GABA, gamma-aminobutyric acid; LH, lateral hypothalamus; MC4-R, melanocortin 4 receptor; MCH, melanin-concentrating hormone; NPB, neuropeptide B; NPW, neuropeptide W; NPBWR, neuropeptide B/W receptor; NPY, neuropeptide Y; PAG, periaqueductal gray; PF, perifornical area; PP, pancreatic polypeptide; pSTAT3, phosphorylated-STAT3; PVN, paraventricular nucleus; PYY, peptide YY; SCN, suprachiasmatic nucleus; SubI, subincertal nucleus; TH, tyrosine hydroxylase; VMH, ventromedial hypothalamus; VTA, ventral tegmental area; ZI, zona incerta; ZT, zeitgeber.

Abstract

Neuropeptide B and W (NPB and NPW) are cognate peptide ligands for NPBWR1 (GPR7), a G-protein coupled receptor. In rodents, they have been implicated in the regulation of energy homeostasis, neuroendocrine/autonomic responses, and social interactions.

Although localization of these peptides and their receptors in adult rodent brain has been well documented, their expression in mouse brain during development is unknown. Here we demonstrate the transient expression of NPW mRNA in the dorsomedial hypothalamus (DMH) of postnatal mouse brain and its co-localization with neuropeptide Y (NPY) mRNA.

Neurons expressing both NPW and NPY mRNAs begin to emerge in the DMH at about postnatal day 0 (P-0) through P-3. Their expression is highest around P-14, declines after P-21, and by P-28 only a faint expression of NPW and NPY mRNA remains. In P-18 brains, we detected NPW neurons in the region spanning the subincertal nucleus (SubI), the lateral hypothalamic (LH) perifornical (PF) areas, and the DMH, where the highest expression of NPW mRNA was observed. The majority of these postnatal hypothalamic NPW neurons co-express NPY mRNA. A cross of NPW-iCre knock-in mice with a Cre-dependent

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7 tdTomato reporter line revealed that more than half of the reporter-positive neurons in the
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10 adult DMH, which mature from the transiently NPW-expressing neurons, are sensitive to
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13 peripherally administrated leptin. These data suggest that the DMH neurons that transiently
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16 co-express NPW and NPY in the peri-weaning period might play a role in regulating energy
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19 homeostasis during postnatal development.
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35 Keywords: NPY, dorsomedial hypothalamus, postnatal development, leptin
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7 Neuropeptide B (NPB) and neuropeptide W (NPW) are endogenous neuropeptide ligands
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10 for NPBWR1 and NPBWR2, which are members of the G protein-coupled receptor
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13 (GPCR) family, originally termed GPR7 and GPR8, respectively (Tanaka et al., 2003,
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17 Sakurai, 2013). Whereas humans and other primates express both NPBWR1 and NPBWR2,
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20 a functional NPBWR2 ortholog has not been demonstrated in rodents. The physiological
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24 function of NPBWR1 has been characterized by the phenotypical analysis of NPBWR1 null
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27 mice, which showed late onset obesity (Ishii et al., 2003), abnormal social interaction, and
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31 an aberrant autonomic response to physical stress (Nagata-Kuroiwa et al., 2011). A single
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35 nucleotide polymorphism of the human NPBWR1 gene has been shown to influence the
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39 ability to evaluate facial expressions (Watanabe et al., 2012). In contrast, the physiological
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42 function of NPBWR2 in primates has not been elucidated.

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45 NPB and NPW are encoded by two separate genes and together with NPBWR1 (and
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49 NPBWR2 in primates) constitute a neuropeptide-receptor system (Tanaka et al., 2003,
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53 Sakurai, 2013). NPB is a 29-amino acid peptide with a unique brominated N-terminal
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7 brain, NPB mRNA is expressed in multiple nuclei in the hypothalamus, hippocampus,
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10 midbrain, and brain stem (Tanaka et al., 2003). Phenotypical analysis of NPB null mice has
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13 shown that the neuropeptide is implicated in metabolism and pain sensation (Kelly et al.,
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17 2005).

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20 As the mature peptide, NPW exists in two forms with 23 and 30 amino acids, named
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24 NPW23 and NPW30, respectively. Both peptides also have tryptophan at the N-terminus,
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27 but neither is brominated, and they activate NPBWR1 and NPBWR2 with similar
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31 nanomolar affinities (Shimomura et al., 2002, Tanaka et al., 2003). In contrast to the
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34 relatively widespread distribution of NPB mRNA throughout mouse brain, the neurons
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37 expressing NPW mRNA are primarily located in specific midbrain regions of the adult
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41 mouse, encompassing the periaqueductal gray (PAG), the ventral tegmental area (VTA), the
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44 Edinger-Westphal nucleus, and the dorsal part of the dorsal raphe nucleus (Tanaka et al.,
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48 2003). We have observed a similar expression pattern of NPW in adult rat brain (Kitamura
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52 et al., 2006) and this expression pattern implies a potential role for NPW in the regulation
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56 of limbic function in the adult rodent. In this regard, we also observed a diffuse expression
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7 of NPW mRNA in the CA3 region of adult mouse hippocampus (Kelly et al., 2005).
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10 Interestingly, although NPW mRNA expression is localized in the midbrain and CA3
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12 region in adult mouse brain, additional expression of NPW is reported in regions of the
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14 adult rat hypothalamus including the paraventricular nucleus (PVN), lateral hypothalamus
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17 (LH), arcuate nucleus (ARC), ventromedial hypothalamus (VMH) and dorsomedial
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20 (DMH) (Dun et al., 2003, Date et al., 2010, Takenoya et al., 2010). Such a
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23 hypothalamus (DMH) (Dun et al., 2003, Date et al., 2010, Takenoya et al., 2010). Such a
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26 species difference in the expression pattern in mouse and rat brains is also observed in the
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29 hypothalamic expression of NPY mRNA: i.e., NPY mRNA is detected in the adult rat
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32 DMH, but not detected in the adult mouse DMH (see below). Thus, the species difference
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35 in the expression of these neuropeptides between mouse and rat may underlie differences in
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38 their species-specific behaviors and metabolism.
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49 NPY was discovered in porcine intestine in 1982 and, since then, its molecular properties,
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52 physiological function and neurocircuitry have been extensively characterized in adult
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56 rodents (Tatemoto et al., 1982, Chambers and Woods, 2012, Sohn et al., 2013). NPY is a
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7 36-amino acid peptide with structural similarities to peptide YY (PYY) and pancreatic
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10 polypeptide (PP), forming a neuropeptide family. Five receptors for NPY have been cloned
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13 from mammals: Y1, Y2, Y4, Y5 and Y6. Y6 is functional in rabbits and mice, but not in
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16 humans. Among these receptors, Y1 and Y5 are widely distributed throughout the brain,
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19 including the hypothalamus, and are thought to mediate primarily the orexigenic effects of
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22 NPY (Kamiji and Inui, 2007, Pedragosa-Badia et al., 2013).
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27 NPY is one of the most abundant neuropeptides and is widely distributed throughout the
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30 central nervous system including the cortex, limbic system, and hypothalamus. Within the
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33 hypothalamus of adult rodents, the highest expression of NPY mRNA is observed in the
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36 ARC where it is co-expressed with Agouti-related protein (AgRP) (Schwartz et al., 2000).
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39 Functionally, NPY is a potent orexigenic peptide in rat as indicated by the observations that
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42 intracerebroventricular (ICV) injection of NPY stimulates robust increases in food intake
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45 and body weight (Clark et al., 1984), and that food restriction increases NPY expression in
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48 the ARC (Brady et al., 1990, White and Kershaw, 1990).
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56 In addition to constitutive expression in the ARC, NPY mRNA expression is also
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7 observed in the DMH of adult rat and rhesus monkey (White and Kershaw, 1990,
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10 Schroeder et al., 2009, Bi et al., 2012). In rat, the expression of NPY mRNA in the DMH
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13 is upregulated under conditions of increased energy demand accompanied by chronic
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17 hyperphagia: such conditions include early development during the postnatal period (Singer
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20 et al., 2000, Grove and Smith, 2003) and lactation in the adult (Li et al., 1998).
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24 However, NPY mRNA expression is not observed in the DMH of normal adult mice.
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27 Indeed, published data indicate that NPY mRNA is only observed in the DMH of obese
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30 mice: both in mice with melanocortinergetic obesity, a model in which signaling through the
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33 melanocortin 4 receptor (MC4-R) is antagonized or deleted (Kesterson et al., 1997) and in
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36 mice that were fed a high-fat diet (Guan et al., 1998, Lee et al., 2013). Postnatal
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39 expression of NPY mRNA is also demonstrated in NPY hrGFP mice, which harbor
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42 humanized renilla reniformis Green Fluorescent Protein (GFP) under the NPY promoter
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45 (Draper et al., 2010). Interestingly, the distribution pattern of NPY mRNA in the DMH is
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48 different in mouse and rat. In mouse DMH, NPY mRNA is expressed only in the dorsal and
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52 ventral part of the DMH (DMHd and DMHv) and not in the compact part of the DMH
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(DMHc) (Draper et al., 2010, Lee et al., 2013). In rat DMH, however, NPY mRNA is predominantly observed in the DMHc (White and Kershaw, 1990, Li et al., 1998, Grove et al., 2001, Bi et al., 2003, Schroeder et al., 2009) and NPW mRNA expression in the non-compacta DMH is only induced under chronic hyperphagic conditions (Li et al., 1998, Grove et al., 2001). Although the effects of NPY in the ARC with respect to the control of energy balance have been well studied, relatively few studies have addressed the function of NPY in the DMH.

The postnatal period is critical for rodent pups not only for their development *per se*, but also in preparation for weaning and independence from the dams. Behaviorally at this stage, the rodent pup severs bonding with the dam and leaves her protection, while metabolically they prepare for a profound change in their energy source, i.e. from milk suckling to solid food intake. Thus, the mechanisms, which underlie these significant transitions, may be highly specialized and differ from those in adult animals. The specific distributions of NPW in rodent brain suggest limbic and metabolic functions for NPW, and it is therefore

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7 important to investigate the expression of the neuropeptide during the postnatal period.
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10 However, although the expression of NPW in adult rodent brain has been well characterized,
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13 its expression during development in rodent brain remains unknown.
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17 Here, we demonstrate the transient expression of NPW mRNA in a distinct
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20 subpopulation of hypothalamic neurons in postnatal mouse brain. We generated *NPW-iCre*
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23 knock-in mice and by mating these mice with Cre-dependent fluorescence reporter mice
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27 (*tdTomato* mice), we further characterized these transiently NPW-positive neurons in the
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30 postnatal DMH.
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EXPERIMENTAL PROCEDURES

Animals

Unless otherwise indicated, all mice were provided with food and water *ad libitum*, maintained under a 12-hour light/dark cycle, and were housed under conditions that controlled for temperature and humidity. All animal procedures were approved by the Institutional Animal Care and Research Advisory Committee (IACRAC) of the University of Texas Southwestern Medical Center, and were strictly in accordance with National Institutes of Health guidelines. The *FLPe* mouse (Stock No. 009086) and *tdTomato* mouse (Stock No. 007909) were purchased from Jackson Laboratory (Bar Harbor, ME). The *GAD67-GFP* knock-in mouse (Tamamaki et al., 2003) was kindly provided by Y. Yanagawa (Gunma University, Maebashi, Japan).

In situ hybridization histochemistry

Sectional *in situ* hybridization histochemistry (ISH) using digoxigenin-labeled riboprobes was performed as previously described (Benjamin et al., 1997, Mieda et al., 2006). A 0.45-

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6 kb coding region of the mouse *NPW* cDNA spanning from the mature peptide to the stop
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10 codon and a 0.29-kb coding region of the mouse *NPY* cDNA were subcloned into
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13 pBluescript (Stratagene, La Jolla, CA). Antisense riboprobes were then generated with T7
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17 phage RNA polymerases, using the Maxiscript kit (Ambion, Austin, TX) with DIG RNA
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20 Labeling Mix (Roche, Germany). Colorimetric staining was performed with nitro blue
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24 tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Roche). For dual-label
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27 NPW or NPY ISH/DsRed immunohistochemistry, sections were processed first for *in situ*
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31 hybridization using 2-hydroxy-3-napthoic acid-2'-phenylanilide phosphate (HNPP) and
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35 Fast Red (Fluorescent Detection Set, Roche). Since tdTomato fluorescence was bleached
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38 out during ISH procedures and no spontaneous tdTomato red fluorescence could be
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42 detected, sections were rinsed with PBS and processed for DsRed immunohistochemistry
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45 after the fluorescent NPW or NPY ISH (Hasegawa et al., 2014). Following incubation with
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49 primary anti-DsRed (i.e., anti-tdTomato) antiserum, sections were directly incubated with
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53 anti-rabbit IgG conjugated with fluorescent dye Alexa Fluor 488 (1:200, Invitrogen,
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Immunohistochemistry

Immunostaining was performed as previously described (Chemelli et al., 1999). Briefly, floating brain sections (35 μ m in thickness) were incubated in anti-DsRed rabbit polyclonal antibody (632496; 1:1,000 dilution, Clontech, Mountain View, CA), anti-MCH rabbit polyclonal antibody (H-070-47; 1:200 dilution, Phoenix Pharmaceuticals, Inc., Burlingame, CA), anti-tyrosine hydroxylase (TH) mouse monoclonal antibody (MAB318; 1:2,500 dilution, Chemicon International, Temecula, CA), and anti-phospho-STAT3 rabbit polyclonal antibody (#9131; 1:4,000 dilution, Cell Signaling Technology, Inc., Danvers, MA) for 1-3 overnight periods at 4°C or room temperature. In the case of immunostaining with the anti-phospho-STAT3 rabbit polyclonal antibody, floating sections were pre-treated with 1% NaOH and 1% hydrogen peroxide in water for 20 min, followed by 0.3% glycine in PBS for 10 min, and 0.03% sodium dodecyl sulfate in PBS for 10 min (Gautron et al., 2013). After the incubation with primary antiserum, sections were rinsed with PBS and directly incubated with anti-rabbit or anti-mouse IgG conjugated with fluorescent dye Alexa Fluor 488 (1:200, Invitrogen).

Production of *NPW-iCre* mutant mice

The *NPW-iCre* mutant mouse line was produced by homologous recombination of the *NPW* gene (*NPW*) with the iCre recombinase gene inserted into the translational initiation site of the *NPW* gene in frame and by electroporation into the C57BL/6N ES cell line RENKA (Mishina and Sakimura, 2007). We constructed a targeting vector in which the iCre gene had been inserted into the translation initiation site of the *NPW* in frame. The 1.8-kb DNA fragment, which carried the polyA signal sequence and Pgk1 promoter-driven neomycin phosphotransferase gene (neo) flanked by two flippase recognition target (FRT) sites (Takeuchi et al., 2002), was inserted into the targeting vector downstream of iCre. The targeting vector contained the iCre-frt-neo-frt cassette, 4.75 kb upstream and 6.64 kb downstream genomic sequences, and 4.3 kb pMC1DTpA (Kitayama et al., 2001). First, the heterozygous mutant mouse was crossed to the *FLPe* mouse (Stock No. 009086, Jackson Laboratory) to remove the Pgk1-neo sequence: the *NPW-iCre* mouse, in which the Pgk1-neo sequence was successfully deleted, was then used to make a reporter mouse line. iCre-expressing cell types were tested by mating this *NPW-iCre* mouse with the floxed

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7 fluorescence reporter *tdTomato* mouse (Stock No. 007909, Jackson Laboratory, Bar Harbor,
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10 ME). When the *tdTomato* mouse is crossed with the *NPW-iCre* mouse, an iCre
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13 recombination event excises the STOP cassette to allow the constitutive expression of
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17 *tdTomato* selectively in NPW-expressing neurons of *NPW-iCre;tdTomato* mice. The
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20 genetic background of *NPW-iCre;tdTomato* mice was an admixture of C57BL/6 and
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32 **Leptin administration**

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36 Adult male *NPW-iCre;tdTomato* mice (20 weeks old) were fasted for 16 hours and injected
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39 intraperitoneally with recombinant murine leptin (5 mg/kg, National Hormone and Peptide
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42 Program, Harbor-UCLA Medical Center, Torrance, CA, n=4) or PBS (n=3). Injections were
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46 performed between ZT3 and ZT5 [zeitgeber time (ZT); ZT0 is defined as the lights-on time
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49 and ZT12 as the lights-off time]. Forty-five minutes after injections, mice were anesthetized
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53 with intraperitoneal injections of a ketamine/xylazine (100 mg/kg;10 mg/kg) mixture, and
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57 perfused transcardially with 4% paraformaldehyde. Brains were harvested, sectioned at 35
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7 μm intervals, and processed for immunostaining with the anti-phospho-STAT3 antibody.
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RESULTS

1. Transient expression of NPW in postnatal mouse hypothalamus

Sectional ISH using the NPW anti-sense probe demonstrated the expression of NPW mRNA in (1) the midbrain periaqueductal gray (PAG) of adult brain and (2) the midbrain and hypothalamic regions of postnatal brain (Fig. 1A). The observation of NPW mRNA expression in the adult and postnatal midbrain region is consistent with previous results reported by several authors, including ourselves (Tanaka et al., 2003, Kitamura et al., 2006). However, the transient expression of NPW mRNA in the postnatal hypothalamic region was novel and unexpected. Thus, we examined the detailed time course of NPW mRNA expression in postnatal hypothalamus (Fig. 1B). Neurons expressing NPW mRNA (NPW neurons) were detected in the hypothalamic region at postnatal day 0 (P-0) and were clearly observed in the dorsomedial hypothalamic (DMH) region at P-3. The expression of NPW mRNA in the DMH increased during postnatal development, becoming highest at about P-14. After P-21, NPW expression in the DMH decreased; only a trace expression was observed at P-28 (Fig. 1B) and this completely disappeared in the adult (Fig. 1A).

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7 We also noted a characteristic spatial expression pattern of NPW mRNA in postnatal
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10 mouse brain (Fig. 1D). In the rostral hypothalamic coronal section from P-18 brain, in
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13 which the paraventricular nucleus (PVN) and suprachiasmatic nucleus (SCN) were
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17 diminishing and the arcuate nucleus (ARC) had started to appear, NPW neurons were
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20 detected in the lateral region of the subincertal nucleus (SubI), localized ventrally to the
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23 zona incerta (ZI). As increasingly caudal coronal brain sections were examined, the
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27 distribution of NPW neurons in the SubI shifted from lateral to medial, dispersed around
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30 the LH/PF, and then shifted further medially to the DMH region. The highest expression of
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34 NPW mRNA was observed in the DMH and expression had dissipated at about the level at
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38 which the DMH was no longer visible. No NPW neurons were detectable in more caudal
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42 hypothalamic regions.

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45 Interestingly, the developmental expression pattern of NPW mRNA in the postnatal
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48 DMH (Fig. 1B) was very similar to that of NPY, which has a well-established expression
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52 pattern in the postnatal rodent hypothalamus (Singer et al., 2000, Grove and Smith, 2003).
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56 We therefore performed ISH using an NPY anti-sense probe in order to evaluate the
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transient expression of NPY mRNA in postnatal mouse brain (Fig. 1C). The time course of NPY mRNA expression was essentially the same as the expression of NPW mRNA in the DMH: its expression in this area started at about P-3 with the maximum NPY mRNA expression also observed at about P-14. After P-21, NPY mRNA expression decreased and only a faint expression of NPY mRNA was observed at P-28. These results are essentially identical to those reported previously in rat DMH (Singer et al., 2000). In adult mouse brain, we could not detect either NPW mRNA or NPY mRNA in the DMH region.

2. tdTomato fluorescence in *NPW-iCre;tdTomato* reporter mouse brain

Noting the highly similar developmental expression pattern of NPW and NPY mRNA in postnatal hypothalamus, we examined the co-localization of these two neuropeptides. For this purpose, we generated *NPW-iCre* knock-in mice, in which iCre expression is driven under the control of the NPW promoter (Fig. 2A). We crossed this mouse with a mouse harboring a *ROSA26-loxP-stop-loxP-tdTomato* reporter allele, producing a mouse line (*NPW-iCre;tdTomato*), in which tdTomato expression is activated through the action of the

NPW promoter-driven iCre recombinase. We first investigated the expression of tdTomato fluorescence in adult *NPW-iCre;tdTomato* mouse brain. Surprisingly, however, only a few tdTomato positive neurons (tdTomato+ neurons) were detected in the PAG of adult mouse brain (Fig. 2B), where the expression of NPW mRNA has been consistently verified by ISH (Fig. 1A) (Tanaka et al., 2003, Kitamura et al., 2006). Two possible explanations for this expression pattern will be the subject of future research: (1) the strength of the ROSA promoter driving the tdTomato fluorescence could vary depending on brain region; or (2) the naive NPW promoter-driven iCre recombinase activity could vary the brain region.

In contrast to the low expression of tdTomato fluorescence in the PAG region in adult mouse brain, tdTomato+ neurons were extensively observed only in three regions of the whole brain: (1) cortex, (2) hippocampus, and (3) hypothalamus including the DMH (Fig. 2B, C).

Following observation of the tdTomato+ neurons in adult cortex and hippocampus, we re-examined postnatal and adult brains to investigate whether NPW+ neurons were revealed in these regions by ISH. In the postnatal or adult cortex (Fig. 3), however, we

could not detect any NPW+ neurons using ISH. Possibly, tdTomato fluorescence in adult cortical neurons was caused by a leaky expression of iCre. Alternatively, some adult cortical neurons might have expressed NPW mRNA at a level below that detectable by ISH. Of note, some tdTomato+ neurons were already observed at P-7 (Fig. 2D), raising a further possibility that some postnatal cortical neurons had expressed NPW early in development but then ceased expression in the adult.

Based on morphology and location in the hippocampal structure, we assumed that the tdTomato+ neurons observed in adult hippocampus comprise a distinct population of pyramidal neurons in the CA3 region. In our previous study, we observed a diffuse expression of NPW mRNA in the CA3 region of adult mouse brain and the present finding is thus consistent with that observation (Kelly et al., 2005). However, we were unable to detect any distinct expression of NPW mRNA in the postnatal or adult hippocampus (Fig. 3). In view of our previous result, we assume that a certain subpopulation of pyramidal neurons in the CA3 might express NPW mRNA but at a level below the present ISH detection limit. Interestingly, tdTomato+ neurons were not observed in P-7 hippocampus

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7 whereas they were detected in the cortex and DMH at this age (Fig. 2D). They were also
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10 observed in P-14 hippocampus (data not shown), suggesting that some pyramidal neurons
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13 in the CA3 region may start expressing NPW mRNA between P-7 and P-14.
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17 Using ISH, we observed the expression of NPW mRNA in a distinct subpopulation of
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20 postnatal, but not adult, hypothalamic neurons (Fig. 1A, B). Since the iCre-mediated
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23 excision of the loxP-stop-loxP sequence in the reporter construct results in the permanent
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26 expression of tdTomato in the iCre-positive cells, our interpretation is that the tdTomato+
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29 neurons observed in the adult hypothalamus (Fig. 2B, C) had matured from those postnatal
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32 neurons that had transiently expressed a high level of NPW mRNA but then ceased that
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35 expression at about the time of weaning. Indeed, the distribution pattern of NPW neurons
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38 was essentially identical to that of tdTomato+ neurons in the postnatal hypothalamus at the
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41 age of P-14 (Figs. 4 and 5).
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49 In order to verify the reliability and usefulness of the *NPW-iCre;tdTomato* mouse for
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52 investigating the postnatal expression of NPW mRNA in the hypothalamus, the number of
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55 neurons which co-expressed NPW mRNA and tdTomato was quantified. We counted
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neurons in an area of 650 μm x 770 μm , in which the whole region of the DMH of P-14 *NPW-iCre;tdTomato* brain was encompassed (this area is defined by the white dotted line in Fig. 5B). tdTomato fluorescence was bleached out by ISH procedures. Therefore, after the fluorescent NPW ISH using HNPP/Fast Red (red), we performed immunohistochemistry with the anti-DsRed (i.e., anti-tdTomato) antibody and Alexa 488 Fluor (green) to detect tdTomato positive neurons. Approximately 95% of NPW mRNA expressing neurons were tdTomato positive and nearly 100% of tdTomato positive neurons expressed NPW mRNA (Fig. 5B, C). We thus adopted this *NPW-iCre;tdTomato* mouse for the co-localization studies of NPW neurons with NPY and other neurotransmitters in postnatal hypothalamus. We also used this mouse line to study the response of adult tdTomato neurons to peripherally administrated leptin.

3. Co-localization with other neurotransmitters

The characteristic spatial and temporal distribution pattern of NPW neurons in postnatal hypothalamus encouraged us to investigate the co-localization of NPW not only with NPY,

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7 but also with other neuropeptides and neurotransmitters, including melanin-concentrating
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10 hormone (MCH), gamma-aminobutyric acid (GABA), and dopamine, the expression
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13 patterns of which have already been well documented in the hypothalamus.
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21 **3.1 NPY**

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24 In order to determine the co-localization of NPY in postnatal NPW neurons in
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27 hypothalamus, we performed NPY ISH on brain slices from P-14 *NPW-iCre;tdTomato* mice.
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31 After fluorescent NPY ISH using HNPP/Fast Red (red), NPW neurons were detected
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34 immunohistochemically using the anti-tdTomato antibody and Alexa 488 Fluor (green). As
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37 shown in Fig. 6, the majority of tdTomato⁺ neurons (green), both in the rostral SubI (Fig.
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41 6A) and caudal DMH (Fig. 6B), expressed NPY mRNA (red). The number of neurons co-
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44 expressing NPW and NPY was counted in the specified area of 650 μ m x 770 μ m in the
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47 DMH (white dotted line in Fig. 6B). Nearly 95% of tdTomato⁺ NPW neurons (green) in
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51 this area were NPY mRNA positive (red), while approximately 87 % of NPY mRNA
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55 expressing neurons were tdTomato⁺ (Fig. 6B, C). Because NPW mRNA had not yet
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disappeared in these hypothalamic neurons at this postnatal age (P-14) (Fig. 1B), we concluded that a specific population of postnatal hypothalamic neurons transiently co-express NPW and NPY.

3.2 MCH

Melanin-concentrating hormone (MCH) is a 19-amino acid cyclic neuropeptide: MCH signaling is implicated in the regulation of multiple physiological functions, especially in relation to feeding behavior and energy balance (Saito and Nagasaki, 2008, Chung et al., 2011). Because neurons containing MCH are predominantly localized to the LH and adjacent zona incerta (ZI) regions (Elias et al., 2001, Croizier et al., 2013), we performed MCH immunohistochemistry on brain slices from P-28 *NPW-iCre;tdTomato* mouse brains. Fig. 7A displays a section, which contains the LH and rostral region of the DMH. In the LH, both MCH neurons (green) and tdTomato+ NPW neurons are observed, but no overlap is detected. Fig. 7B shows a proximal caudal section, which contains the caudal region of the DMH. At this level, the majority of tdTomato+ neurons are localized in the DMH and only

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7 a few tdTomato⁺ neurons are detected around the lateral hypothalamic (LH) perifornical
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10 (PF) region. In contrast, a robust expression of MCH is observed in the LH/PF region at
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13 this level. Thus, as demonstrated in these figures, no overlap was observed between MCH
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17 neurons and tdTomato⁺ neurons, indicating that distinct populations of hypothalamic
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21 neurons express MCH and NPW in postnatal mouse brain.
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27 **3.3 GABA**

31 γ -aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the CNS. Its
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34 distribution in rodent brain has been extensively studied and its localization pattern in the
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38 hypothalamus is well characterized (Tamamaki et al., 2003). For example, although
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42 GABAergic neurons are barely detectable in the ventromedial hypothalamic neurons
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45 (VMH), substantial numbers of GABAergic neurons are noted in the DMH (Tamamaki et
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48 al., 2003). Furthermore, Horvath and collaborators have reported the co-localization of
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52 GABA in adult ARC NPY neurons in rat brain (Horvath et al., 1997). In order to examine
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56 the co-localization of GABA and NPW in postnatal hypothalamic neurons, we mated
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7 *GAD67-GFP* knock-in mice (Tamamaki et al., 2003) with a *NPW-iCre;tdTomato* mouse to
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10 generate a *GAD67-GFP;NPW-iCre;tdTomato* line, in which GFP fluorescence is driven by
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13 the *GAD67* promoter in GABAergic neurons and tdTomato fluorescence identifies NPW
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17 neurons. Although a distinct population of NPY neurons in the adult mouse ARC is
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20 reported to be GABAergic (Horvath et al., 1997), we did not observe any co-localization of
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24 GFP fluorescence and tdTomato fluorescence in postnatal hypothalamic neurons from P-28
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27 *GAD67-GFP;NPW-iCre;tdTomato* mice (Fig. 8). We thus conclude that NPW neurons in
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31 the postnatal hypothalamus are not GABAergic, which would be specifically labeled by the
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35 *GAD67* promoter-driven GFP. However, this still leaves the possibility that a minor
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38 subpopulation of GABAergic neurons, not expressing *GAD67*, could co-localize with NPW.
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42 Further studies using ISH for *GAD65* and/or *GAD67* mRNA are therefore required before
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46 we can unequivocally confirm this conclusion.
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52 **3.4 Dopamine**

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56 The diencephalic dopaminergic (DA) neurons are divided into four groups in rodents,
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identified as A11 (caudal hypothalamus), A12 (ARC), A13 (medial ZI) and A14 (rostral third ventricle) (Albanese et al., 1986, Qu et al., 2006). Amongst these four groups of neurons, DA neurons of the A13 group are widely distributed from the ventral side of the ZI to the mammillothalamic tract, where some NPW neurons were observed (Figs. 1D and 4A). Furthermore, most DA neurons of the A12 group are localized to the ARC region, but subpopulations of this group are also observed between the DMH and the third ventricle. Moreover, we found that the majority of NPW neurons in the adult mouse midbrain PAG co-express dopamine (Motoike et al. unpublished observations). We therefore performed TH immunohistochemistry on brain slices from P-28 *NPW-iCre;tdTomato* mice to determine whether postnatal hypothalamic NPW neurons also co-express dopamine (Fig. 9). No tdTomato⁺ neurons in the rostral SubI and caudal DMH regions were co-stained with the TH antibody, indicating that NPW neurons in the postnatal hypothalamus are not dopaminergic.

4. Responsiveness of tdTomato⁺ neurons to peripherally administrated leptin

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7 The plasma leptin level of young postnatal mice (at about P-10) is 5-10-fold higher than
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10 that of adult mice: this is known as the *postnatal leptin surge* (Ahima et al., 1998). Gautron
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13 and collaborators have demonstrated the expression of leptin receptors (LepRb) in a
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16 subpopulation of adult DMH neurons by using *LepRb-Cre* mice, in which Cre recombinase
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19 expression is under the control of the leptin receptor promoter (Gautron et al., 2010). We
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22 described above a specific population of postnatal DMH neurons transiently co-expressing
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25 NPW and NPY. Therefore, in order to examine the response of these NPW neurons to leptin,
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28 we administered leptin peripherally to adult *NPW-iCre;tdTomato* mice that had been fasted
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31 for 16 h. and then examined immunohistochemically the phosphorylation of STAT3, which
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34 is a marker for direct leptin signaling. Sixteen hours of fasting during the dark period (i.e.,
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37 from ZT12 to ZT4 the next day) lowered the background phosphorylation of STAT3 in
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40 DMH neurons. In fact, almost no phosphorylated STAT3 (pSTAT3) was detected in the
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43 DMH region 45 min after a control injection of PBS (Fig. 10A). In contrast, leptin
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46 administration induced a vigorous phosphorylation of STAT3 in numerous cells in the
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49 DMH region 45 min after the injection (Fig. 10B, C). In an area encompassing the whole
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7 DMH, which is the same size as that used for data displayed in Figs. 5 and 6, the number of
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10 tdTomato+ neurons stained for pSTAT3 was quantified. Robust pSTAT3 induction was
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13 observed in more than 60% of tdTomato+ neurons, while approximately 35% of pSTAT3-
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17 positive neurons expressed tdTomato (Fig. 10D). These results indicate that DMH
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20 tdTomato+ neurons, i.e., the neurons that had matured from those transiently co-expressing
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24 NPW and NPY in the neonatal period, may be involved in leptin-dependent energy
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28 homeostasis in the adult.
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DISCUSSION

NPB and NPW are newly discovered neuropeptides, which were identified by several laboratories including ours (Fujii et al., 2002, Shimomura et al., 2002, Brezillon et al., 2003, Tanaka et al., 2003). To date, no developmental studies on these two neuropeptides have been performed. In previous studies, we reported the primary localization of NPW mRNA in midbrain regions of adult rodents (Tanaka et al., 2003, Kitamura et al., 2006) and diffuse expression of NPW mRNA in the adult mouse hippocampus (Kelly et al., 2005). Other groups have also reported NPW expression in adult rat hypothalamus (Dun et al., 2003, Date et al., 2010, Takenoya et al., 2010), but we have not observed NPW expression in this region of the adult mouse. Here, we demonstrated a robust but transient expression of NPW mRNA in the postnatal mouse DMH region, peaking during the peri-weaning period. This is consistent with our previous finding that NPW mRNA is not detected in the adult DMH (Tanaka et al., 2003). Furthermore, this observation is supported by results from microarray analysis using RNA from adult mouse DMH in which no expression of NPW was detected (Draper et al., 2010, Lee et al., 2012).

The characteristic expression pattern of NPW mRNA in postnatal mouse hypothalamus described here can be summarized as showing a gradient of NPW mRNA expressed from lateral to medial in coronal sections moving from rostral to caudal. A physiological role for these NPW neurons in postnatal hypothalamus is currently unknown. We have also examined the co-localization of other neurotransmitters with well-characterized distribution patterns and functions, such as MCH, GABA, and DA in postnatal tdTomato+ neurons. However, we did not observe any co-localization of these neurotransmitters in tdTomato+ neurons. In the present study, we did not examine the co-expression of cocaine amphetamine-regulated transcript (CART) in postnatal NPW neurons. CART is a neuropeptide that is highly expressed in several rat hypothalamic regions such as the PVN, DMH, LH and ARC, and is associated with the regulation of food intake (Elias et al., 2001). Lee and collaborators have recently shown that NPY mRNA was detectable in the DMH of adult mice after 10-weeks of high-fat diet and essentially all of these NPY neurons co-expressed CART (Lee et al., 2013). Significantly, however, these authors also reported that CART was not expressed in DMH NPY neurons during development, suggesting that NPW

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7 neurons in the postnatal DMH might not co-express CART. Furthermore, several other
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10 neurotransmitters, including neurotensin, galanin and orexin are also known to show
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13 specific distributions in the DMH, PF and LH, i.e. those regions in which postnatal NPW
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17 neurons were observed in the present study (Leininger et al., 2011, Laque et al., 2013).
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21 Currently, any co-localization of these neurotransmitters with NPW remains unknown and
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24 further studies will be required to elucidate the function(s) of these postnatal NPW neurons.
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28 We noted that a majority of hypothalamic NPW neurons co-expressed NPY during the
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31 postnatal period in mouse brain. One of the most rigorously established roles of NPY is the
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34 regulation of feeding and body weight (Chambers and Woods, 2012, Sohn et al., 2013). In
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37 adult rodents, the highest expression of NPY is observed in the ARC, where 95% of NPY
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41 neurons co-express AgRP mRNA and these neurons are able to sense and integrate
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45 peripheral hormonal signals, such as leptin, ghrelin and insulin to modulate food intake and
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48 energy balance (Schwartz et al., 2000, Sohn et al., 2013). In addition to ARC neurons, rat
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52 DMH neurons also express NPY (White and Kershaw, 1990, Schroeder et al., 2009) and the
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56 expression of this neuropeptide is upregulated under the specific conditions of chronic
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7 hyperphagia such as during postnatal development (Singer et al., 2000, Grove and Smith,
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10 2003), lactation (Li et al., 1998) or chronic obesity (Schroeder et al., 2009). Whereas most
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13 studies on energy homeostasis have focused on NPY/AgRP neurons in the ARC, little is
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16 known about the role of NPY in the DMH in the control of food intake and energy balance
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20 (Bi et al., 2012). Singer and collaborators quantified the transient expression of NPY
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23 mRNA in non-ARC hypothalamic neurons from postnatal rat brain and reported that the
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26 peak expression of NPY mRNA in rat DMH was observed at about P-15 to P-16 (Singer et
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29 al., 2000). This observation is consistent with our present results with postnatal mouse brain,
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32 in which the maximum expression of NPY mRNA, as well as NPW mRNA, was observed
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35 at about P-14. Neonatal rat pups are known to begin the ingestion of solid food at about P-
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38 15 to P-16, helping to initiate the process for weaning (Swithers, 2003). A robust increase
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41 of NPY and NPW in this postnatal period may thus be related to the important transition
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44 from milk suckling to solid food intake, with concomitant changes in fat energy intake.
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52 Using a *LepRb-Cre* mouse, Gautron and collaborators have demonstrated that leptin
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55 receptors are expressed in mouse DMH (Gautron et al., 2010). The distribution pattern of
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7 leptin receptor-positive neurons in mouse DMH reported by these authors was similar to
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10 that of tdTomato+ neurons which we observed here: both tdTomato+ neurons and leptin
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13 receptor-positive neurons are observed in the dorsal and ventral part of the DMH (DMHd
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16 and DMHv), but are not detectable in the compact part of the DMH (DMHc). We
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20 demonstrated here that approximately 60% of tdTomato+ neurons in the adult DMH
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24 responded to peripherally administrated leptin as shown by a robust induction of pSTAT3.
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27 These tdTomato+ neurons were the neurons that had transiently expressed NPW and NPY
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30 mRNA during the postnatal period. Lee and collaborators have reported that NPY mRNA
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34 was re-expressed in the adult DMH after 10-weeks of high-fat diet and that approximately
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38 64% of NPY neurons in the DMH of these obese mice exhibited robust phosphorylation of
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41 STAT3 after systemic leptin treatment (Lee et al., 2013), suggesting that they comprised the
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45 same specific subpopulation of DMH neurons that had transiently expressed NPW/NPY.
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48 Furthermore, in view of the high plasma leptin level during the early postnatal period, these
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52 results raise the possibility that NPW/NPY neurons in postnatal DMH are also under the
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56 control of leptin signaling. It will therefore, be important to identify the direct signal(s),
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6 activated under high-fat diet-induced obesity, and causing the re-induction of NPY
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10 expression in these DMH neurons. It will also be worth examining whether such signal(s)
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14 could re-induce NPW expression in adult DMH neurons.
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17 In conclusion, we report here that the specific postnatal expression of NPW mRNA in the
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20 DMH region is essentially identical to the tdTomato expression pattern in *NPW-*
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23 *iCre;tdTomato* mice. The DMH has long been implicated in multiple physiological
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26 functions such as autonomic responses, thermogenesis, circadian rhythmicity and energy
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29 homeostasis (Bellinger and Bernardis, 2002, Chou et al., 2003, Dimicco and Zaretsky, 2007,
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CONFLICT OF INTEREST

The authors declare that no competing interests exist.

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FIGURE LEGENDS

Fig. 1. Transient expression of NPW and NPY mRNA in mouse brain.

(A) Expression of NPW mRNA in the periaqueductal gray (PAG) and the dorsomedial hypothalamus (DMH) at postnatal day 18 (P-18) and adult (15 week old). Note that NPW mRNA is detectable in the young, but not in the adult DMH. (B) Expression of NPW mRNA in the DMH during development: sections from postnatal day 0 (P-0) to P-28. (C) Expression of NPY mRNA in the DMH during development: sections from postnatal day 0 (P-0) to P-28. Note that NPY mRNA is consistently detectable in the ARC during the immediate postnatal period. (D) Characteristic spatial expression pattern of NPW mRNA in hypothalamic regions at P-18: sections from rostral to caudal. mt, mammillothalamic tract; f, fornix; ZI, zona incerta; VMH, ventromedial hypothalamus; ARC, arcuate nucleus; ME, median eminence; 3v, 3rd ventricle. Approximate distance of each section from bregma is shown in white letters. Scale bars = 200 μ m.

Fig. 2. Expression of tdTomato fluorescence in *NPW-iCre;tdTomato* mouse brain.

(A) Strategy for generation of a *NPW-iCre* mouse line: schematic representation of wild-type NPW locus, targeting vector, and targeted locus. The targeting vector was designed to insert the iCre recombinase gene cassette into the translational initiation site of the NPW gene. Gray boxes represent 2 exons (Ex1 and Ex2) and the white box represents 3' non-coding sequence. Flippase recognition target (FRT) sites are indicated by open half-circles. pgk-gb2Neo p(A), pgk-promoter-neo-pA cassette; DT, diphtheria toxin cassette. (B) Expression of tdTomato fluorescence in the PAG, cortex, hippocampus and DMH from adult *NPW-iCre;tdTomato* mouse. tdTomato fluorescence positive neurons are extensively observed in the cortex, hippocampal CA3 region, and DMH, but only a few neurons in the PAG express tdTomato fluorescence. (C) Characteristic expression pattern of tdTomato fluorescence in hypothalamic regions from adult *NPW-iCre;tdTomato* mouse brain: sections from rostral to caudal. (D) Expression of tdTomato fluorescence in the PAG, cortex, hippocampus and DMH from a P-7 *NPW-iCre;tdTomato* mouse. Note that no neurons in the PAG or hippocampal CA3 region express tdTomato fluorescence at this age, although some neurons in the cortex and DMH do express tdTomato fluorescence. mt,

mammillothalamic tract; f, fornix; ZI, zona incerta; ARC, arcuate nucleus; 3v, 3rd ventricle.

Scale bars = 200 μ m.

Fig. 3. Expression of NPW mRNA in adult and P-18 mouse brains.

(A) Adult brain. Expression of NPW mRNA is observed only in the PAG, but is not detectable in the primary somatosensory cortex (a), ectorhinal cortex (b), piriform cortex (c), or hippocampal CA3 region (d). (B) P-18 brain. Expression of NPW mRNA is observed only in the DMH (c), but is not detectable in the hippocampal CA3 region (a) or ectorhinal cortex (b). Scale bars = 200 μ m.

Fig. 4. Expression pattern of NPW mRNA and tdTomato fluorescence in P-14

hypothalamic regions: sections from rostral to caudal.

Note that the expression pattern of NPW mRNA in hypothalamic regions from a wild type mouse (A) is essentially identical to that of tdTomato fluorescence from a *NPW-*

iCre;tdTomato mouse (B). mt, mammillothalamic tract; f, fornix; ZI, zona incerta; VMH,

ventromedial hypothalamus; ARC, arcuate nucleus; ME, median eminence. Scale bars = 200 μ m.

Fig. 5. Co-localization of NPW mRNA (red) and tdTomato (green) in the postnatal hypothalamus.

The rostral lateral hypothalamus (A) and the DMH (B) from a P-14 *NPW-iCre;tdTomato* mouse. NPW mRNA is detected using ISH with HNPP/Fast Red (red), and tdTomato is detected immunohistochemically with the anti-tdTomato antibody and Alexa Fluor 488 (green). The number of DMH neurons co-expressing NPW mRNA and tdTomato was quantified in a rectangular area (650 μ m x 770 μ m), defined by the white dotted line. Red and green linear structures observed in the figures are non-specifically stained capillary blood vessels. (C) The number of DMH neurons co-expressing NPW mRNA and tdTomato in the area defined by the white dotted line in B. Values are displayed as mean $\pm$ SEM (n=3). Scale bars = 200 μ m.

Fig. 6. Co-localization of NPY mRNA (red) and tdTomato (green) in the postnatal hypothalamus.

The rostral lateral hypothalamus (A) and the DMH (B) from a P-14 *NPW-iCre;tdTomato* mouse. NPY mRNA is detected using ISH with HNPP/Fast Red (red), and tdTomato is detected immunohistochemically with the anti-tdTomato antibody and Alexa Fluor 488 (green). The number of DMH neurons co-expressing NPY mRNA and tdTomato was quantified in a rectangular area (650 μ m x 770 μ m), defined by the white dotted line. Red and green linear structures observed in the figures are non-specifically stained capillary blood vessels. (C) The number of DMH neurons co-expressing NPY mRNA and tdTomato in the area defined by the white dotted line in B. Values are displayed as mean $\pm$ SEM (n=3). Scale bars = 200 μ m.

Fig. 7. No overlap of tdTomato fluorescence (red) and MCH (green).

A section containing the LH and rostral region of the DMH (A) and a proximal caudal section containing the LH/PF and caudal region of the DMH (B) from a P-28 *NPW-*

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7 *iCre;tdTomato* mouse. MCH is detected immunohistochemically with the anti-MCH
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10 antibody and Alexa Fluor 488 (green). Note that no co-localization is detectable between
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13 tdTomato red fluorescence and MCH immunoreactivity (green). Scale bars = 200 μ m.
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21 **Fig. 8. No overlap of tdTomato fluorescence (red) and GAD67GFP.**

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24 The rostral lateral hypothalamus (A) and the DMH (B) from a P-28 *GAD67-GFP;NPW-*
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27 *iCre;tdTomato* mouse. (C) High magnification view of a subregion of the DMH defined by
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30 the yellow dotted line in B. Note that no co-localization is detectable between tdTomato red
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42 **Fig. 9. No overlap of tdTomato fluorescence (red) and TH (green).**

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52 488 (green). Note that no co-localization is detectable between tdTomato red fluorescence
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56 and TH immunoreactivity (green). Scale bars = 200 μ m.
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Fig. 10. Co-localization of tdTomato fluorescence (red) and pSTAT3 (green) after leptin administration.

The DMH from an adult *NPW-iCre;tdTomato* mouse 45 minutes after systemic PBS (A) or leptin (B) administration. pSTAT3 is detected immunohistochemically with the anti-pSTAT3 antibody and Alexa Fluor 488 (green). Note that almost no pSTAT3 immunoreactivity is observed after PBS administration but a robust induction of pSTAT3 is observed after leptin administration. The number of DMH neurons co-expressing tdTomato and pSTAT3 after leptin treatment was quantified in a rectangular area (650 μm x 770 μm), defined by the white dotted line. (C) High magnification view of a subregion of the DMH defined by the yellow dotted line in B. (D) The number of DMH neurons co-expressing tdTomato and pSTAT3 in the area defined by the white dotted line in B. Values are displayed as mean $\pm$ SEM (n=4). Scale bars = 200 μm .

Figure 1.

Figure 2.

Figure 3.

Figure 4.

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Figure 5.

Figure 6.

Figure 7.

Figure 8.

Figure 9.

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Figure 10.