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Arcuate Nucleus Expression of NKX2.1 and DLX, and Lineages Expressing These Transcription Factors in NPY⁺, POMC⁺ and TH⁺ Neurons in Neonatal and Adult Mice

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Abstract

Despite its small size, the arcuate nucleus of the hypothalamus has a critical role in regulating energy homeostasis. We have embarked on defining genetic approaches to express genes in specific cell types within the developing arcuate nucleus, to enable precise molecular perturbations of these cells. Furthermore, we our analysis aims to contribute to defining the transcriptional networks that regulate the development of function of the arcuate neurons. Herein, we define the neuronal cells types within the arcuate that express *Nkx2.1* and *Dlx* homeobox genes. In addition, we used mice expressing Cre recombinase from the *Dlx5/6* intergenic enhancer (*Dlx5/6i*) and from the *Nkx2.1* locus to follow the fate of embryonic cells expressing these genes within the arcuate nucleus. We demonstrate that NKX2.1⁺ cells, and their lineages, are broadly expressed in arcuate neurons (GABA⁺, NPY⁺, POMC⁺, TH⁺) and glia (tanycytes). On the other hand, DLX⁺ cells, and their lineages, mark only GABA⁺ and TH⁺ (dopaminergic) neurons, and *Dlx1*^{-/-} mutants have fewer TH⁺ neurons. These results have implications for the genetic control of arcuate development and function, and for the utility of the *Nkx2.1-Cre* and *Dlx5/6i-Cre* mouse lines to alter gene expression in the developing arcuate.

Keywords

arcuate nucleus; hypothalamus; transcription factors; Dlx; Nkx2.1; VMH; NPY; POMC; mouse; development

Introduction

Many of the cellular components and circuits that control feeding within the central nervous system have been identified (Schwartz et al., 2000; Saper et al., 2002; Horvath and Diano, 2004; Cone, 2005). Among these, nuclei in the hypothalamus have salient roles. Direct

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inputs to the hypothalamus come through the median eminence, where humoral inputs affect activity of the arcuate nucleus (ARN). The ARN then projects to the paraventricular hypothalamic nucleus (PVH) and the lateral hypothalamic and perifornical areas (LHA and PFA). Other major hypothalamic nuclei that participate in regulating appetite include the ventromedial and dorsomedial hypothalamus (VMH and DMH), as well as circadian inputs through the retina to the superchiasmatic nucleus. The DMH, VMH and PVN largely promote negative energy balance, whereas the lateral hypothalamus and perifornical areas largely promote positive energy balance (Horvath and Diano, 2004).

Several peptides are expressed in subsets of neurons in the ARN and play a role in homeostatic regulation of feeding behavior. Some of the key regulators of homeostasis in the ARN include NPY, POMC, CART, and AgRP. Each of these peptides plays a role in stimulating or inhibiting food intake. Leptin is a key humoral factor that regulates the activity of many of these subsets of neurons in the ARN nucleus. Leptin inhibition of NPY⁺ cells is thought to send signals to the paraventricular nucleus that stimulate food intake (orexigenic signal), whereas activation of POMC⁺ neurons sends signals to the paraventricular nucleus that suppress food intake (anorexigenic).

Within the ARN nucleus, most of the NPY⁺ neurons are GABAergic, whereas most POMC neurons are not GABAergic (Horvath et al., 1997; Ovejero et al., 2001; Hentges et al., 2004). GABA can stimulate feeding behaviors in the rat (Kalra et al., 1999). Therefore, GABA and NPY signaling from ARN neurons activate appetite, whereas POMC suppresses appetite.

A subset of ARN neurons expresses tyrosine hydroxylase (TH), a key enzyme in dopamine synthesis (Phelps et al., 2003). These “tuberoinfundibular” neurons project to the median eminence, where their release of dopamine into the portal vasculature inhibits prolactin secretion from the anterior pituitary (Voogt et al., 2001). Roughly 15% of dopamine-containing ARN neurons also contain growth hormone releasing hormone (GHRH) (Phelps et al., 2003). Analysis of Dopamine receptor 2 mutant mice demonstrates that dopamine signaling through this receptor is required for normal levels of growth hormone release and growth (Diaz-Torga et al., 2002). Dopamine is also a potent orexin (reviewed in Volkow and Wise, 2005). It is clear that some of its orexigenic effects are controlled through mesencephalic dopaminergic innervation of the basal ganglia (Szczycka et al., 2001; Cannon et al., 2004). It is worth considering the hypothesis that dopamine signaling in the hypothalamus could contribute to its orexigenic activities. For instance, leptin positively regulates dopamine levels in the ARN; treatment of leptin deficient mice with dopamine agonists reverses their obesity (reviewed in Pijl, 2003). Treatment of humans with dopamine D2-receptor antagonists can induce obesity and diabetes; dopamine agonists can reduce these problems (Pijl, 2003).

The transcriptional control of the development and function of ARN neurons is beginning to be elucidated largely from studies in the mouse. Expression of the *Nkx2.1* homeobox gene in progenitor cells of the ventral hypothalamus is required for specification of this region (Marin et al., 2002). *Nkx2.1* expression persists in hypothalamic neurons, where it is required for promoting expression of *LHRH* and *KISS*, and in regulating onset of puberty and reproductive function (Mastroratti et al., 2006).

Mash1 is also expressed in ventral hypothalamic progenitor cells; *Mash1* mutants have hypoplastic ARN and ventromedial nuclei, due to defects in neurogenesis and apoptosis (McNay et al., 2006). Furthermore, *Mash1* promotes expression of *Gsh1*, a transcription factor required for *GHRH* expression in the ARN (Li et al., 1996). *Mash1* suppresses expression of both *tyrosine hydroxylase (TH)* and *neuropeptide Y (NPY)* (McNay et al., 2006).

Mash1 represses expression of the *Dlx* homeobox genes (Casarosa et al., 1999; Yun et al., 2002). The *Dlx* transcription factors are expressed in the region of the developing and adult ARN nucleus (Eisenstat et al., 1999; Saino-Saito et al., 2003), where, as described herein, they are expressed in GABA⁺ and TH⁺ neurons. The *Dlx* genes have key roles in the differentiation of forebrain GABA⁺ and TH⁺ neurons (Andrews et al., 2003; Cobos et al., 2007; Long et al., 2007; Petryniak et al., 2007; Qiu et al., 1995).

Finally, the ARN also expresses the *Otp* and *Nhlh2* transcription factors (Acampora et al., 1999; Bardet et al., 2008; Jing et al., 2004). There is evidence that *NHLH2* is expressed in POMC⁺ neurons where it promotes production of the anorexigenic peptides alpha-melanocyte-stimulating hormone and thyrotropin-releasing hormone (Jing et al., 2004). More recently, it has been demonstrated that *Dmbx1* regulates the response of brainstem nuclei to signals arising in the hypothalamus (Fujimoto et al., 2007).

Herein, we define the neuronal cells types within the ARN that express *Nkx2.1* and the *Dlx* genes. In addition, we used mice expressing Cre recombinase from the *Dlx5/6* intergenic enhancer (*Dlx5/6i*) and from a BAC containing the *Nkx2.1* locus to follow the fate of embryonic cells expressing these genes within the ARN nucleus. We demonstrate that NKX2.1⁺ cells, and their lineages, are broadly expressed in ARN neurons (GABA⁺, NPY⁺, POMC⁺, TH⁺) and glia (tanycytes). On the other hand, DLX⁺ cells, and their lineages, mark only GABA⁺ and TH⁺ (dopaminergic) neurons. *Dlx1*^{-/-} mutants have reduced numbers of these TH⁺ neurons. These results have implications for the genetic control of ARN development and function, and for the utility of the *Nkx2.1-Cre* and *Dlx5/6i-Cre* mouse lines to alter gene expression in the developing ARN.

Materials and Methods

Gad1 (*Gad67*)-EGFP (Δ neo) mice (Tamamaki et al., 2003) were provided by Kunihiro Obata and Yuchio Yanagawa (Gunma University, Japan). In these mice, EGFP was inserted into one allele of *Gad67*; therefore these mice are heterozygous for a functional *Gad67* allele.

A BAC DNA containing *Nkx2.1* was used for generating the transgenic *Nkx2.1-cre* mouse (Xu et al., 2008). This BAC was modified by inserting the Cre-polyA fragment into the exon II of *Nkx2.1* at the ATG site. *Dlx5/6i-Cre* mice carrying a transgene consisting of the *Dlx5/6* intergenic (*Dlx5/6i*) enhancer driving Cre (Monory et al., 2006; Kohwi et al., 2007) were used for fate-mapping studies; expression of the transgene largely recapitulates *Dlx5* expression (Stuhmer et al., 2002a), although in some cases there is ectopic expression in the caudoventral pallium; recombination in the region of the ARN reflects normal expression of *Dlx5*.

We used two lines of mice that express reporter genes following Cre recombination; the *Z/EG* reporter mice express GFP following Cre recombination (Novak et al., 1999), and the *ROSA26* reporter strain expresses *LacZ* (Soriano, 1999).

Animals

Brains were obtained from 26 adult and 17 postnatal day 0 (PO) mice. The University of California San Francisco's Institutional Animal Care and Use Committee approved the handling and use of all animals for these studies. Adult mice were anesthetized with avertin and perfused transcardially with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS; per 1 liter: 0.8g of NaCl, 0.02g of KCl, 0.144g of Na₂HPO₄, 0.024g of KH₂PO₄, pH to 7.4). PO mice were anesthetized on ice; brains were removed and placed in 4% PFA.

Immunocytochemistry and histochemistry

Following postfixation (2–4 hours) and cryoprotection in 10% sucrose for at least four hours and 20% sucrose in PBS overnight, PO and adult brains were sectioned coronally (20 μ m) on a cryostat and collected onto slides (FisherBrand Superfrost Slides, Fisher Scientific). Slides were washed in PBS, 3 $\times$ 10 minutes, and incubated for one hour at room temperature in blocking solution (1% bovine serum albumin, 5% normal goat serum, 0.3% Triton X-100 in PBS). After pretreatment, sections were placed overnight at 4°C in primary antisera diluted in blocking solution (For a list of antibodies, see Table 1). The following day, after three ten-minute rinses in PBS, sections were incubated for 1–2 hours at room temperature in the appropriate fluorescently labeled secondary antibodies (Alexa- 594 or Alexa-488). After three ten-minute rinses in PBS, sections were cover-slipped using Fluoromount G (Fisher Scientific).

Free-floating Immunohistochemistry: Following intracardial perfusion with 4% paraformaldehyde in PBS the brains were removed and postfixed overnight in the same fixative and cryoprotected by immersion in 30% sucrose. Free-floating cryostat sections (40 μ m) were processed using standard procedures. Briefly, sections were treated with 1% hydrogen peroxide in PBS for 30 minutes, and then washed for 30 minutes in PBS. Sections were blocked in 10% goat serum in PBST (0.1% TritonX-100 in PBS) for 2 hours, and then incubated with Tyrosine hydroxylase primary antibody (1:1000) overnight at 4°C. After primary incubation, sections were washed for 1 hour in PBS, and then incubated in biotinylated rabbit secondary antibody (Vector Labs, Burlingame, CA; 1:500) for 2 hours at room temperature. Sections were then washed for 1 hour in PBS, incubated with ABC solution (Elite kit, Vector) for 2 hours, washed for 1 hour in PBS, and developed in 0.5mg/ml diaminobenzamine (Sigma, St. Louis, MO) and 0.005% peroxide. Sections were mounted, dehydrated with alcohol and xylene washes, and cover slipped with Mount-Quick (Ted Pella, Redding, CA).

Immunocytochemical Controls: Omission of primary antibodies eliminated all immunoreactivity reported below. Elimination of one of the primary antibodies with application of both secondary antibodies confirmed secondary antibody specificity, i.e. the secondary antisera reacted only with IgG of the appropriate species. In cases where null mutant mice are available, antibodies were used on mutant tissue and staining was not detected (See Table 1).

Imaging

Images were collected using a Zeiss Meta 510 scanning microscope. For each double label immunohistochemical image, the two channels were collected separately with single wavelength excitation and then merged to produce the composite image. All images were composed in Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA) adjusting only brightness and contrast.

Nuclear profile counts and statistics

Cells on one side of the ARN were counted. A square area that began at the third ventricle was defined and used for profile counts. For TH immunoreactive profiles and double labeled profiles, the area of the ARN above the third ventricle on both the left and right side were chosen for analysis. Nuclear profiles were counted in a single section plane ($n=3$, where n = number of sections). The number of antibody⁺ profiles, GFP⁺ or β -galactosidase⁺ profiles and antibody⁺/GFP⁺ or β -galactosidase⁺ profiles were counted in PO and adult mice. The percent of antibody double labeled and GFP double labeled profiles was determined ($n=3$) and the over-counting of profiles was corrected for by applying Abercrombie's formula: $T/T+h$, where T = section thickness and h = mean diameter of the objects along the z axis (20/

20+ 6.785). The numbers of cell profiles labeled by immunohistochemistry were counted; the average number of labeled cells, and the percentage of double labeled cells were determined. The Abercrombie correction factor was applied and the standard deviation (variance) was determined for each value (Tables 2–4).

Results

Gad67 (Gad1)-GFP is co-expressed with DLX, NKX2.1, NPY and TH in the neonatal and adult ARN

We studied *Gad67 (Gad1)* expression in the ARN and adjacent regions of the hypothalamus using GFP expression from the *GAD67* locus (Tamamaki et al., 2003) on the day of birth (P0) (Fig. 1) and in adulthood (Fig. 2). We compared expression of *Gad67* (GFP) to the expression of the *DLX* and *NKX2.1* transcription factors, the *NPY* and *POMC* neuropeptides and the tyrosine hydroxylase (*TH*) enzyme. In the neonatal brain (P0) *Gad67*-GFP was detected in cell bodies and nerve fibers, whereas the VMH was largely devoid of GFP⁺ cells but contained some GFP⁺ processes (Figure 1 B, F, J, N and R). The pan distalless antibody, which binds *DLX* proteins from invertebrates and vertebrates (Panganiban et al., 1995; Stuhmer et al., 2002a), detected *DLX* expression in a subset of *Gad67* (GFP)⁺ P0 ARN cells (Figure 1, A–D); *DLX* expression was not detected in the VMH. 92.4% *DLX*⁺ (pan distalless) cells are *Gad67* (GFP)⁺ (Figure 1 C and D, arrowheads, Table 2). *NKX2.1* was expressed in a subset of *Gad67*-GFP⁺ cells in the P0 ARN (Figure 1 E–H) and a subset of *Gad67*-GFP[−] VMH cells (Lee et al., 2001; Marin et al., 2002; Kim et al., 2006). *NKX2.1* was also expressed in cells lining the ventricle (which become ependymal cells, or tanycytes, in the mature hypothalamus) (Lee et al., 2001).

POMC is expressed in anorexigenic neurons (activated in response to leptin; Cowley et al., 2001). *POMC*⁺ neurons in the P0 ARN did not exhibit detectable *Gad67* (GFP)⁺ immunoreactivity (Figure 1 I–L) (*POMC*⁺ cells were not detected in the VMH). *NPY*, which is expressed in orexigenic cells (inhibited by leptin; Inui et al., 1998; Serguei et al., 2004), are largely *Gad67* (GFP)⁺ (Figure 1 M–P). There were a few *NPY*⁺ cells in the VMH that are *Gad67* (GFP)⁺ (Figure 1 O, asterisk). Dopaminergic cells in the P0 ARN express tyrosine hydroxylase (*TH*). *TH*⁺ cells were present in similar distribution to *DLX*⁺ cells (Figure 1 Q). *TH*⁺ cells that are large in diameter clearly expressed *Gad67* (GFP) (Figure 1 S and T, arrowheads), whereas smaller diameter *TH*⁺ cells did not appear to be *GAD67*⁺ (GFP) (Figure 1 S and T, arrows).

In the adult ARN, *Gad67* (GFP) showed a similar pattern of co-expression with *DLX*, *NKX2.1*, *NPY* and *TH* as in the P0 ARN (Fig. 2). There were a few salient differences. First, there were a subset of *POMC*⁺ cells that were *Gad67* (GFP)⁺ (Figure 2 I–L, arrowhead), however the majority of *POMC* expressing cells (~95%) in the adult ARN lacked *GAD67* (GFP) immunoreactivity. Thus, like Hentges et al., 2004, we found evidence that a subset of *POMC*⁺ cells are GABAergic, although we observed roughly 6-fold fewer double-positive cells, perhaps because of differences in our methods (they used double *in situ* hybridization). Second, *NPY* immunoreactivity was difficult for us to detect in cell bodies in the adult mice, making it difficult to determine whether the *NPY*-expressing neurons also expressed *Gad67* (Figure 2 M–P).

Fate mapping of *Nkx2.1*-expressing cells in the P0 ARN using the *Nkx2.1-Cre BAC* transgene

We crossed mice with the *Nkx2.1-Cre BAC* transgene (Xu et al., 2008) with the *Z/EG* “recombination reporter” mice (Novak et al., 1999) to identify cells that had expressed *Nkx2.1* at some point in their development (Figure 3 B–D, F–H, J–L, N–P, R–T and V–X).

Cre mediated recombination activates GFP expression in the *Z/EG* mice. First, we compared GFP and NKX2.1 protein expression at P0 (Figure 3 A–D). The regional pattern of expression in the rostroventral hypothalamus was very similar (particularly in the ARN; Figure 3 C and D), although there were some differences. There were fewer GFP⁺ ependymal cells (arrow in Figure 3 B), and there was less GFP labeling in the VMH (Figure 3 B). In the ARN, there is a small population of NKX2.1⁺ cells (14.7%) that did not display GFP immunoreactivity (Figure 3 C and D, arrows), and 25.3% of GFP⁺ cells lack NKX2.1 immunoreactivity (Figure 3 C and D, asterisk, Table 3). Presumably some of the GFP⁺ only cells expressed *Nkx2.1* at an earlier point in their lifetime. Lack of GFP-expression in some NKX2.1⁺ cells suggests that the *Nkx2.1-Cre BAC* may lack regulatory elements that drive expression in these regions, or could be due to under-reporting because of incomplete recombination.

We next assayed whether *Nkx2.1*-lineage cells co-expressed DLX, POMC, NPY and TH pan-DLX antibody at PO. Cells co-expressing DLX and GFP (*Nkx2.1* lineage) were detected in lateral areas of the ARN (Figure 3G, boxed region), although 55.2% of DLX⁺ cells were GFP-negative (Figure 3G and H, arrows). Approximately 76.6% of POMC⁺ cells were GFP⁺ (Figure 3 K and L, arrowheads). Approximately two-thirds of NPY⁺ cells arise from the *Nkx2.1* lineage (Figure 3 O and P); NPY⁺;GFP[−] cells tend to be closer to the ventricle (Figure 3 O,P, arrow). 54% of TH⁺ cells were GFP⁺ (Figure 1 S and T, arrowheads), many cells that display only TH immunoreactivity were present in lateral areas of the ARN (Figure 3 S and T, arrows).

Fate mapping of *Nkx2.1*-expressing cells in the adult ARN using the *Nkx2.1-Cre BAC transgene*

Analysis in the adult involved crossing the *Nkx2.1-Cre BAC* transgene (Xu et al., 2008) with the *Rosa-LacZ* reporter mouse. All NKX2.1⁺ cells were also β-gal⁺ (i.e. had expressed Cre), while some NKX2.1[−] cells were also β-gal⁺ indicating they had expressed *Nkx2.1* at an earlier point in their development (Figure 4 C and D). Approximately 56.5% of ARN β-gal⁺ cells were pan-DLX⁺; 29% of ARN pan-DLX⁺ cells were β-gal[−] (Figure 4 E–H). *Nkx2.1-Cre* descendants contributed to 85.8% of the POMC⁺ cells (Figure 4 I–L) and 56.6% of the TH⁺ cells (Figure 4 M–P).

***Dlx 5/6i* lineage in the P0 ARN**

Dlx5 and *Dlx6* are expressed during development of the hypothalamus (Eisenstat et al., 1999; Liu et al, 1999; Ghanem et al., 2007; Puelles and Rubenstein, unpublished); their expression is driven by at least two intergenic enhancers (I5/6i and I5/6ii) (Zerucha et al., 2000; Ghanem et al., 2007) and by an uncharacterized extragenic element(s) (Dye and Rubenstein, unpublished). We have generated a transgenic mouse that expresses Cre under the control of *Dlx5/6i* (Monory et al., 2006; Kohwi et al., 2007), which largely recapitulates the expression of transgenic mice that express *LacZ* under the control of I5/6i (Zerucha et al., 2000; Stuhmer et al., 2002).

We crossed the *Dlx5/6i-Cre* transgene mouse with the *Z/EG* “recombination reporter” mice to identify cells that had expressed the *Dlx5/6* intergenic enhancer (i) at some point in their development (“*Dlx5/6i* lineage”). GFP⁺ cells of the *Dlx5/6i* lineage were present throughout the ARN, but were absent from the VMH (Figure 5 B, F, J, N and R). GFP⁺ axons were also apparent in the ARN and median eminence (Figure 5B, light green staining, open arrow). Pan-DLX and GFP double immunostaining revealed that there were cells that displayed only DLX immunoreactivity (Figure 5 C and D, asterisks), suggesting that *Dlx5/6i* was not expressed in all DLX⁺ ARN neurons. There were also some GFP⁺;DLX[−] cells suggesting

that DLX expression was down-regulated in some cells that had expressed *Dlx5/6i* at an earlier point in their lifetime.

Double immunohistochemical labeling of NKX2.1⁺ and GFP⁺ (I5/6i lineage) in the ARN (Figure 5 E–H) revealed that 66.5% of NKX2.1⁺ cells were derived from the I5/6i lineage (Figure 5 G and H, arrowheads, Table 4). Cells that displayed either POMC or NPY immunoreactivity were GFP[−] (Figure 5 I–K; M–P, arrows). On the other hand, TH was expressed in 20.8% of the *Dlx5/6i* lineage population in the ARN (Figure 5 Q–T, arrowheads in S and T, Table 4).

***Dlx5/6i* lineage in the adult ARN**

Similar to the *Dlx5/6i* lineage in the ARN at PO, NKX2.1⁺ cells were present in 46.3% of *Dlx5/6i-Cre*⁺ descendants in the adult ARN (Figure 6 A–D, arrowheads pointing to white cells in C and D, Table 4). NPY immunoreactivity was mainly present in nerve fibers and was largely absent from cell bodies (Figure 6 E, G and H). NPY immunoreactive nerve fibers were in close contact with *Dlx5/6i-Cre* descendants in the ARN (Figure 6 G and H, arrowheads). POMC was not present in *Dlx5/6i-Cre* descendants (Figure 6 I–L, arrows in K and L). 57.6% of TH⁺ cells were present in *Dlx5/6i-Cre* descendants. (Figure 6 M–P, arrowheads in O and P, Table 4).

***Dlx1* Regulates the number of tyrosine hydroxylase⁺ ARN Neurons**

Given DLX expression of TH⁺ cells of the ARN, we studied these cells in adult *Dlx1*^{−/−} mutants (*Dlx2*^{−/−} and *Dlx5*^{−/−} die the day of birth precluding this analysis). We found a ~2-fold reduction in the number of TH⁺ ARN neurons in postnatal day 60 *Dlx1*^{−/−} mutants (*Dlx1*^{+/+}: 54.67±6.74; *Dlx1*^{−/−}: 25.67±2.44) (Figure 7; Supplemental Figure 1). There was also an obvious reduction in the TH⁺ neuropile in the ARN, but not in the median eminence. TH was also reduced in dorsomedial hypothalamus (arrow, Figure 7), but not in the ventral midbrain (not shown), or its projection through the cerebral peduncle (Figure 7).

DISCUSSION

***Dlx* and *Nkx2.1* expression in the hypothalamus**

Previous studies in the mouse have focused on the expression of *Nkx2.1* and the *Dlx* genes (*Dlx1*, 2, 5 and 6) in progenitors and immature neurons of the prenatal telencephalon and diencephalon (Eisenstat et al., 1999; Marin et al., 2002; Sussel et al., 1999). However, these genes are also expressed postnatally in the forebrain (Cobos et al., 2005; Lee et al., 2001; Marin et al., 2000; Mastronardi et al., 2006; Saino-Saito et al., 2003). Within the diencephalon, *Nkx2.1* is broadly expressed in the basal hypothalamus, including the mammillary, ventromedial and ARN nuclei (Puelles and Rubenstein, 2003). In the diencephalon, the *Dlx* genes are expressed in a transverse alar domain that extends from the prethalamus (ventral) thalamus (reticular nucleus, zona incerta) through the posterior entopeduncular area (PEP) to the anterior hypothalamus and the suprachiasmatic area (SCH) (Puelles and Rubenstein, 2003). *Dlx* expression is also present ventral to the PEP-SCH domain, where the dorsomedial hypothalamic and tuberomammillary and ARN areas are *Dlx*⁺.

In the telencephalon, there is evidence that *Dlx* gene expression marks virtually all differentiating GABAergic and dopaminergic neurons (Kohwi et al., 2007; Saino-Saito et al., 2003; Stuhmer et al., 2002a,b); *Nkx2.1* expression marks a subset of differentiating GABAergic and cholinergic neurons derived from the basal telencephalon (Marin et al., 2000).

Here we focused on *Nkx2.1* and *Dlx* expression in the neonatal and adult ARN and ventromedial hypothalamic nuclei, exploring their expression in GAD67-, NPY-, POMC- and TH-expressing neurons. *Nkx2.1* conditional mutants show differentiation defects in the region of the ventromedial hypothalamus and ARN nucleus, with alterations in reproductive functions (Mastronardi et al., 2006). Ongoing studies are investigating the effects of mutations in the *Dlx* genes on ARN development.

Validation of the *Dlx5/6i-Cre* and *Nkx2.1-Cre* transgenic lines

Throughout our work we found a high concordance of the recombination of patterns of both the *Dlx5/6i-Cre* and *Nkx2.1-Cre* transgenic lines and the endogenous expression of DLX and NKX2.1 in the ARN. However, we did detect some cells that underwent recombination and that did not express the transcription factors (Figure 3 D, Figure 4 D, Figure 5 D, Figure 6D). This could be due to earlier expression of the transgene, in a correct pattern, and subsequent of the down-regulation of the endogenous gene; however, we can't rule out whether there is ectopic expression of the transgene. Likewise, we also observed some DLX⁺ cells that did not show *Dlx5/6i-Cre* activity. This could reflect the fact that there are multiple *Dlx5/6* and *Dlx1/2* enhancers, which could drive DLX expression more broadly in the ARN (Ghanem et al., 2007). None-the-less, the recombination patterns driven by the *Dlx5/6i-Cre* and *Nkx2.1-Cre* transgenic lines provide useful information for future studies aiming to use these Cre lines to alter gene expression in the developing ARN.

Dlx lineage marks GABAergic and dopaminergic ARN neurons, and *Dlx1* regulates the number of TH⁺ ARN neurons

Over 90% of DLX⁺ (pan-Distal-less antibody-reactive) cells in the neonatal and adult ARN nucleus express *Gad67* (*Gad1*) (Figure 1; Table 2) showing that DLX-expressing cells are GABAergic (as in the telencephalon). However, without demonstrating that *Gad65* (*Gad2*) and *Gad67* (*Gad1*) expression are the same, we can't be certain that we have labeled all GABAergic neurons. We did not observe POMC co-expression in neurons of the *Dlx56i-Cre* lineage (Figures 5 and 6; Table 4). While roughly 50% of TH⁺ cells were in the *Dlx56i-Cre* lineage (Figures 5 and 6; Table 4). Thus, we conclude that *Dlx* expression in the ARN is restricted to GABAergic and dopaminergic (TH⁺) neurons. Surprisingly, NPY⁺ neurons, which are GABAergic, do not appear to be in the *Dlx5/6i-Cre* lineage. Furthermore, the distribution of pan-Distal-less⁺ and NPY⁺ cells appears different (Figure 1 and 2), suggesting that most ARN NPY⁺ cells do not express *Dlx* genes, although further analyses are needed to be certain. The observation that POMC⁺ ARN neurons are not in the *Dlx5/6i* lineage is expected given that these neurons are not GABAergic. In sum, *Dlx* expression in the ARN is largely limited to dopaminergic (TH⁺) neurons, and GABAergic neurons that are NPY negative; future analysis is needed to define the identity and properties of these GABAergic neurons, including establishing whether they are projection or local circuit neurons. Consistent with these analyses, we found that adult (P60) *Dlx1*^{-/-} mutants have ~2-fold fewer TH⁺ ARN neurons (Fig. 7). This adds to previous studies demonstrating the requirement of the *Dlx* genes for olfactory bulb and diencephalic TH⁺ neurons (Andrews et al., 2003; Long et al., 2007; Qiu et al., 1995). Future studies will define the mechanisms through which the *Dlx* genes control TH neuron numbers.

Nkx2.1 lineage marks subsets of POMC, GABAergic and dopaminergic ARN neurons

In the ARN, 60–70% of NKX2.1⁺ cells are GAD67⁺ (Figure 1; Table 2). Analysis of the *Nkx2.1-Cre* lineage shows that many of these cells are DLX⁺ and NPY⁺ (Figures 3 and 4; Table 3), supporting a model that NKX2.1 is expressed during the development of several types of GABAergic neurons. In addition, *Nkx2.1-Cre* induces recombination in TH⁺ and POMC⁺ neurons (Figures 3 and 4; Table 3); thus, NKX2.1 may be expressed in most subtypes of ARN neurons. However many GAD67⁺, TH⁺ and POMC⁺ cells do not arise

from NKX2.1⁺ lineage, as assessed by Cre recombination. This is either because *Nkx2.1* is not expressed in all cells, or due to incomplete recombination induced by the *Nkx2.1-Cre* transgene.

Furthermore, *Nkx2.1* is broadly expressed in the ventromedial hypothalamus (a glutamatergic structure), which lacks *Dlx* expression (Figures 3 and 4). Thus, expression of *Nkx2.1*, and cells in this lineage, are not restricted to GABAergic and dopaminergic neurons of the ARN and contribute to many cell populations within the different hypothalamic regions.

Utility of *Dlx5/6i-Cre* and *Nkx2.1-Cre* mice for modifying gene expression in the ARN nucleus

Our analysis provides insights into the origins, transcriptional regulation and molecular properties of ARN neurons. Furthermore, this study demonstrates the utility of the *Dlx5/6i-Cre* and *Nkx2.1-Cre* mouse lines for modifying gene expression in ARN neurons.

The *Nkx2.1-Cre* allele will induce recombination in all known ARN neuronal cell types (POMC, NPY, GABA and TH). As *Nkx2.1* expression begins in the neuroepithelium, it is likely that it can induce loxp recombination in progenitor cells; as a result, the ependyma also shows recombination (Figures 3 and 4). Thus, gene expression in ependymal tanycytes of the ARN and median eminence can be modified; tanycytes are implicated in regulating many hypothalamic functions (Rodríguez et al., 2005). Based on the recombination pattern at P0 and in the adult, *Nkx2.1-Cre* may not induce recombination in all ARN neurons; this may pose a problem for phenotypic analyses. Furthermore, phenotypic analysis of mutants generated using *Nkx2.1-Cre* need to consider the fact that *Nkx2.1* is also expressed in the telencephalon (Xu et al., 2008), other regions of the hypothalamus, the thyroid and the lung.

The *Dlx5/6i-Cre* allele will induce recombination in only a subset of ARN neuronal cell types (GABA and TH). As *Dlx5/6* expression begins in the subventricular zone, it is likely that it can induce loxp recombination in these secondary progenitor cells; as a result, cells can have genetic changes during midembryogenesis (~E11) when *Dlx5/6* expression begins. Phenotypic analysis of mutants generated using *Dlx5/6i-Cre* need to consider the fact that this enhancer is also expressed in the telencephalon and other regions of the hypothalamus (Kohwi et al., 2007; Monory et al., 2006).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. GAD67-GFP co-expression with DLX, NKX2.1, NPY and TH in the ARN at postnatal day 0 (PO)

DLX⁺ cells in the ARN are purple (A, C and D, arrow in C and D). GFP⁺ (expressed from the GAD67 gene) cells and nerve fibers in the ARN are green (B, co-localized with DLX in C and D). DLX/GAD67 double-positive cells are white (C and D, arrowheads). NKX2.1⁺ cells in the ARN, VMH and in cells lining the third ventricle are purple (E, G and H, arrow in G and H). GFP (GAD67) (F) co-localized with NKX2.1 in G and H. NKX2.1/GAD67 double-positive cells are white (G and H, arrowheads). POMC⁺ cells in the ARN are purple (I, K and L, arrow in K and L). GFP⁻ (GAD67) is green (J, K and L). Merged images (K and L) show no POMC/GFP co-localization in the ARN. NPY⁺ cells in the ARN are purple (M, O and P, arrow in O and P). GFP⁺ (GAD67) is green (N, co-localized with NPY in O and P). NPY/ GAD67 double-positive cells are white (O and P, arrowheads). TH⁺ cells in the ARN are purple (Q, S and T, arrow in S and T). GFP⁺ (GAD67) cells are green (R, co-localized with TH in S and T). TH/ GAD67 double-positive cells are white (S and T, arrowheads). The boxed regions in C, G, K, O and S are the part of the ARN shown at higher magnification in D, H, L, P and T, respectively. Magnification is 20× unless otherwise specified (10× I-L) and 40× (D, H, L, P and T). Scale bars= 50μm in G,K, O and S. Scale Bars= 20μm in D, H, L, P and T.

Figure 2. GAD67-GFP co-expression with DLX, NKX2.1, NPY and TH in the adult ARN
 DLX⁺ cells in the adult ARN are purple (A, C and D, arrow). GFP⁺ (GAD67) cells and nerve fibers in the ARN and median eminence (ME) are green (B, co-localized with DLX in C and D). Cells that display both DLX⁺ and GAD67⁺ are white (C and D, arrowheads). NKX2.1⁺ cells in the ARN, VMH, cells lining the third ventricle and ependymal cells in the adult are purple (E, G and H, arrow in G and H). GFP⁺ (GAD67) cells are green (F, co-localized with NKX2.1 in G and H). Cells that display both NKX2.1⁺ and GAD67⁺ are white (G and H, arrowheads). POMC⁺ cells and nerve fibers in the adult ARN are in purple (I, K and L, arrows). GFP⁺ (GAD67) is green (J, K and L). Merged images (K and L) show rare co-localization of POMC⁺ and GFP⁺ in the ARN (arrowheads). NPY⁺ in nerve fibers in the adult ARN are purple (M, O and P, arrows). GFP⁺ (GAD67) cells are green (N, merged with NPY in O and P). Cells that display GAD67⁺ surround and are found adjacent to NPY⁺ nerve fibers (O and P). TH⁺ cells and nerve fibers in the adult ARN and median eminence are purple (Q, S and T, arrows). GFP⁺ (GAD67) cells are green (R, co-localized with TH in S and T). Cells that display both TH⁺ and GAD67⁺ are white (S and T, arrowheads). The boxed regions in C, G, K, O and S are the part of the ARN shown at higher magnification in D, H, L, P and T, respectively. Magnification is 20× unless otherwise specified (40× in panels D, H, L, P and T). Scale bars= 50μm in G,K, O and S. Scale Bars= 20μm in D, H, L, P and T.

Figure 3. *Nkx2.1-Cre* induced GFP⁺ co-expression with DLX, NKX2.1, POMC, NPY and TH in the ARN at PO

NKX2.1⁺ cells in the ARN, VMH, cells lining the ventricle and ME are purple (A, C and D). *Nkx2.1-Cre* induced GFP⁺ cells (*Nkx2.1* lineage) in the ARN, VMH, cells lining the third ventricle and ME are green (B, co-localized with DLX in C and D). Cells that are both NKX2.1⁺ and GFP⁺ are white (C and D). Several cells that display *Nkx2.1*⁺ but lack apparent GFP⁺ are purple (C, shown best in D). Cells that display GFP⁺ but lack NKX2.1⁺ are green (C and D). DLX⁺ cells in the ARN purple (E, G and H). GFP⁺ is green (F, co-localized with DLX in G and H). Cells that display both DLX⁺ and GFP⁺ are white (G and H). POMC⁺ cells in the ARN are purple (I, K and L, arrow in K and L). *Nkx2.1-Cre* induced GFP⁺ cells are green (J, K and L). Merged images (K and L) show a subset of *Nkx2.1-Cre* induced GFP⁺ cells exhibit POMC⁺ in the ARN. NPY⁺ cells in the ARN are purple (M, O and P, arrow in O and P). *Nkx2.1-Cre* induced GFP⁺ cells are green (GFP) (N, co-localized with NPY in O and P). A subset of cells that display both NPY⁺ and GFP⁺ are white (O and P). TH⁺ cells in the ARN are purple (Q, S and T, arrow in S and T). GFP⁺ cells are green (R, co-localized with TH in S and T). Cells that display both TH⁺ and GFP⁺ are white (S and T, arrowheads). The boxed regions in C, G, K, O and S are the part of the ARN shown at higher magnification in D, H, L, P and T, respectively. Magnification is 20× unless otherwise specified and 64× (D, H, L, P and T). Scale bars= 50μm in G, K, O and S. Scale Bars= 20μm in D, H, L, P and T.

Figure 4. *Nkx2.1-Cre* induced β -galactosidase co-expression with DLX, NKX2.1, POMC and TH in the adult ARN

NKX2.1⁺ cells in the adult ARN and VMH are purple (A, C and D). β -galactosidase⁺ cells (*Nkx2.1* lineage) in the ARN and VMH are green (B, co-localized with DLX in C and D). Several cells that are both Nkx2.1⁺ and β -galactosidase⁺ are white (C and D). Cells that are β -galactosidase⁺, but lack NKX2.1⁺ are green (C and D). DLX⁺ ARN cells are purple (E, G and H). β -galactosidase⁺ is green (F, co-localized with DLX in G and H). Cells that display both DLX⁺ and β -galactosidase⁺ are white (G and H). POMC⁺ cells in the ARN are purple (I, K and L). *Nkx2.1-Cre* lineage cells are green (J, K and L). Merged images (K and L) show a subset of *Nkx2.1-Cre* lineage cells that are POMC⁺ in the ARN. TH⁺ cells in the ARN (M, O and P). *Nkx2.1-Cre* lineage cells are green (GFP) (N, co-localized with TH in O and P). The boxed regions in C, G, K and O are the part of the ARN shown at higher magnification in D, H, L, P, respectively. Magnification is 20 $\times$ unless otherwise specified 64 $\times$ (D, H, L and P). Scale bars= 50 μ m in G, K and O. Scale Bars= 20 μ m in D, H, L, and P.

Figure 5. *Dlx5/6i*-Cre induced GFP co-expression with DLX, NKX2.1 and TH in the ARN at PO DLX⁺ cells in the ARN at PO (purple) (A, C and D, arrow in C and D). *Dlx5/6i*-Cre induced GFP cells (*Dlx5/6i* lineage) in the ARN are green (B, co-localized with DLX in C and D). Cells that display both DLX⁺ and *Dlx5/6i*-Cre induced GFP⁺ cells are white (C and D, arrowheads). DLX⁺ cells that lack GFP are C and D (arrow) and the occasional cell that expresses GFP but lacks DLX expression is shown (C and D, asterisk). NKX2.1⁺ cells in the ARN, VMH (purple) (E, G and H, arrows). GFP⁺ is green (F, co-localized with NKX2.1 in G and H). Cells that display both NKX2.1⁺ and *Dlx5/6i*-Cre induced GFP⁺ cells are white (G and H, arrowheads). POMC⁺ cells in the ARN are purple (I, K and L, arrow in K and L). *Dlx5/6i*-Cre induced GFP⁺ cells is green (J, K and L). Merged images (K and L) show no co-localization of POMC⁺ and GFP⁺ in the ARN. NPY⁺ cells in the ARN at PO (purple) (M, O and P, arrow in O and P) does not co-localize with *Dlx5/6i*-Cre induced GFP⁺ cells are green (N, O and P). TH⁺ cells and nerve fibers in the ARN are purple (Q, S and T, arrow in S and T). *Dlx5/6i*-Cre induced GFP⁺ cells are green (R, S and T, asterisks). Cells that display both TH⁺ and asterisk in S and T are white with green nuclei (S and T, arrowheads). The boxed regions in C, G, K, O and S are the part of the ARN shown at higher magnification in D, H, L, P and T, respectively. Magnification is 20× unless otherwise specified 64× (D, H, L, P and T). Scale bars= 50μm in G, K, O and S. Scale Bars= 20μm in D, H, L, P and T.

Figure 6. *Dlx5/6i-Cre* induced GFP co-expression with NKX2.1, POMC and TH in the adult ARN

NKX2.1⁺ cells in the ARN (purple) (A, C and D, arrow in C and D). *Dlx5/6i-Cre* induced GFP⁺ cells (*Dlx5/6i* lineage) in the ARN (green) (B, co-localized with NKX2.1 in C and D). Cells that display both NKX2.1⁺ and *Dlx5/6i-Cre* induced GFP⁺ cells are white (C and D, arrowheads). POMC⁺ cells in the ARN (purple) (E, G and H, arrows). *Dlx5/6i-Cre* induced GFP⁺ lineage is green (F, G and H). Merged images (G, H and L) show no co-localization of POMC⁺ and GFP⁺ in the ARN. TH⁺ cells and nerve fibers in the ARN and ME are purple (I, K and L, arrow). *Dlx5/6i-Cre* induced GFP⁺ is green (J, K and L). Cells that display both TH⁺ and *Dlx5/6i-Cre* induced GFP⁺ (asterisk in S and T) are white with green nuclei (K and L, arrowheads). The boxed regions in C, G, and K are the part of the ARN shown at higher magnification in D, H, and L, respectively. Magnification is 20× unless otherwise specified, 64× (D, H, and L).

Figure 7.

Dlx1 Regulates the Number of Tyrosine Hydroxylase⁺ ARN Neurons.

TH immunohistochemistry of coronal P60 sections through wild type (A, A') and *Dlx1*^{-/-} mutants (B, B'). The boxed regions in A and B are shown at higher magnification in A' and B'. C: histogram showing the reduced number of TH⁺ neurons in the *Dlx1*^{-/-} mutant ARN.

Abbreviations: ARN nucleus: ARN; Cerebral Peduncle: CP; Dorsomedial Hypothalamus: DMH; Median Eminence: ME; Ventromedial Hypothalamus: VMH. Scale bar: A, B: 1 mm; A', B': 250 μ m.

Table 1

Primary Antibodies Used for Double Label Immunohistochemical Analysis

Antibody, host; dilution Source Source location Catalog number Lot number	Antibody Specificity
GFP , chicken; 1:1000 Aves labs Tigard, Oregon GFP-1020 1223FP03	Antigen: Purified recombinant GFP. Specificity: No immunofluorescence is detected in mice that lack the GFP transgene (wild type mice). Antibodies were analyzed by western blot analysis on various tissue using a blocking reagent (BlokHen, Aves labs), negative control and GFP positive tissue.
NKX2.1 , rabbit; 1:2500 Biopat Caserta, Italy PA-0100 b-I	Nkx2.1 antiserum was raised against a synthetic peptide corresponding to amino acids 110–122 at the amino terminus of rat TTF-1 (thyroid transcription factor-1, also known as, NKX2.1). Immunoreactivity is lost in the <i>Nkx2.1</i> ^{-/-} mutant brain (Yee and Rubenstein, unpublished).
NPY , rabbit; 1:1000 Immunostar Hudson, Wisconsin 22940 208001	Antigen: Synthetic porcine NPY. Specificity: Analyzed by the manufacturer by using the biotin-streptavidin/ horseradish peroxidases (HRP) detection method on rat brain tissue. All staining is blocked by preabsorption of the diluted antiserum with excess NPY, while other peptides in excess including Peptide YY, avian pancreatic peptide, beta endorphin, vasoactive intestinal peptide, CCK or SS) had no effect on the staining intensity
POMC , rabbit; 1:1000 Phoenix Pharmaceuticals Belmont, CA H-029-30 00338 and 00583	The antibody is made against the POMC precursor (27–52). Double label immunohistochemical analysis in POMC-mice show complete co-localization of GFP and POMC.
Pan distaless (DLX) , rabbit; 1:1000 Grace Boekhoff-Falk University of Wisconsin-Madison Medical School	Antigen: N-terminal 200 aa and 61 aa homeodomain of butterfly DLX protein Specificity: Immunohistochemistry to vertebrate tissues show expression patterns that are indistinguishable from the sum of the <i>Dlx1,2,5,6</i> RNA expression patterns (Stuhmer et al., 2002; Stuhmer et al., 2002) Zhao et al., 2008, show a co-labeling experiment using the DLX2 and pan-DLX antibodies in an embryonic mouse brain section. This data strongly suggests that all the DLX2 ⁺ cells are also pan-DLX ⁺ and vice versa.
TH , rabbit 1:1000 Chemicon Temecula, CA AB152 25040712	Immunized against denatured tyrosine hydroxylase from rat pheochromocytoma (denatured by sodium dodecyl sulfate). Specificity: Western blot analysis on rat brain demonstrates a single band at 62kD; no band is detected in rat liver, negative control tissue.

Table 2Percent of double labeled DLX⁺, NKX2.1⁺, POMC⁺, NPY⁺ and TH⁺ cells in the ARN nucleus of GAD67-GFP mice

Age	Antibody	# antibody labeled cells	#GFP labeled cells	#of double labeled cells	% antibody double labeled cells	% GFP double labeled cells
PO	DLX	49.1±1.5	57.3±2.7	45.4±1.97	92.4±4.5	79.2±0.4
	NKX2.1	63.9±6.7	55.6±1.9	37.0±5.5	58.7±14.9	66.6±10.2
	POMC	23.8±7.9	56.5±3.4	0.0	0.0	0.0
	NPY	31.7±2.4	60.0±4.1	20.3±1.6	64.1±1.8	34.1±4.73
	TH	19.3±2.0	43.7±1.1	12.4±0.9	64.1±6.5	28.4±1.9
Adult	DLX	54.06±5.78	72.91±6.82	50.8±4.78	94.0±3.9	69.7±5.5
	NKX2.1	43.9±7.3	77.3±16.2	32.2±2.3	74.5±10.23	42.7±8.5
	POMC	19.3±1.3	71.9±3.35	1.0±.4	5.1±2.0	1.4±0.7
	NPY	0.0	68.0±8.5	0.0	0.0	0.0
	TH	14.14±1.29	62.5±2.2	14.1±1.3	100.0	22.6±1.4

Table 3

Percent of double labeled DLX⁺, NKX2.1⁺, POMC⁺, NPY⁺ and TH⁺ cells in the Nkx2.1-Cre lineage in the ARN nucleus

Age	Antibody	# antibody labeled cells	# GFP labeled cells	# of double labeled cells	% antibody double labeled cells	% GFP/βgal double labeled cells
PO	DLX	66.5 ± 3.5	63.5 ± 6.2	29.8 ± 1.3	44.8 ± 0.6	47.3 ± 6.4
	NKX2.1	55.8 ± 4.1	63.7 ± 1.1	47.6 ± 2.2	85.4 ± 2.4	74.7 ± 2.8
	POMC	19.1 ± 2.8	56.3 ± 5.6	14.6 ± 2.8	76.3 ± 4.9	26.1 ± 5.6
	NPY	28.5 ± 2.4	52.82 ± 4.5	15.4 ± 2.6	54.6 ± 2.6	29.4 ± 6.7
	TH	34.0 ± .4	54.0 ± 3.5	18.6 ± 1.6	54.0 ± 4.4	33.9 ± 33.9
Adult	DLX	51.1 ± 6.0	63.0 ± 7.9	35.7 ± 5.6	71.0 ± 15.5	56.5 ± 1.8
	NKX2.1	52.8 ± 5.2	64.5 ± 3.8	37.5 ± 7.9	71.0 ± 14.2	57.9 ± 10.9
	POMC	14.1 ± 3.4	58.5 ± 5.2	12.2 ± 3.1	85.8 ± 2.2	21.0 ± 6.0
	TH	14.4 ± 1.1	56.3 ± 5.8	8.2 ± 1.5	56.6 ± 6.1	14.7 ± 3.2

Table 4

Percent of double labeled DLX⁺, NKX2.1⁺, POMC⁺, NPY⁺ and TH⁺ cells the *Dlx5/6i-Cre* lineage in the ARN nucleus

Age	Antibody	# antibody labeled cells	# GFP labeled cells	# of double labeled cells	% antibody double labeled cells	% GFP double labeled cells
PO	DLX	54.1 ± 4.9	50.1 ± 4.8	40.4 ± 3.1	74.9 ± 4.6	81.0 ± 6.4
	NKX2.1	63.5 ± 3.1	58.0 ± 3.0	42.2 ± 1.1	66.5 ± 3.8	72.8 ± 4.9
	POMC	44.6 ± 2.2	52.3 ± 5.5	0.0	0.0	0.0
	NPY	51.8 ± 3.7	55.6 ± 4.4	0.00	0.00	0.0
	TH	22.0 ± 2.8	56.1 ± 2.3	11.7 ± 1.1	53.7 ± 10.9	20.8 ± 1.2
Adult	NKX2.1	42.7 ± 0.8	37.0 ± 1.1	17.1 ± 3.4	40.2 ± 8.6	46.4 ± 10.1
	POMC	18.3 ± 0.9	46.1 ± 3.9	0.0	0.0	0.0
	TH	16.4 ± 1.5	44.1 ± 7.1	9.4 ± 2.6	57.5 ± 14.4	21.6 ± 6.1