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Molecular phenotyping of transient postnatal tyrosine hydroxylase neurons in the rat bed nucleus of the stria terminalis.

David A. Carter

School of Biosciences, Cardiff University, Cardiff, UK.

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Correspondence:

David Carter PhD, School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3AX. UK.

Tel: 029-20-874095

Fax: 029-20-876328

Email: smbdac@cardiff.ac.uk

Abstract

The bed nucleus of the stria terminalis (BNST) is a complex integrative centre in the forebrain, composed of multiple sub-nuclei, each with discrete populations of neurons. Progress in understanding BNST function, both in the adult and during postnatal maturation, is dependent upon a more complete characterization of neuronal phenotypes in the BNST. The aim of the current study was to define the molecular phenotype of one postnatal BNST neuronal population, in order to identify molecular factors that may underlie both (protein marker-related) immaturity, and secondly, the transience of this phenotype. This BNST population was originally identified by high, but transient expression of the EGR1 transcription factor (TF) in postnatal rat lateral intermediate BNST (BNSTLI). The current results confirm a high level of Egr1 activation in postnatal day 10 (PN10) male BNSTLI that is lost at PN40, and now demonstrate a similar pattern of transient activation in female brains. Apparent cellular immaturity in this population, as indicated by low levels of the adult neuronal marker NeuN/RBFOX3, was found to be uncorrelated with both key neuronal regulator protein expression (SOX2 and REST), and also RBFOX2 protein levels. The BNSTLI neurons have a partial catecholaminergic phenotype (tyrosine hydroxylase-positive/dopa decarboxylase-negative; TH+ve/DDC-ve) that is lost at PN40. In contrast, the co-expressed neuropeptide, somatostatin, is maintained, albeit at lower levels, at PN40. The transcriptional basis of the transient and partial catecholaminergic phenotype was investigated by analysing TFs known to maintain adult dopaminergic (TH+ve/DDC+ve) neuronal phenotypes. The BNSTLI neurons were shown to lack forkhead TFs including FOXA1, FOXA2 and FOXO1. In addition, the BNSTLI neurons had low, primarily cytoplasmic, expression of NR4A2/ NURR1, an orphan nuclear receptor that is critical for adult maintenance of midbrain dopamine neurons. These results detail the molecular

features of an immature neuronal phenotype, and reveal TF deficiencies that may underlie postnatal transience of the phenotype.

Introduction

The postnatal period can be considered a second phase of mammalian brain development, when the maturation of adult brain function is additionally responsive to sensory experience. Postnatal development occurs at many organizational levels, including transcriptomic; cell-specific transcription factors (TFs) direct the maturation of a transcriptome that is appropriate for the required adult phenotype. For example, one TF, PET-1, acts postnatally to direct acquisition of specific neurotransmitter receptors in serotonergic neurons (Wyler et al, 2016). A global overview of the molecular events that underlie these processes has been obtained by the analysis of brain transcriptome dynamics across postnatal development (see Carter et al, 2016). One early study of the postnatal period in rat brain revealed extensive changes in transcriptome content (Stead et al, 2006), and more recent work has documented similarly extensive changes in both mouse (Shimogori et al, 2010), and primate brain (Bakken et al, 2016).

Our laboratory has studied the postnatal activity of one TF in the rat, early growth response factor-1 (EGR1, NGFI-A, Zif268) that is highly expressed during brain development (Wells et al, 2011), and exhibits cell-specific changes in expression during the postnatal period (Wells et al, 2011; Man et al, 2007). In general, levels of EGR1 decrease in the postnatal brain; specific exceptions include a neuronal population in the intermediate region of the lateral bed nucleus of the stria terminalis (BNSTLI), where a high level of EGR1 is maintained until postnatal day 20 (PN20), but thereafter declines to a negligible level on PN40 (Man et al, 2007). The BNST is a complex limbic system structure that integrates different aspects of physiology and behaviour including the

stress response and emotional states, and may have aberrant function in human psychiatric conditions (see Lebow and Chen, 2016). The marked change in EGR1 expression during the period PN20-40 parallels developmental changes in limbic system functionality, for example, the development of fear conditioning in the rat (Deal et al, 2016). Because of the potential importance of this neuronal population in the postnatal maturation of BNST function, it will be important to further characterize the phenotype of these neurons in the rat.

The BNSTLI neurons also have other unusual phenotypic characteristics that have an unknown molecular basis. In addition to high levels of EGR1, these neurons have unusually low expression of the adult neuronal marker NeuN/RBFOX3 (Man et al, 2007), but abundant expression of the rate-limiting, catecholamine synthesis enzyme, tyrosine hydroxylase (TH; Man et al, 2007). The most notable aspect of this neuronal phenotype, however, is temporal; the described pattern of protein expression is maintained for a limited postnatal period of around one month. It is now recognized that these neurons are likely identical to a BNST population shown previously to transiently express TH (Verney et al, 1988; Beltramino et al, 1996). The apparent transience of this cellular phenotype is intriguing given that we have demonstrated extensive innervation of this group by substance P (*Tac1* gene product) and a corresponding adundance of a substance P receptor on TH/EGR1 BNST neurons (NK-1; Man et al, 2007), indicating a functional innervation that would be inconsistent with imminent cell death. A previous study has also documented an amygdaloid input onto these cells in postnatal rats (Beltramino et al, 1996).

It is now recognized that adult neuronal phenotype is actively maintained by cell-type-specific TF activity (Deneris and Hobert, 2014), and, moreover, that differentiated

mammalian neurons can switch phenotype (Spitzer, 2015). In the current study, further characterization of the BNSTLI EGR1 neuronal population has been performed in order to identify aspects of the molecular phenotype that may underlie phenotypic transience. In particular, the expression of key TFs that are known to be involved in the maintenance of TH+ve (dopaminergic) neurons were investigated. The current experimental analysis focused on the more caudal group of BNSTLI neurons as this region has the greatest density of EGR1+ve neurons (see Man et al, 2007). In addition, because the BNST is a sexually dimorphic structure (Hines et al, 1992) exhibiting sex-hormone dependent changes of gene expression (Carter & Murphy, 1993), potential sex differences in the BNSTLI neuronal population have also been examined.

Material and Methods

Animals

Transgenic *Egr1*-d2EGFP rat models (male and female) were used under the UK Animals (Scientific Procedures) 1986 Act of Parliament, and in accordance with Cardiff University ethical review. Health status was monitored, and assessed by a veterinarian, if required. Animals were maintained in standard laboratory conditions (14:10 light:dark cycle, lights on: 05.00h) with *ad libitum* access to standard rat food and water. Hemizygous transgenic rats were identified by PCR analysis of tissue biopsies as described (Man et al, 2007), and killed (Schedule 1 method) for experimental analysis on specific postnatal days at 17.00h, designating the day of birth as postnatal (PN) day 1. For PN10 rats, brains were rapidly dissected prior to fixation in 4% paraformaldehyde in 0.1M phosphate buffer (PFA, 24h, 4^{o}C) and cryoprotection in 20% sucrose in 0.1M phosphate buffer (24h, 4˚C). For PN40 rats, these were terminally anaesthetized with sodium pentobarbitone (150 mg/kg, i.p), and perfused via the ascending aorta with phosphate buffered saline (PBS) followed by 4% PFA. Dissected brains were then post-

fixed in PFA overnight at 4˚C, and cryoprotected in 20% sucrose in 0.1M phosphate buffer at 4˚C, overnight. Brains were stored briefly at –80˚C prior to sectioning.

Immunohistochemical analysis.

Sections of rat brain (12µm) were cut in the transverse plane (Leica CM1900 Cryostat; Leica Imaging Solutions Ltd., Cambridge, UK), and mounted on glass slides (SuperFrost Plus, VWR International Ltd., East Grinstead, West Sussex, UK). Brain sections were collected from across the BNST region previously shown to express the GFP+ve cell group (Bregma 0.2mm to -1.2mm; Paxinos & Watson, 2005). Slides were dried briefly, and stored at -80 ^{o}C prior to immunohistochemical analysis.

Transgene-derived GFP and a variety of endogenous antigens were detected by standard fluorescence immunohistochemistry using described procedures, and a number of primary antibodies, some of which have been validated in previous studies from this laboratory (Table 1; Man et al, 2007; Hoefflin and Carter, 2014; Man et al, 2014). Antibodies used for the first time in this laboratory were validated firstly by confirming sub-cellular/brain region detection accuracy, and also through reference to previous use in peer-reviewed publications (Table 1). All antibodies were further validated by confirming both excitation light-specific detection of signal, and an absence of detection when the primary antibody was omitted (eg. Fig.3B' inset).

Primary antisera (Table 1) were used in combination with species-specific, fluorophore-conjugated, secondary antisera (diluted 1/500): Alexa Fluor 488-conjugated goat anti-chicken IgG, Abcam, Cambridge, UK; Alexa Fluor 488-conjugated goat anti-rabbit IgG, Molecular Probes Inc, Eugene, OR, USA; Alexa Fluor 488-conjugated donkey anti-mouse IgG, Molecular Probes; Cy3-conjugated donkey anti-mouse IgG, Jackson

Immunoresearch Laboratories Inc., West Grove, PA, USA; Cy3-conjugated sheep anti-rabbit IgG, Sigma Aldrich, Dorset, UK). Following the final washing step, sections were mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA), and stored at 4 ^{o}C.

Rat brain sections were viewed with a fluorescence microscope (Leica DM-LB, Leica). Images were captured with a Leica DFC-300FX digital camera and Leica QWin software (V3), and assembled in Photoshop (CS2, Adobe Systems Inc., San Jose, CA, USA). Immunohistochemical analyses were conducted on a minimum of three brain sections each, sampled from at least two different animals of the Z27B *Egr1-d2EGFP* line (Man et al, 2007). For the purposes of the current study, the majority of analyses were conducted on female neonates. However, in a quantitative comparison of BNSTLI neurons between male and female PN10 brains, the numbers of TH+ve neurons were counted in 8 caudal BNSTLI sections, sampled from each of 3 rats of each sex. The cell counts were compared using Student's t test for unpaired samples (IBM SPSS Statistics version 20; IBM, New York, USA)

Results

BNSTLI neurons were characterized in Egr1-d2EGFP transgenic rats which provide both enhanced visualization and identification of EGR-1+ve cells (Man et al, 2007; Man et al, 2008; Wells et al, 2011). For the purposes of the current study, BNSTLI expression patterns were compared between the PN10 and PN40 time points; these were selected as representing established (PN10), and diminished (PN40) d2EGFP/EGR1 expression (Man et al, 2007). It was firstly shown that the pattern of transgene/EGR1 expression identified previously in the BNSTLI of male neonates is also observed in females (Fig.1). Cells in this neuronal group displayed a similar general phenotype (TH+ve/low NeuN;

Fig.1A-F) as described previously for males (Man et al, 2007), and additionally, a similar morphology (uni/bipolar; Fig.1G,H) that is less complex than other multipolar d2EGFP-ve/TH+ve neurons (eg. dorsal hypothalamic area; Fig.1I). Microscopic analysis of the d2EGFP fluorescence also indicates the presence of processes associated with varicosities in the BNSTLI region that may represent axons arising from these neurons (Fig.1H). Identity with the transient BNST TH+ve neurons previously documented by Verney et al (1988) was supported by confirming an absence of TH+ve cells in female PN40 BNSTLI (data not shown), as previously documented in male PN40 BNSTLI in our laboratory (Man et al, 2007). In a subsequent analysis, identity with the Verney et al (1988) neuronal population was further supported by showing that these neurons also extensively co-express SST with d2EGFP (and by extension TH; Man et al, 2007; Fig.2A-D). Rarely, examples of SST+ve/low d2EGFP, or conversely d2EGFP+ve/SST-ve cells were observed (Fig.2D). With respect to BNSTLI SST, it was also found that whereas the cellular level of this neuropeptide clearly decreased in PN40 BNSTLI compared with PN10 brains, there was nevertheless clear evidence of SST in neuronal soma at this time point (Fig.2E,F). This finding also concurs with Verney et al (1988) who recognized a relative sparing of SST activity compared with TH at later postnatal time points in their study. In agreement with our previous analysis of PN40 males (Man et al, 2007), levels of d2EGFP are negligible in female PN40 BNSTLI (Fig.2F); as a control for this observation, given differential brain fixation, for example, images of PN40 medial preoptic area from the same brains are shown (Fig.2G-I) where the transgene is highly expressed at this time point in a population of neurons adjacent to the third ventricle, distinct from periventricular SST neurons (Fig.2H,I). Extensive Egr1 transcription in these PN40 preoptic neurons is interesting in the context of pubertal development at this stage of development.

In order to address potential sex differences in the postnatal BNSTLI population, the numbers of TH+ve neurons in the caudal group of BNSTLI neurons were counted in matching sections from different brains sampled at the PN10 time point. Despite an indication of higher TH+ve cell numbers in the female samples, no significant sex difference was found in this analysis (Male 20.5±1.4; Female 23.2±0.9; values are mean ± SEM of distinct TH+ve cells per individual caudal BNSTLI section; n = 3 brains/group, p = 0.176, unpaired Student's t test).

The general phenotype of BNSTLI EGR1/TH/SST neurons was further characterized by examining co-/aberrant expression of additional proteins related to the currently defined phenotype (Fig.3). Firstly, it was shown that the transient TH/*Egr1*+ve neurons of the BNSTLI do not express dopa decarboxylase (DDC; Fig.3A-C), and are therefore unable to convert cellular levodopa to dopamine. As a control for this detection, DDC+ve neurons in the dorsomedial area of the SCN (Ishida et al, 2002) were shown to express abundant cytoplasmic DDC (Fig.3A). Second, the potential deficiency of nuclear transcript splicing regulation indicated by low NeuN (Rbfox3) levels was shown to be potentially compensated by another Rbfox RNA binding protein family member (RBFOX2), that was expressed in d2EGFP+ve neurons at levels similar other adjacent neurons (Fig.3D-F). Using two other antisera, it was also shown that the BNSTLI neurons are Sox2-ve (Fig.3G-I; unlike NeuN-ve/Sox2+ve neurons of the SCN, Hoefflin & Carter, 2014), and also do not exhibit consistent under- or over-expression of the neuron-restrictive silencing factor, REST (Fig.3J-L). Finally, with regard to the cellular activation indicated by high EGR1 levels, it was shown that the BNSTLI neurons do not exhibit detectable levels of the neuronal activation marker, c-FOS (Fig.3M-O). As a control for this detection, isolated c-FOS+ve neurons of the adjacent cerebral cortex are shown (Fig. 3M).

To compare the TH+ve BNSTLI neurons with adult TH+ve (dopamine–synthesizing) neurons, a number of key TFs involved in the maintenance of these neurons were investigated. NR4A2 (NURR1) was detected in PN10 BNSTLI (Fig.4), but at markedly lower levels than in the PN10 subplate area, for example (Fig.4A). For direct semi-quantitative comparison of these regions, the same camera exposure was maintained for the images in 4A, and 4B&C. NR4A2 was co-expressed in d2EGFP+ve neurons (Fig.4D-F), but here NR4A2 was predominantly cytoplasmic, in marked contrast to subplate cells where expression of NR4A2 was predominantly nuclear as indicated by coincident DAPI staining (Fig.4A). The FOXO1 factor was abundantly expressed in PN10 striatum (Fig.5A; Hoekman et al, 2006) but could not be detected in PN10 BNSTLI d2EGFP neurons (Fig.5B&C). Two other Forkhead Box TFs associated with the maintenance of dopaminergic neurons, FOXA1 and FOXA2, were shown to be robustly expressed in PN2 subplate cells, with, respectively, nuclear, and perinuclear localization (Fig.5D&G), but were not detectable in PN10 BNSTLI neurons (Fig.5E&F, H&I).

Discussion

Recent studies using single-cell transcriptome analysis have broadened our view of neuronal diversity by identifying novel sub-classifications of neurons (Poulin et al, 2014; Tasic et al, 2016; Romanov et al, 2017). However, phenotypic definition of neuronal sub-types also demands *in-situ* profiling in order to confirm the presence or absence of key proteins, including TFs, that partly define (and maintain) cellular phenotypes. In the current study, a specific neuronal phenotype in the postnatal rat brain has been investigated in order to identify molecular factors that might underlie both immaturity, and secondly, the transience of these neurons. The results show that a population of BNST neurons expressing high levels of the EGR1 TF with conversely low levels of the

adult neuronal marker NeuN/RBFOX3 is common (and quantitatively similar) to both male and female rats during the postnatal period. The current results confirm previously published data in male rats (Man et al, 2007), but now reveal details of the molecular phenotype of these neurons. NeuN/RBFOX3 immunoreactivity-deficient neuronal immaturity has been characterized in an adult neuronal population (suprachiasmatic nucleus, SCN; Geoghegan & Carter, 2008; Hoefflin & Carter,2014), but BNSTLI neurons do not similarly co-express SOX2, and are therefore distinct from the NeuN/RBFOX3-ve cells of the SCN. However, with respect to transience, the current study has also shown that the BNSTLI neurons are almost certainly identical to a population of transient TH/SST+ve BNST neurons initially identified by Verney et al (1988). Thus, the current results show that BNSTLI EGR1/TH+ve neurons also extensively co-express SST, and that expression of this neuropeptide is maintained at low levels on PN40 whereas EGR1/TH expression is negligible (Man et al, 2007; current study). Maintenance of SST expression at PN40 is in agreement with the observations of Verney et al (1988) and also with adult expression in this area (Gray & Mogenson, 1987; Herbison & Theodosis, 1993), indicating that the BNSTLI neurons have not died, but are maintained with a different phenotype. This transition may represent an additional example of neurotransmitter switching/selection in neurons (see Spitzer, 2015), and provide a physiological model for the analysis of this phenomenon.

A key feature of the postnatal BNSTLI neuronal phenotype is high (relative to other nuclei), and maintained (up to PN30) expression of the TF, EGR1. Use of the *Egr1-d2EGFP* transgenic model demonstrates that this pattern of expression is driven at the transcriptional level, and is presumably due to chronic synaptic activation/depolarization as indicated by previous studies of *Egr1* induction (Cole et al, 1989; Xie et al, 2014). The neurotransmitter system(s) mediating this synaptic input onto BNSTLI neurons is

undefined, but may involve substance P and NK-1 receptors on d2EGFP+ve neurons (Al-Sharraj and Thiel, 2002; Man et al, 2007). Notably, the requirement for EGR1 in these neurons is limited to the postnatal period (see below); the finding that expression rapidly terminates after PN20 is consistent with studies showing low *Egr1* expression in adult rodent BNST (Cullinan et al, 1995; Rosen et al, 2005; Bupesh et al, 2014), and may be related to postnatal changes in substance P receptor levels (Quirion & Dam, 1986). With regard to potential postnatal functions of this factor, EGR1 may regulate genes involved in neurite development (Ravni et al, 2008), and/or neurotransmission, including TH (Akiba et al, 2009; Nakashima et al, 2003), but the functional repertoire of EGR1 in these neurons is currently unknown. TH expression is activity-dependent (Aumann, 2016), perhaps explaining the abundance of TH in the postnatal BNSTLI neurons (see below).

In addition to high levels of *Egr1* transcription, another defining feature of the transient BNSTLI neurons is low expression of the adult neuronal 'marker' NeuN, compared with adjacent postnatal neurons (Man et al, 2007; current study). This aspect of the BNSTLI phenotype may also relate to chronic synaptic activation because Weyer and Schilling (2003) have shown that chronic depolarization lowers NeuN/RBFOX3 expression. In the current study, levels of another RBFOX family member, RBFOX2, were shown to be similar in BNSTLI neurons and adjacent neurons. This finding could be interpreted to indicate that RBFOX2 does not functionally compensate for potential deficiencies in RBFOX-related splice regulation (see Darnell, 2013) due to low BNSTLI RBFOX3. However, other recent studies in this laboratory (Partridge & Carter, unpublished) have identified distinct *Rbfox2* transcript profiles in NeuN-ve neurons of the SCN that are not revealed with current N-terminal-specific RBFOX2 antisera. Further studies are required to investigate whether a similar change in *Rbfox2* expression profile, and potentially

function, is found in BNSTLI neurons. The presence of low RBFOX3/ NeuN in the TH+ BNSTLI neurons is of general interest because recent work has shown that RBFOX3 levels in dopaminergic neurons vary markedly in both normal (Cannon and Greenamyre, 2009), and disease contexts (Duan et al, 2016). BNSTLI neurons could therefore provide a model for investigating the causes and relevance of low neuronal RBFOX3 expression.

The key finding of the present study is that the BNSTLI neurons have deficiencies in TFs known to be important for the maintenance of dopaminergic neurons: NR4A2, FOXO1, FOXA1 and FOXA2 (see Deneris and Hobert, 2014). Levels of these factors differ among dopaminergic neuronal subtypes, but some are consistently expressed (Poulin et al, 2014). A deficiency/absence of these factors may therefore contribute to both an insufficiency in the complement of dopamine-related genes in these neurons (Verney et al, 1988), and the eventual loss of TH. NR4A2 is required for both the development, and maintenance of a dopaminergic phenotype (see Kadkhodaei et al, 2009) and is now known to act together with the Forkhead pioneer proteins FOXA1 and FOXA2 (Yi et al, 2014; Pristera et al, 2015). Another Forkhead factor, FOXO1, has also been shown to regulate TH expression and dopaminergic phenotype (Doan et al, 2016). The Forkhead factors are not universally expressed in adult dopaminergic neurons (Pristera et al, 2015), but their complete absence in PN10 BNSTLI neurons, together with the demonstrated low levels of NR4A2 is indicative of a transcriptional basis for the transient phenotype. Additionally, the current study has also revealed that the low levels of NR4A2 in the BNSTLI are primarily cytoplasmic, with a consequent loss of transcriptional activity (Baron et al, 2012; Boldingh Debernard et al, 2012). With respect to the surprising lack of additional dopamine synthesis enzymes in the TH+ BNSTLI neurons (Verney et al, 1988), the present study has confirmed an absence of DDC immunoreactivity. Distinct transcriptional regulatory mechanisms control, for example,

dopamine beta-hydroxylase (*Dbh)* and *Th* (Kilbourne et al, 1991; Kim et al, 2003). Of note, EGR1 has contrasting effects on *Dbh* (Cheng et al, 2008) and *Th* expression (Akiba et al, 2009; Nakashima et al, 2003); and may similarly discriminate *Th* and *Ddc* gene regulation.

The functional role of the transient EGR1/TH neurons of the BNSTLI is currently unknown. Transient postnatal neuronal phenotypes/circuits are documented in many species, including humans (see Verney, 2003), and contribute to functional development in hypothalamus (Grove and Smith, 2003), hippocampus (Chen et al, 2004) and sensory cortex (Chen et al, 2015; Marques-Smith et al, 2016). Much remains to be learnt about the functional development of the BNST (see Lebow & Chen, 2016), but current evidence indicates that components of this complex region are responsive to postnatal experience (Fenoglio et al, 2006) that may fix lifelong differences in anxiety/fear responses (Dunarci et al, 2009). There is also published data showing that changes in BNST neuronal phenotype can result from postnatal social stress (Wommack and Delville, 2002), but extensive further studies are required to define any relationship between postnatal experience and an adaptive (re)organization of the BNST. The discrete population of BNSTLI neurons characterized in the current study may represent one tractable component for analysis of this relationship.

In summary, the current study has characterized a neuronal molecular phenotype that is distinct from other known TH/SST+ve phenotypes, including recently described TH/SST+ve hypothalamic neurons (Romanov et al, 2017), which have an alternative TF profile but differ from the BNSTLI neurons in co-expressing *Ddc.* These findings further reveal the diversity of neuronal phenotypes and emphasize the value of molecular profiling in phenotypic analysis.

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Figure Legends

Fig.1. Characteristics of intense GFP/Egr1+ve neurons in the intermediate lateral region of the rat bed nucleus of the stria terminalis (BNSTLI). Representative fluoresence microscopic images of female PN10 brain showing the distribution, morphology and protein co-localization of GFP+ve neurons as detected with immunohistochemical analysis. **A-C.** Extensive co-localization of GFP with TH across the BNSTLI**.** Arrow indicates the course of the stria terminalis (st)**. D-F.** Intense BNSTLI GFP/Egr1+ve neurons have low expression of NeuN. Arrowhead highlights one neuron with characteristic high GFP, and low NeuN immunoreactivity. **G-I.** Details of GFP+ve (G&H) and TH+ve (I) processes in BNSTLI (G&H), and isolated dorsal hypothalamic (I) neurons. Arrowheads indicate cellular processes and individual varicosities. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; GFP, d2EGFP green fluorescent protein; NeuN, neuronal nuclei antigen/RBFOX3; st, pathway of the stria terminalis; TH, tyrosine hydroxylase. Scale bars: A-C = 100mm; D-I = 50mm.

Fig.2. Postnatal day 10 (PN10) GFP/Egr1+ve neurons in the lateral ventral region of the bed nucleus of the stria terminalis (BNSTLI) also express SST, but only SST is retained at PN40. Representative fluoresence microscopic images of rat brain showing the distribution and protein co-localization of GFP+ve neurons, as detected with immunohistochemical analysis. **A-C.** Extensive co-localization of GFP with SST across PN10 BNSTLI**.** Arrow indicates the course of the stria terminalis (st). Box indicates the area shown in panel D. **D.** Detail of panel C showing co-localization of GFP and SST, and two SST+ve/GFP-ve neurons (arrowheads). **E&F.** SST+ve/GFP-ve neurons in PN40 BNSTLI. **G-I.** GFP+ve neurons in PN40 medial preoptic area, adjacent to the third ventricle (3v). Note the distinct, periventricular SST+ve neurons. Abbreviations: as Fig.1.

3v, third ventricle; SST, somatostatin; Scale bars: A-C = 100mm; D-F = 50mm; G-I=100mm.

Fig.3. Molecular phenotype of BNSTLI GFP/EGR1 neurons. Representative fluoresence microscopic images of PN10 rat brain showing protein absence/co-localization in GFP+ve neurons as detected with immunohistochemical analysis. **A-C.** Presence of dopa decarboxylase (DDC) in the dorsomedial area of the SCN (A) but absence in BNSTLI neurons (B,C). The boxed area in (B') is an inset image selected from a similar analysis of a similar brain section where the primary antibody was omitted. **D-F.** Abundant expression of RBFOX2 in the BNSTLI, including GFP+ve neurons. **G.** Abundant expression of SOX2 in both neuroepithelial cells surrounding the third ventricle (3v) and adjacent hypothalamic cells. **H-I.** SOX2 is expressed in BNSTLI cells but is absent from GFP+ve neurons. **J-L.** REST is widely expressed in BNSTLI cells including GFP+ve neurons (indicated by arrowheads). **M.** c-FOS is expressed in isolated PN10 cortical cells, including cells expressing a low level of GFP in this region. **N-O.** c-FOS is absent from BNSTLI GFP+ve neurons. Abbreviations: as Fig.2. DDC, dopa decarboxylase. Scale bars: A-F = 50mm; G = 100mm;; H-O = 50mm.

Fig.4. NR4A2 is expressed at low levels in the cytoplasm of GFP+ve BNSTLI neurons. Representative fluoresence microscopic images of PN10 rat brain showing protein co-localization in GFP+ve neurons as detected with immunohistochemical analysis. **A.** High levels of nuclear NR4A2 in developing PN10 cortical subplate neurons. **B-C.** Low levels of NR4A2 in GFP+ve BNSTLI neurons. Box indicates the area shown in panels D-F. **D-F.** Cytoplasmic NR4A2 in GFP+ve BNSTLI neurons. The (cytoplasmic) NR4A2+ve region of one GFP+ve neuron is highlighted in D-F. Abbreviations: as Fig.2. Scale bars: A-F = 50mm.

Fig.5. Absence of specific forkhead transcription factors in BNSTLI GFP+ve neurons. Representative fluoresence microscopic images of postnatal rat brain showing protein expression as detected with immunohistochemical analysis. **A.** Abundant FOXO1 expression in PN10 striatum. **B-C.** Absence of FOXO1 expression in PN10 BNSTLI GFP+ve neurons. **D.** Nuclear FOXA1 in PN2 subplate neurons. **E-F.** Absence of FOXA1 expression in PN10 BNSTLI GFP+ve neurons. **G.** Perinuclear FOXA2 in PN2 subplate neurons. **H-I.** Absence of FOXA2 expression in PN10 BNSTLI GFP+ve neurons. Abbreviations: as Fig.2. Scale bars: A-F = 50mm.

Table 1. Source and validation of primary antibodies used in this study.

Antigen	Antibody	Source	Validation
AADC	10166-1-AP	Proteintech	IHC, current study
EGR1	15F7	CST	Wells et al (2011)
FOXA1	sc-514695	SCBT	IHC, current study
FOXA2	sc-374376	SCBT	IHC, current study
c-FOS	Ab-2	Millipore	Derbyshire & Ludwig (2004)
FOXO1	C29H4	CST	Yang & McKnight (2015)
GFP	Ab13970	Abcam	Man et al (2011)
NeuN	MAB377	Millipore	Man et al (2007)
NR4A2	sc-5568	SCBT	Wells et al (2011)
RBFOX2	IHC-00199	Bethyl	IHC, Bethyl Labs.
SOX2	39823	Active Motif	Hoefflin & Carter (2014)
SST	IHC8001	Penninsula	Slade et al (2001)
TH	MAB318	Millipore	Man et al (2007)

IHC, immunohistochemical detection
Sources: Abcam, Cambridge, UK; Active Motif, Carlsbad, CA, USA; Bethyl Laboratories Inc., Montgomery TX, USA; Biomol International, Plymouth Meeting, PA, USA; Chemicon International, Temecula, CA, USA; CST (Cell Signaling Technology, Beverly, MA, USA); Millipore, Temecula, CA, USA); Proteintech, Rosemont, IL, USA; SCBT (Santa Cruz Biotechnology, Santa Cruz, CA, USA).