Generation of neuropeptidergic hypothalamic neurons from human pluripotent stem cells

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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Generation of neuropeptidergic hypothalamic neurons from human pluripotent stem cells

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ABSTRACT

Hypothalamic neurons orchestrate many essential physiological and behavioral processes via secreted neuropeptides, and are relevant to human diseases such as obesity, narcolepsy and infertility. We report the differentiation of human pluripotent stem cells into many of the major types of neuropeptidergic hypothalamic neurons, including those producing pro-opiolemelanocortin, agouti-related peptide, hypocretin/orexin, melanin-concentrating hormone, oxytocin, arginine vasopressin, corticotropin-releasing hormone (CRH) or thyrotropin-releasing hormone. Hypothalamic neurons can be generated using a 'self-patterning' strategy that yields a broad array of cell types, or via a more reproducible directed differentiation approach. Stem cell-derived human hypothalamic neurons share characteristic morphological properties and gene expression patterns with their counterparts *in vivo*, and are able to integrate into the mouse brain. These neurons could form the basis of cellular models, chemical screens or cellular therapies to study and treat common human diseases.

KEY WORDS: Differentiation, Hypothalamus, Narcolepsy, Neuropeptide, Pluripotent, Stem cell, Mouse, Human

INTRODUCTION

Although it makes up only 0.3% of the adult human brain, the hypothalamus is one of its most complex and essential regions (Blinkov and Glezer, 1968; Maroof et al., 2013). In their central location in the ventral forebrain (Fig. 1A,B), hypothalamic neurons are positioned to sense both neural and physiological signals and to respond by releasing neurotransmitters and peptide neuromodulators into the brain. Furthermore, they can induce the release of hormones from the endocrine glands to regulate physiology and behavior. These activities are relegated to different subtypes of hypothalamic neurons that secrete distinct neuropeptides to regulate puberty, reproduction, stress, circadian rhythms and immune function, as well as more complex behaviors, including sleep, mood and social behavior (Fig. 1C; supplementary material Table S1). As these functions are fundamental, the dysfunction of human hypothalamic neurons has been linked to common diseases, such as obesity, hypertension, mood disorders and sleep disorders, that place a substantial burden on Western healthcare systems (Meyer-Lindenberg et al., 2011; Spiegelman and Flier, 2001; Swaab, 1999, 2006; Wataya et al., 2008) (supplementary material Table S1).

To enable the study and therapeutic use of human hypothalamic neurons, we aimed to generate these cells *in vitro* from human pluripotent stem cells (hPSCs) using two distinct approaches: 'self-patterning' and directed differentiation. The self-patterning approach permits organ-like tissue development *in vitro* via the cell-cell and paracrine interactions that pattern tissues *in vivo* (Ludwig and Thomson, 2007; Sasai et al., 2012). Self-patterning is a rational choice for hypothalamic differentiation as pluripotent stem cells are predisposed to generate anterior neural structures such as the hypothalamus (Puelles and Rubenstein, 2003; Watanabe et al., 2007) by default (Kamiya et al., 2011; Wilson and Rubenstein, 2000) (Fig. 1A). Directed differentiation of hPSCs in the presence of inhibitors of the TGFβ/NODAL/activin and BMP signaling pathways leads to the efficient production of neural progenitors (Blinkov and Glezer, 1968; Chambers et al., 2009) that can be patterned into ventral forebrain neurons by the early inhibition of the WNT signaling pathway followed by activation of the sonic hedgehog (SHH) pathway (Maroof et al., 2013; Meyer-Lindenberg et al., 2011; Spiegelman and Flier, 2001; Swaab, 1999, 2006). We reasoned that a similar approach could be taken to generate human hypothalamic neurons (Fig. 1D).

Here, we report the differentiation of both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) into hypothalamic neurons using complementary self-patterning and directed differentiation approaches. The neuropeptide-expressing cells we observed are highly enriched or exclusively localized in the hypothalamus and were morphologically similar to their *in vivo* counterparts. The efficiency with which these rare neuropeptidergic cell types were produced *in vitro* rivaled their prevalence in the human hypothalamus *in vivo*. Furthermore, human hypothalamic neurons survived transplantation into the mouse brain and continued to express hypothalamic neuropeptides in ectopic locations. The resources described here lay the foundation for the study of human hypothalamic neurons in health and disease.

RESULTS

Self-patterning of hPSCs into hypothalamic progenitors

To generate hypothalamic progenitors from hPSCs, we took advantage of the natural propensity of pluripotent stem cells to differentiate into anterior neural structures, such as the hypothalamus (Fig. 1A-C), in the absence of inductive signals (Fig. 1D). We first implemented a previously described self-patterning protocol (Sasai et al., 2012; Wataya et al., 2008) to confirm the generation of hypothalamic progenitors from mouse embryonic stem cells (mESCs) plated in a growth factor-free,

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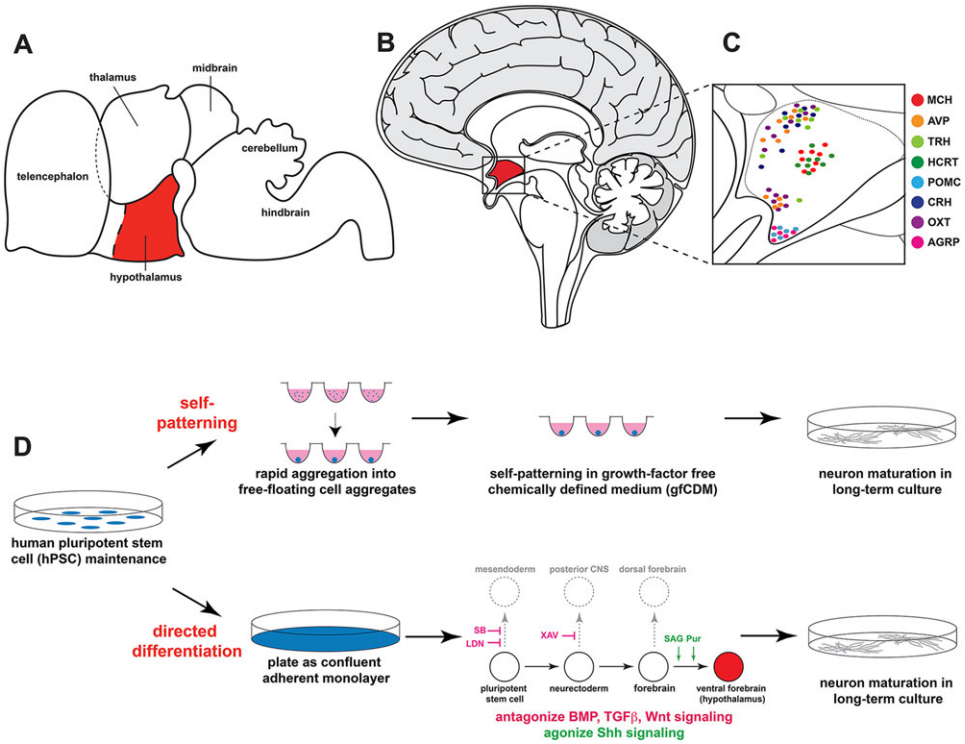

Fig. 1. *In vivo* location and *in vitro* generation of hypothalamic neurons. (A,B) Schematic lateral views of the embryonic mammalian brain (A) and adult human brain (B) showing the anterior and ventral location of the hypothalamus (red). (C) Enlarged schematic of the human hypothalamus showing the approximate distribution of select neuropeptidergic cell types (colored dots). MCH, melanin-concentrating hormone; AVP, arginine vasopressin; TRH, thyrotropin-releasing hormone; HCRT, hypocretin (also known as orexin); POMC, pro-opiomelanocortin; CRH, corticotropin-releasing hormone; OXT, oxytocin; AGRP, agouti-related protein. (D) Self-patterning and directed differentiation strategies for hypothalamic differentiation from hPSCs. gfCDM, growth factor-free chemically defined minimal medium; LDN, 100 nM LDN-193189; SB, 10 µM SB435142; XAV, 2 µM XAV939; SAG, 1 µM smoothened agonist; Pur, 1 µM purmorphamine; BMP, bone morphogenetic protein; TGFβ, transforming growth factor β; WNT, wingless-related MMTV integration site; SHH, sonic hedgehog.

chemically defined medium (gfCDM). We observed that mESCs seeded into gfCDM differentiated into neurons that expressed hypothalamus-specific neuropeptides, including hypocretin (supplementary material Fig. S1).

To adapt the mESC self-patterning protocol to hPSCs, we grew two hESC and two hiPSC lines (Fig. 2A) under feeder-free conditions in the defined medium mTeSR (Ludwig and Thomson, 2007; Puelles and Rubenstein, 2003). When hPSCs were dissociated and plated into gfCDM, they died within several days, even in the presence of the Rho kinase (ROCK) inhibitor Y27632 (Kamiya et al., 2011; Watanabe et al., 2007; Wilson and Rubenstein, 2000). We found that cell aggregates efficiently formed and survived in mTeSR with ROCK inhibitor (Fig. 2B), but died within 1 week when transferred to gfCDM. To overcome this cell death, we supplemented gfCDM with insulin and a small molecule Akt inhibitor, as Akt signaling has previously been shown to inhibit hypothalamic differentiation under similar conditions (Wataya et al., 2008).

After 30 days (D30) in this medium, 93±4% of hPSC-derived cells were immunopositive for the early neural transcription factor SOX1 (supplementary material Table S2). Morphologically, D30 cell aggregates formed neuroepithelial-like structures that enclosed ventricle-like vesicles (Fig. 2C). We cryosectioned and immunostained hPSC-derived cell aggregates for the apical marker atypical protein kinase C (aPKC) (Djiane et al., 2005; Manabe et al., 2002), and found that aPKC localized to the inner (apical) surface of the ventricle-like structures (Fig. 2D), as in the embryonic neuroepithelium. Next, we immunostained for the mitotic marker phosphorylated histone H3 (pHH3), and observed that over 70% of pHH3-immunopositive cells were located within 20 µm of the apical surface of the ventricle-like structures (Fig. 2E), consistent with the division of neural stem cells at the apical surface of the ventricle *in vivo* (Taverna and Huttner, 2010). Finally, we immunostained cell aggregates for neuron-specific class III β-tubulin (TUJ1) and found that most TUJ1-expressing cells were separated from the ventricle-like structures by at least 50 µm (Fig. 2D,E), as is seen in the embryonic nervous system (Marín and Rubenstein, 2003). Together, our findings indicated that hPSCs could self-pattern into aggregates that resembled the embryonic neuroepithelium.

To determine whether cells within self-patterned cell aggregates adopted a hypothalamic identity, we cryosectioned cell aggregates at D30 and performed immunostaining for the transcription factors forkhead box G1 (FOXG1), which is expressed throughout the telencephalon but is absent from the hypothalamus (Tao and Lai, 1992), and NK2 homeobox 1 (NKX2.1), which is expressed in the hypothalamus as well as in the medial ganglionic eminence (MGE) of the telencephalon. Over 15% of cells we analyzed expressed NKX2.1 but not FOXG1, indicating their likely hypothalamic identity (Fig. 2F,G,L). To confirm and extend these results, we immunostained for the transcription factors retina and anterior neural fold homeobox (RAX) (Furukawa et al., 1997), orthopedia homeobox (OTP) (Simeone et al., 1994) and single-minded homolog 1 (SIM1) (Fan et al., 1996) (Fig. 2H-J). RAX is exclusively expressed in the hypothalamus and retina (Furukawa et al., 1997). SIM1 and OTP are expressed in a subset of hypothalamic progenitors where they cooperatively specify certain neuropeptidergic cell types (Acampora et al., 1999; Michaud et al., 1998). We found that each of these genes that were indicative of hypothalamic identity were expressed in self-patterned cell aggregates (Fig. 2L), but were absent from control cell aggregates that were patterned to a caudal and ventral neural identity by exposure to retinoic acid (RA) and smoothened agonist (SAG) (Wichterle et al., 2002). We also noticed that immunopositive cells were often clustered together, suggesting that self-patterning produced distinct progenitor domains (arrowheads in Fig. 2F,I,J).

To support these results, we isolated RNA from D30 cell aggregates and performed quantitative RT-PCR for genes that are regionally expressed in the cerebral cortex, hypothalamus or midbrain/hindbrain (Fig. 2K; supplementary material Table S3).

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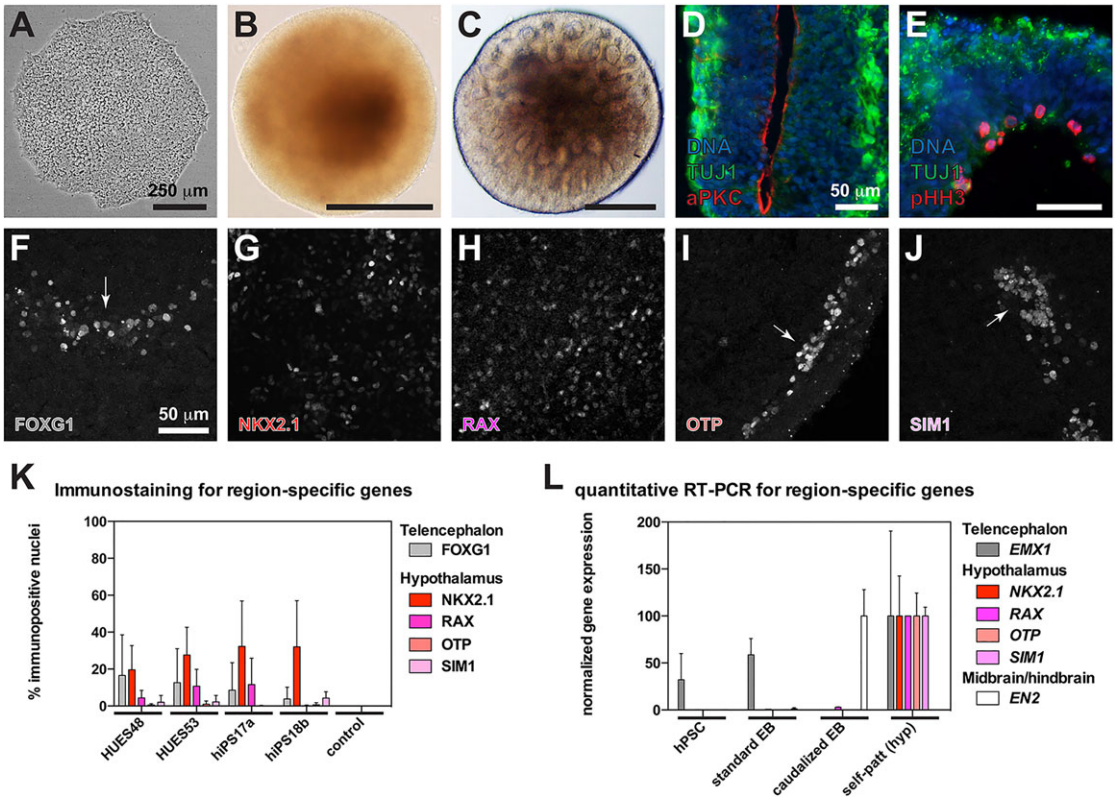

Fig. 2. Self-patterning of hPSCs to hypothalamic progenitors. (A) Human pluripotent stem cell (hPSC) colony grown in feeder-free conditions. (B) D1 hPSC aggregate. (C) D30 self-patterned cell aggregate displaying ventricle-like structures. (D) Ventricle-like structures (D30) show apical-basal polarity as indicated by immunostaining for the apically expressed atypical protein kinase C (aPKC) and the neuronal gene TUJ1. (E) The majority of cell divisions in self-patterned aggregates (D30) occur at the apical (ventricle-like) surface, as indicated by immunostaining for phosphorylated histone H3 (pHH3). (F-J) hPSC-derived cell aggregates contain clusters of cells (arrows) immunopositive for genes indicative of forebrain (FOXG1) or hypothalamic (NKX2.1, RAX, OTP, SIM1) identity. (K,L) Quantification of immunostaining (K) and quantitative RT-PCR (L) for region-specific transcription factors. Scale bars: 250 μm in A-C; 50 μm in D-J. Error bars in K,L show s.d. EB, embryoid body; EMX1, empty spiracles homeobox 1; EN2, engrailed 2; FOXG1, forkhead box G1; NKX2.1, NK2 homeobox 1; RAX, retina and anterior neural fold homeobox; OTP, orthopedia homeobox; SIM1, single-minded homolog 1; Self-patt (hyp), self-patterned hypothalamic cell aggregates.

Relative to control cell populations, self-patterned aggregates weakly expressed the telencephalic marker gene empty spiracles homeobox 1 (*EMX1*) (Simeone et al., 1992a,b) but strongly expressed markers of the ventral telecephalon and hypothalamus, including *NKX2.1* and *OTP*. By contrast, transcripts of the midbrain/hindbrain marker gene engrailed 2 (*EN2*) (Joyner and Martin, 1987) were barely detectable in self-patterned aggregates. These results suggest that most self-patterned hPSCs adopted a forebrain identity composed of hypothalamic and telencephalic cells.

Self-patterning of hPSCs into neuropeptidergic hypothalamic neurons

Having established that hPSCs can be differentiated into hypothalamic progenitors, we tested whether they could give rise to physiologically important populations of hypothalamic neurons. Cultures were matured and assayed for the presence of subpopulations of neuropeptidergic hypothalamic neurons that exert potent effects on behavior and physiology, and are highly enriched or exclusively located in the hypothalamus. In particular, we observed cells strongly immunoreactive for α-melanin-stimulating hormone (αMSH/POMC), agouti related peptide (AGRP), hypocretin A/orexin (HCRTA), melanin-concentrating hormone (MCH), oxytocin (OXT), arginine vasopressin (AVP), corticotropin-releasing hormone (CRH) or thyrotropin-releasing hormone (TRH) (Fig. 1C and Fig. 3A-H). These neuropeptide-expressing cells were not seen in caudalized control cell aggregates (supplementary material Fig. S2) and were immunopositive for the neuron-specific genes MAP2 and NEUN (RBFOX3 – Human Gene Nomenclature Committee) (Fig. 3I,J; supplementary material Fig. S3), confirming their neuronal identity. We did not observe neurons that were immunoreactive for growth hormone-releasing hormone (GHRH), kisspeptin (KISS1) or for gonadotropin-releasing hormone (GnRH/LHRH). GnRH neurons are located in the hypothalamus, but are developmentally derived from the olfactory placode (Schwanzel-Fukuda and Pfaff, 1989). The number of neuropeptide-immunopositive cells varied between cell aggregates, and within aggregates immunopostive cells were often clustered together (Fig. 3K). Upon analyzing many aggregates, we found that ∼3.6 per 1000 nuclei expressed one of the assayed neuropeptides, which is similar to the incidence of these rare cells (2.1 in 1000 neurons) in the human hypothalamus (supplementary material Table S4) (Blinkov and Glezer, 1968).

To extend the characterization of *in vitro*-derived hypothalamic neurons, we dissociated cell aggregates at D90 and maintained them in culture for an additional 60 days in the presence of mouse cortical glia (supplementary material Fig. S4). After this extended culture, we continued to observe neurons that expressed neuropeptides such as hypocretin A (HCRTA) or OXT (Fig. 3L). As *in vitro*-derived neurons often adopt similar axonal and dendritic arbors as their counterparts *in vivo* (Muguruma et al., 2010; Su et al., 2006; Tao et al., 2010), we traced the neuropeptide-

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immunopositive neurites of hPSC-derived hypothalamic neurons. *In vivo*, HCRT neurons extend axons widely throughout the cerebrum and even to the sacral spinal cord (Peyron et al., 1998; van den Pol, 1999), whereas the processes of OXT neurons are more restricted to the brain regions adjacent to the hypothalamus (Lee et al., 2009). *In vitro*, we observed that HCRT neurons had strikingly long axonal processes that extended for over 1 cm in culture, and were punctuated by sparse collaterals, as is seen *in vivo*. By contrast, the axonal branches of *in vitro*-derived OXT neurons were highly branched and covered a more restricted area, mimicking their projection pattern *in vivo*.

Directed differentiation of hPSCs into hypothalamic progenitors

The self-patterning approach relies on individually grown three-dimensional aggregates and is not easily scalable, complicating efforts to generate large numbers of neurons needed for disease modeling or chemical screening. We therefore developed a directed differentiation approach to generate human hypothalamic neurons from a monolayer of hPSCs by modulating the signaling pathways leading to neuralization, forebrain specification and ventralization.

As the hypothalamus is an anterior and ventral neural structure (Fig. 1A,B), we differentiated hPSCs in the presence of small molecule inhibitors of the BMP and TGFβ/NODAL/activin signaling pathways to promote neural differentiation (Fig. 1D) (Chambers et al., 2009) and a small molecular inhibitor of the WNT pathway (Huang et al., 2009), because WNTs induce the formation of posterior brain regions (Yamaguchi, 2001). To ventralize these putative forebrain progenitors, we exposed them to small molecule agonists of the SHH pathway, including purmorphamine (Pur) (Sinha and Chen, 2006) and SAG (Chen et al., 2002), which can induce ventral neural characteristics (Wilson and Rubenstein, 2000), particularly when exposed to differentiating hPSCs at early time points (Fasano et al., 2010; Maroof et al., 2013).

To determine the spatial identity of cell types generated in the presence and absence of SHH agonists, we immunostained cultures for the transcription factors FOXG1, NKX2.1, RAX, OTP and SIM1 (Fig. 4A-E). The vast majority cells expressed the transcription factor NKX2.1, and ~80% expressed NKX2.1 but not FOXG1, as observed in ventral hypothalamic progenitors *in vivo*. Next, we assayed for markers that are highly enriched or specifically expressed in subsets of hypothalamic cells, including RAX, OTP or SIM1. We observed that a significant fraction (5-10%) of hPSC-derived cells expressed these markers when directed to a hypothalamic identity, but not in control telencephalic differentiations (Fig. 4F). This efficiency is consistent with the expression of these factors in subsets of hypothalamic progenitors in the mouse brain (Acampora et al., 1999; Furukawa et al., 1997; Mathers et al., 1997; Shimogori et al., 2010; Simeone et al., 1994). We observed the most efficient induction of hypothalamic markers when we treated cultures with a combination of 1 µM Pur and 1 µM SAG from D2-8 (supplementary material Fig. S5).

As NKX2.1 is expressed predominantly in the ventral hypothalamus, the high percentage of NKX2.1-immunopositive

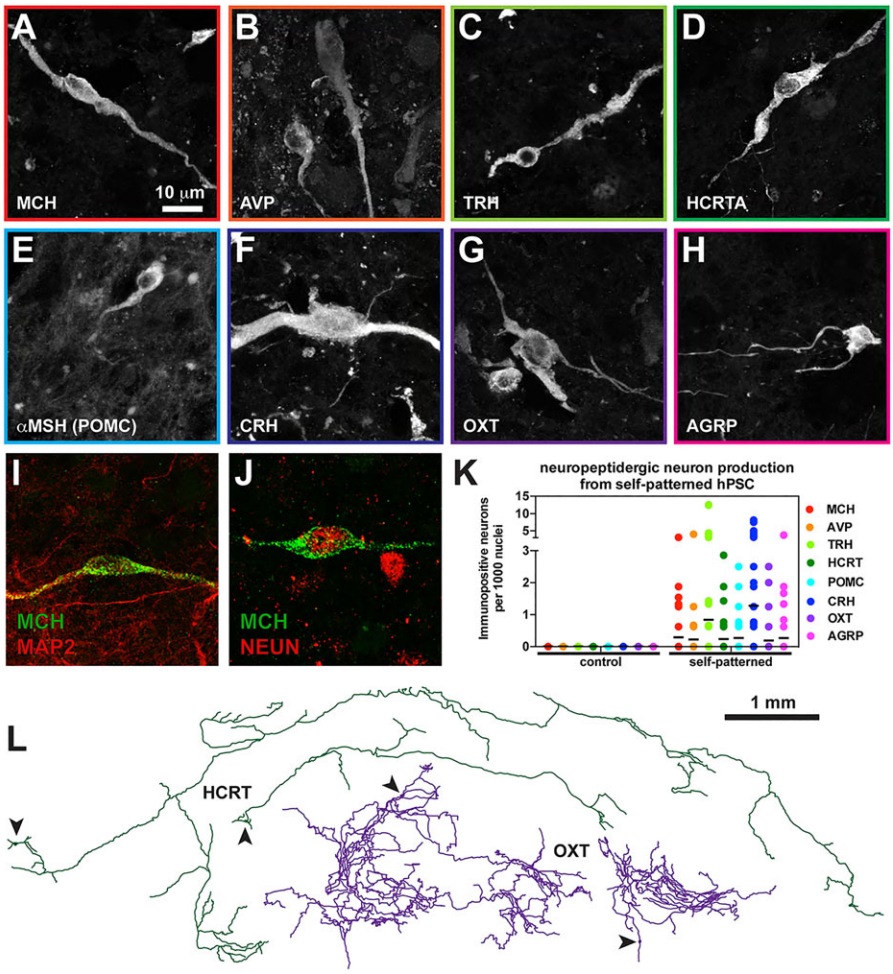

Fig. 3. Self-patterning of hPSCs to neuropetidergic neurons. (A-H) hPSC-derived cells display neuronal morphology and are immunopositive for hypothalamic neuropeptides. HCRTA, hypocretin A; αMSH, α-melanocyte stimulating hormone. (I,J) hPSC-derived neuropeptidergic cells express the neuron-specific genes MAP2 and NEUN. (K) Hypothalamic neuropeptidergic neurons were observed in four different self-patterned hPSC lines but not in a caudalized control line. (L) Hypothalamic neurons survive for long periods of time in culture and develop extensive arbors of neurites resembling their *in vivo* counterparts. Cell bodies are indicated by arrowheads. Analysis was performed on sectioned cell aggregates at D90 for A-K and on dissociated and cultured cells at D150 for L.

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cells suggested that hPSC-derived neural progenitors might have adopted a ventral hypothalamic identity. To gain further insight into the nature of hypothalamic patterning, we differentiated an *NKX2-1::GFP* reporter cell line (Goulburn et al., 2011) to hypothalamic progenitors and immunostained for GFP and hypothalamic transcription factors. This analysis revealed that 68±5% of RAX-immunopositive cells, 15±7% of OTP-immunopositive cells and 51±16% of SIM1-immunopositive cells expressed GFP (supplementary material Fig. S6A-C). These findings are consistent with the overlap of *Nkx2.1* with *Rax*, *Otp* and *Sim1* expression domains in ventral hypothalamus of the embryonic mouse brain (supplementary material Fig. S6D-G), as well as with the expression of these transcription factors in the *Nkx2.1*-negative anterior dorsal hypothalamus.

To confirm and extend these results, we performed quantitative RT-PCR for transcripts indicative of telencephalic, hypothalamic and midbrain/hindbrain identities. We observed the strong induction of the ventral forebrain and hypothalamic marker *NKX2.1* and the largely hypothalamic marker *OTP*, whereas there was relatively low expression of the telencephalic transcript *FOXG1* or the midbrain/hindbrain transcripts *EN2*, suggesting that hPSCs adopted a hypothalamic rather than a telencephalic or midbrain/hindbrain spatial identity (Fig. 4G). Similar results were obtained from 15 distinct hESC and hiPSC cell lines. We conclude that hypothalamic progenitors can be efficiently and reproducibly generated by directed differentiation of hPSCs.

Directed differentiation of hPSCs into neuropeptidergic hypothalamic neurons

In order to promote the generation and maturation of neuropeptide-expressing hypothalamic neurons, hPSC-derived hypothalamic progenitors were passaged on D30 onto a monolayer of mouse cortical glia. Upon immunostaining these cells on D40, we observed cells with neuronal morphology that were strongly immunopositive for MCH, TRH, HCRTA, αMSH, CRH and AGRP (Fig. 5A-F). Neuropeptide-immunopositive cells were not seen in control cultures in which SAG and Pur were omitted. Although no examples of OXT or AVP immunoreactive neurons were observed, neuropeptide-immunopositive cells were generated from hPSCs at a frequency (9.8 per 1000 nuclei) comparable with that observed in the adult human hypothalamus (2.1 per 1000 neurons) (Fig. 5G; supplementary material Table S4).

To confirm the neuronal identity of hPSC-derived neuropeptidergic cells, we immunostained for the neuron-specific marker genes MAP2 and NEUN and found that these markers were co-expressed in all assayed neuropeptide-immunopositive cells (432/432) (Fig. 6A,B; supplementary material Fig. S7). To further characterize the cells produced by directed hypothalamic differentiation, we immunostained cultures on D60 and determined that 87±8% of cells expressed TUJ1, 50±14% expressed NEUN, 21±3% expressed γ-aminobutyric acid (GABA), 12±3% expressed tyrosine hydroxylase (TH), 13±2% expressed glial fibrillary acidic protein (GFAP), but only 0.11±0.1% of cells expressed choline acetyltransferase (CHAT). These findings are consistent with the widespread expression of GABA in the hypothalamus (Tappaz et al., 1983), the existence of multiple hypothalamic dopaminergic populations (Moore, 1987; Moore and Bloom, 1979) and the relative lack of CHAT expression in the hypothalamus (Kasa et al., 1997). We conclude that hPSCs can be efficiently differentiated into subpopulations of rare but physiologically important neuropeptidergic neurons.

We next hypothesized that hPSC-derived hypothalamic neurons express other characteristic genes found in their *in vivo* counterparts. To test this hypothesis, we focused on assayed immuno, as these cells are well characterized and are clinically relevant because their loss causes the sleep disorder narcolepsy (Peyron et al., 2000; Thannickal et al., 2000) (supplementary material Table S1). We first tested the specificity of our immunoreagents and found that 100% (118/118) of immunoassayed neurons were clearly co-stained by separate antibodies raised against different epitopes of HCRTA, a post-translationally modified cleavage product of the HCRT pro-peptide (de Lecea et al., 1998; Sakurai et al., 1998) (Fig. 6C). Antibodies against HCRTA and other hypothalamic neuropeptides gave the punctate staining pattern (Fig. 5A-H and Fig. 6A-F) expected for neuropeptides, which are packaged into dense core vesicles (van den Pol, 2012; Zhang et al., 2010). Next, we tested whether hPSC-derived HCRT-immunopositive neurons express the opiate peptide

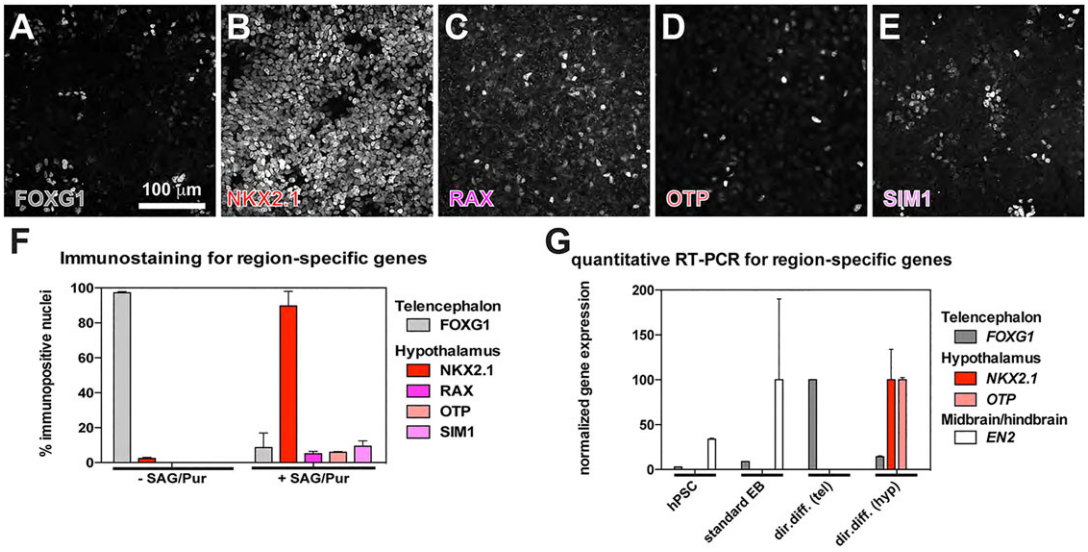

Fig. 4. Directed differentiation of hPSCs to hypothalamic progenitors. (A-E) hPSCs were subjected to directed hypothalamic differentiation and immunostained on D12 for transcription factors indicative of telencephalic identity (FOXG1) or hypothalamic identity (NKX2.1, RAX, OTP, SIM1). (F,G) Quantification of immunostaining data (F) or quantitative RT-PCR data (G) on D12 shows that directed differentiation to telencephalic [dir.diff. (tel)] or hypothalamic progenitors [dir.diff. (hyp)] leads to the expression of expected region-specific genes. Error bars in F,G show s.d.

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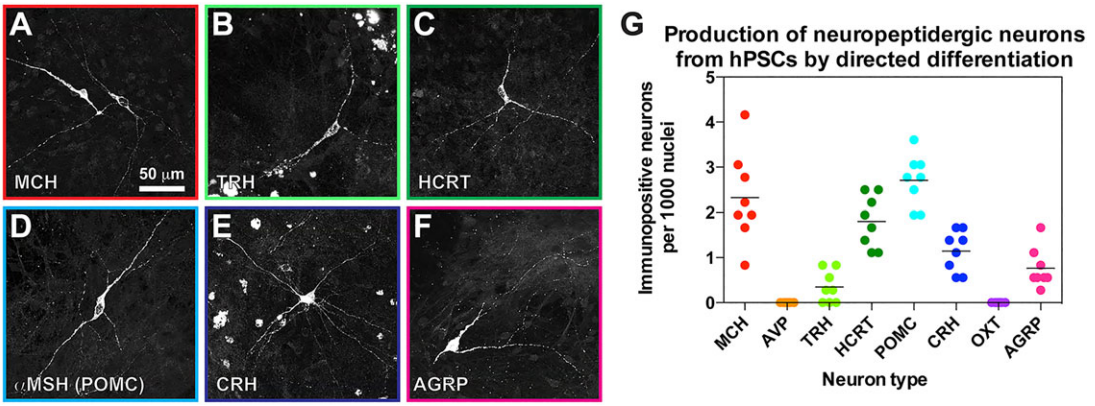

Fig. 5. Directed differentiation of hPSCs to neuropeptidergic neurons. (A-F) Directed differentiation of hPSCs to neurons immunopositive for hypothalamic neuropeptides. Cells were analyzed on D40. (G) Neuropeptidergic neurons are efficiently generated by directed differentiation (see supplementary material Fig. S5 and Table S4). Quantification was performed on D40 and cell counts were normalized to Hoechst-positive nuclei.

dynorphin A (DYNA) and the neuronal pentraxin NPTX2 (NARP), which are expressed in nearly all mouse and human HCRT neurons *in vivo* (Blouin et al., 2005; Chou et al., 2001; Crocker et al., 2005). We observed that 54/55 *in vitro*-derived HCRTA-immunopositive neurons expressed PDYN (Fig. 6D) and that 43/50 of HCRTA-immunopositive cells expressed NPTX2 with the punctate pattern this AMPA receptor-associated protein characteristically shows *in vivo* (O'Brien et al., 1999) (Fig. 6E). We did not observe colocalization of HCRTA and MCH immunoreactivity (0/283 cells) (Fig. 6F), indicating that these *in vitro*-derived hypothalamic neurons restrict neuropeptide expression to a specific cell type as seen *in vivo* (Broberger et al., 1998). Together, these results suggest that hPSC-derived hypothalamic neurons share key morphological and genetic properties with their counterparts in the brain.

In the human brain, HCRT and MCH neurons send extensive processes throughout the brain and spinal cord, whereas the processes of CRH, TRH, AGRP and POMC neurons are more restricted (supplementary material Table S1). To test whether the

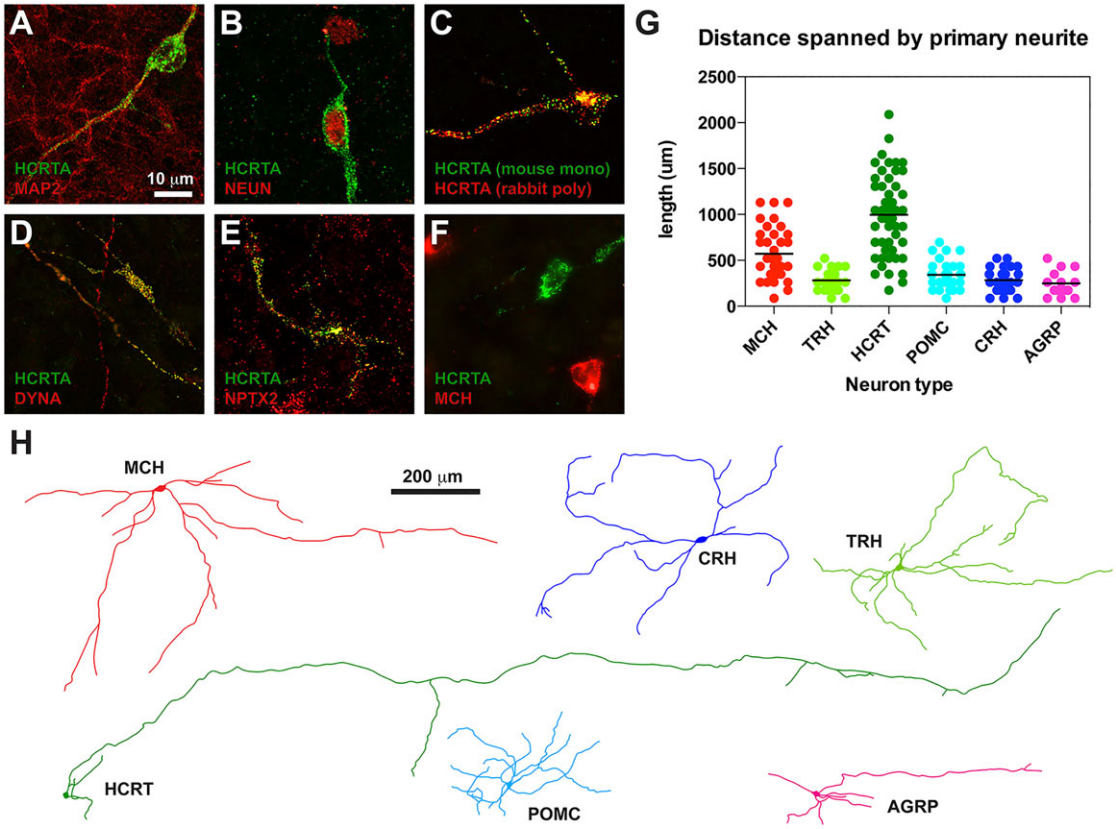

Fig. 6. Characteristics of hPSC-derived neuropeptidergic neurons. (A,B) Neuropeptide-immunopositive cells express the neuron-specific genes MAP2 and NEUN. (C) Neuropeptide immunostaining is specific, as indicated by the co-labeling of HCRT neurons with two independent anti-HCRTA antibodies. (D-F) *In vitro*-derived HCRT neurons express the expected marker genes dynorphin A (DYNA) (D) and neuronal pentraxin II (NPTX2, also known as NARP) (E), but do not aberrantly co-express MCH (F). (G,H) *In vitro*-derived hypothalamic neurons have distinctive morphologies that resemble their counterparts *in vivo*, as indicated by quantification of primary neurite length (G) and representative cell traces (H). Analysis was performed on D40 for A and B, and on D60 for C-H.

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neurite arbors of *in vitro*-derived hypothalamic neurons resembled those of their counterparts *in vivo*, we quantified the maximum distance spanned by their primary neurites. We found that HCRT neurons had significantly longer processes ($P<0.001$) than those of the other neuropeptidergic neuronal subtypes (Fig. 6G). The distinct morphologies can be seen in representative digital traces of the immunopositive neurites for each assayed cell type (Fig. 6H). For example, whereas AGRP and POMC neurons had multiple processes of similar length and one or two longer processes, the morphology of HCRT neurons was consistently dominated by a single, very long process, as seen *in vivo* (van den Pol, 1999) and as seen in HCRT neurons generated *in vitro* by self-patterning (Fig. 3L). These results suggest that *in vitro*-derived hypothalamic neurons adopt not only the gene expression patterns but also the morphological characteristics of their counterparts in the human brain.

Xenotransplantation of human hypothalamic neurons

As hPSC-derived hypothalamic neurons might one day be transplanted therapeutically, we tested whether they could survive, maintain their identity and continue to express neuropeptides after transplantation into the mouse brain. Human embryonic stem cells were differentiated into hypothalamic neurons using the directed differentiation approach described above and matured for 60 days, at which point neuropeptidergic cells could be detected and ∼90% of cells were immunopositive for TUJ1. Differentiated cultures were treated with 1 µM mitomycin C to arrest proliferating cells, enzymatically dissociated and transplanted into the brains of neonatal mice as previously described (Merkle et al., 2007). Neurons were ectopically transplanted into either the lateral ventricle or brain parenchyma to test whether human neurons could maintain their identity and to confidently distinguish between neuropeptidergic cells of human and mouse origin. The brains of transplanted mice were analyzed at 2 (n=1), 15 (n=6), 30-32 (n=6) or 45 (n=4) days post-transplantation, as well as at 6-7 (n=11) and 18 (n=8) months post-transplantation.

Upon immunostaining transplanted mouse brains with antibodies specific for human nuclear (huNuc, MAB1281) or cytoplasmic (huCyt, SC121) antigens (supplementary material Table S2), we observed human cells with neuronal morphology in all analyzed transplanted mouse brains (36/36) (Fig. 7A,B; supplementary material Fig. S5A). We observed abundant HCRT-immunopositive processes from endogenous mouse neurons in regions that contained predominantly huCyt-immunopositive cells (Fig. 7C), indicating that mouse cells robustly innervated human transplants. To assess whether transplanted human neurons integrate into the mouse brain, we immunostained parenchymally transplanted human cells for huCyt and synapsin, an antigen that is concentrated at synapses. In confocal images, we observed synapsin-immunopositive puncta in close proximity to huCyt-immunopositive cells, suggesting the formation of synapses between mouse and human cells (Fig. 7D,E). Cells immunopositive for huCyt extended long processes into the mouse brain parenchyma, including the hypothalamus (Fig. 7F-H). To determine whether transplanted neurons retain neuropeptide expression *in vivo*, we immunostained parenchymally transplanted brains for the hypothalamic neuropeptides MCH and HCRT. We observed cells with neuronal morphology that clearly expressed human nuclear antigen as well as MCH or HCRT (Fig. 7B,I,J; supplementary material Fig. S8). Human HCRT and MCH neurons were found at similar frequencies in transplants and in culture (Fig. 7K), suggesting that human neuropeptidergic neurons do not die in large numbers or down-regulate neuropeptidergic gene expression in ectopic brain regions. We conclude that *in vitro*-generated human hypothalamic neurons continue to express neuropeptides that are normally found only in the hypothalamus and survive for at least 18 months after transplantation into the newborn mouse brain.

DISCUSSION

Here, we report the efficient generation of most of the major human neuropeptidergic hypothalamic neuron types by self-patterning and by directed differentiation. These approaches have distinct advantages and drawbacks, and they enable several new lines of research.

Comparison of hypothalamic differentiation strategies

We generated hypothalamic cells by allowing hPSCs to self-pattern in a growth factor-free chemically defined medium (gfCDM). This finding is consistent with the evolutionarily conserved anterior location of the hypothalamus and with experiments in other model organisms suggesting the preferential differentiation of pluripotent cells into anterior neural structures (Hemmati-Brivanlou and Melton, 1997; Muñoz-Sanjuán and Brivanlou, 2002). We note, however, that gfCDM is composed of a complex mixture of salts, small molecules and proteins that might contribute to hPSC patterning in some unappreciated way.

A principal advantage of the self-patterning approach is the production of a wide variety of cell types, including OXT and AVP neurons. Furthermore, the fact that self-patterned cell aggregates morphologically resembled the developing brain (Fig. 2C-E) suggests that neurons generated from these structures might experience similar developmental histories as their *in vivo* counterparts, thereby producing similar epigenetic modifications and functional properties. However, the efficiency of hypothalamic differentiation by self-patterning was variable. The average standard deviation observed for the expression of hypothalamic transcription factors was ∼70% of the mean value for the self-patterning approach but ∼15% of the mean value for the directed differentiation approach. Similarly, neuropeptidergic hypothalamic neuron types were generated more variably by self-patterning than by directed differentiation, with standard deviations of 250% or 40% of the mean values, respectively (supplementary material Fig. S9). This variability could not be reduced despite extensive efforts to eliminate extrinsic sources of variability by using feeder-free systems, defined media, standardized reagents and by optimizing cell density, media volume and feeding schedules. We speculate that in the absence of strong instructive signals, stochastic variations might lead to slightly different developmental trajectories in each cell aggregate.

To achieve greater control over hypothalamic patterning, we developed a directed differentiation strategy. This monolayer-based protocol identifies an early role for SHH signaling in hypothalamic specification and permits reproducible and efficient hypothalamic differentiation. Progenitors appeared to adopt a largely ventral hypothalamic identity (supplementary material Fig. S7), suggesting that the protocol could be modified to more efficiently produce dorsal or anterior hypothalamic cell types. Indeed, a recent study suggests that activation the SHH pathway followed by inhibition of the NOTCH pathway enables the generation of hypothalamic neuron types typically found in the arcuate nucleus (Wang et al., 2015). Finally, the two-dimensional nature of the protocol is easily scalable, making it an attractive option for applications that require large numbers of neurons, such as drug screening or disease modeling.

The efficiency of generating rare but physiologically important neuropeptidergic hypothalamic neurons by self-patterning (0.36±

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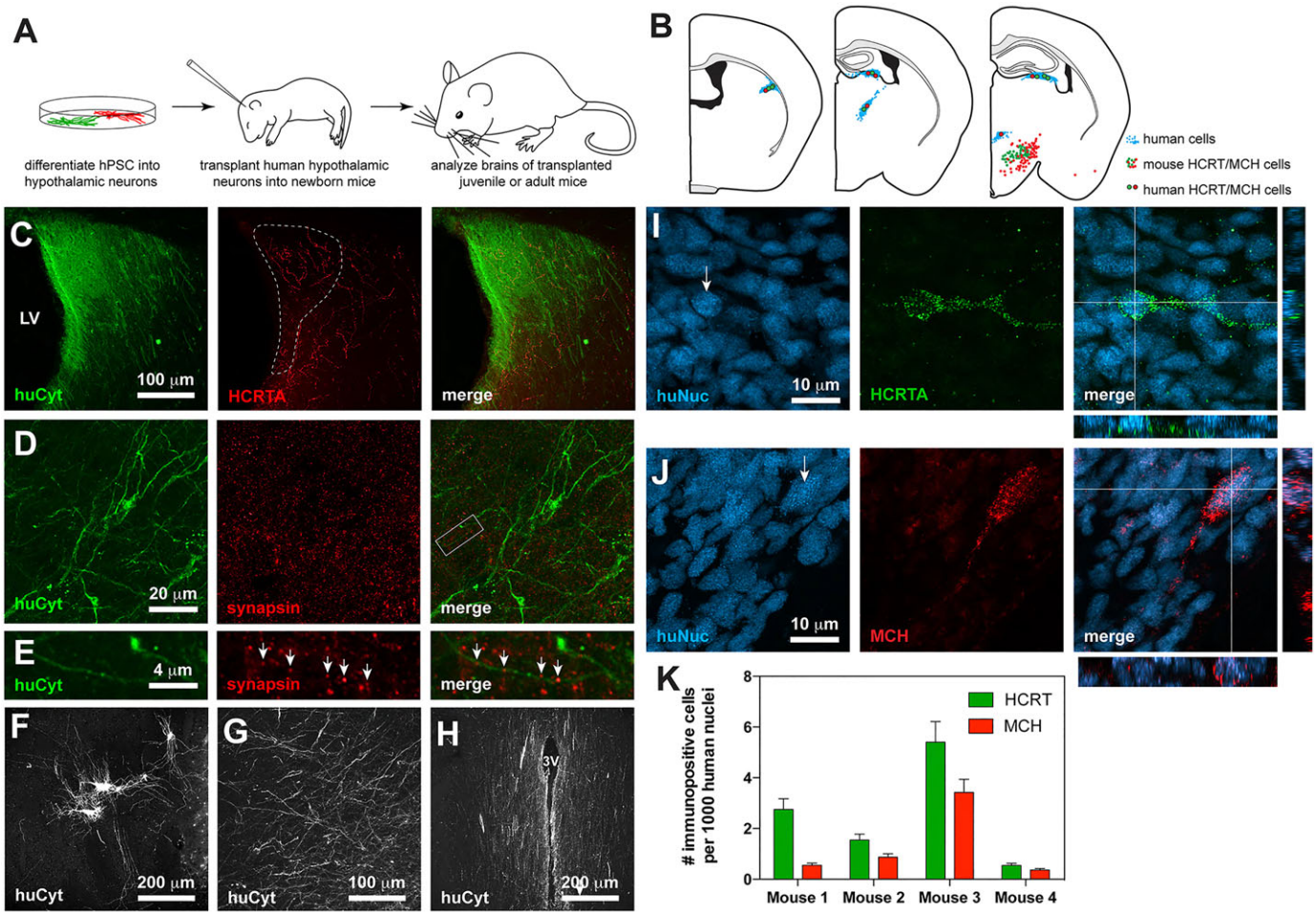

Fig. 7. Survival of hPSC-derived neurons in the mouse brain. (A) Experimental schematic. (B) Schematic of a coronally sectioned adult mouse brain hemisphere showing the distribution of transplanted human cells and endogenous mouse HCRT- or MCH-immunopositive cells. (C) Processes from endogenous mouse HCRT neurons (red) robustly innervate regions of predominantly human cells (outlined region). (D,E) The processes of transplanted human cells are found in close spatial proximity to synapsin-immunopositive puncta. (F-H) HuCyt-immunopositive cells extend elaborate processes into the mouse brain parenchyma in the striatum (F), thalamus (G) and hypothalamus (H) that persist up to 18 months post-transplantation. (I,J) Transplanted cells with huNuc-immunopositive nuclei (arrows) maintain expression of the hypothalamic-specific neuropeptides HCRTA (I) and MCH (J). (K) Quantification of human HCRT- or MCH-immunopositive cells in transplanted mouse brains as a fraction of huNuc-immunopositive cells. huNuc, human nuclear antigen (MAB1281); huCyt, human cytoplasmic antigen (SC121); LV, lateral ventricle; 3V, third ventricle.

0.79%) or by directed differentiation (0.98±0.39%), was similar to that seen in the human hypothalamus *in vivo* (0.18-0.38%). It is conceivable that differentiation efficiencies could be further improved in future studies by systematic modulation of developmentally relevant signaling pathways with small molecule drugs, co-culture with primary neuronal cultures to promote neuronal maturation or by direct programming of progenitors with candidate transcription factors. Even if these approaches fail to further increase differentiation efficiency, many types of studies could be performed if hypothalamic neuropeptidergic cell types could be prospectively identified in reporter cell lines.

Applications of hPSC-derived hypothalamic neurons

The inaccessibility of human hypothalamic neurons has been a major impediment to studying diseases linked to the hypothalamus. Below, we discuss how *in vitro*-generated human hypothalamic neurons enable new lines of investigation, including disease modeling, cell transplantation and drug screening. For clarity, we will discuss these applications using the example of HCRT neurons, the loss of which causes the debilitating sleep disorder narcolepsy (Peyron et al., 2000; Thannickal et al., 2000). The clinical relevance of other *in vitro*-derived neuropeptidergic hypothalamic neuron types is summarized in supplementary material Table S1.

The generation of human hypothalamic neurons from disease-carrying iPSCs would enable diseases of hypothalamic origin to be studied (supplementary material Table S1). For example, narcolepsy is associated with both genetic and environmental factors, but the causes of HCRT neuron loss are not well understood (Chabas et al., 2003; Kornum et al., 2010; Mignot, 1998; Winkelmann et al., 2012). Although *in vitro* systems might be insufficient to recapitulate the complex interactions that likely precipitate an autoimmune attack, they might enable cell-autonomous mechanisms of HCRT neuron loss in narcolepsy to be tested in HCRT neurons generated from the iPS cells of individuals with narcolepsy.

The transplantation of hypothalamic neurons has previously been shown to rescue defects caused by the absence or dysfunction of hypothalamic neurons in rodents (supplementary material Table S1). For example, transplanted AVP neurons restore normal fluid homeostasis after hypothalamic ablation (Gash et al., 1980; Marciano and Gash, 1986), transplanted neurons of the suprachiasmatic nucleus (SCN) can rescue circadian rhythmicity defects (Sollars et al., 1995), transplanted leptin-sensitive hypothalamic neurons can ameliorate the

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obesity phenotype of leptin receptor knockout (db/db) mice (Czupryn et al., 2011) and transplanted HCRT neurons improve the symptoms of narcolepsy (Arias-Carrión et al., 2004, 2006). If *in vitro*-derived human hypothalamic neurons can safely and effectively rescue animal models of disease, it is conceivable that one day these studies could be translated to humans, as the human hypothalamus is accessible by stereoscopic surgery (Wait et al., 2011).

Finally, *in vitro*-generated human hypothalamic neurons could be screened for drugs that stimulate or inhibit their activity, and thereby modulate neuropeptide secretion. For example, drugs that stimulate HCRT neurons to treat excessive sleepiness in individuals with narcolepsy and other sleep disorders, who together make up an estimated 20% the population (Kaplan and Harvey, 2009; Roth, 2007; Young et al., 1993). These studies would require the development of reporters for neuronal activity and screening for specificity, safety and efficacy *in vivo*. In summary, the generation of neuropeptidergic hypothalamic neurons lays the groundwork for studies that have the potential to improve human health.

MATERIALS AND METHODS

All procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Harvard University. Work with human embryonic stem cells was reviewed and approved by the Embryonic Stem Cell Research Oversight (ESCRO) committee at Harvard University.

Pluripotent stem cell culture

All cultured cells in this study were maintained at 37°C in 5% CO_2/20% O_2. Mouse embryonic stem cells were maintained under feeder-free conditions on gelatin-coated plates in medium containing KO-DMEM (Life Technologies), 10% knockout serum replacement (Life Technologies), 2 mM glutamax-I (Life Technologies), MEM-NEAA (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 55 µM β-mercaptoethanol (Life Technologies), ~2000 units/ml LIF (made in house), 0.5 µM PD0325901 (EMD Millipore) and 3 µM CHIR99021 (EMD Millipore). mESCs were passaged at 20% confluence and were used at passage 40 or lower. Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) were maintained on plates coated with hESC-qualified Matrigel (BD Biosciences) in chemically defined mTeSR-1 medium (Stemcell Technologies) and were passaged by manual picking or by enzymatic digestion with TrypLE Express (Life Technologies) in the presence of 10 µM Y27632 (Sigma). HESC lines used for most experiments were HUES48, HUES49 or HUES53 (Chen et al., 2009), and hiPSC lines were hiPS17a and hiPS18b (Boulting et al., 2011). HESCs were passaged at 80% confluence and were used at passage 40 or lower. Media for both mouse and human stem cells were changed daily.

Derivation of cortical glial cultures

Cortical glia were derived from the microdissected cortices of newborn C57BL/6 mice as previously described (Di Giorgio et al., 2008).

Self-patterning to hypothalamic neurons

Mouse ESCs were patterned to hypothalamic neurons as previously described (Wataya et al., 2008). Human PSCs were enzymatically dissociated to a single cell suspension with TrypLE Express (Life Technologies), pelleted and resuspended in mTeSR-1 medium (Stemcell Technologies) with 10 µM Y27632 (Sigma). Cells were plated into Lipidure-coated round-bottomed 96-well plates (NOF Corporation) at a concentration of 5000 hPSCs per well. The following day (D0), cell aggregates had formed at the bottom of the wells and mTeSR was removed by four washes of GMEM, followed by two washes with growth factor-free chemically defined medium (gfCDM) (Wataya et al., 2008) containing 7 µg/ml insulin and 2 µM Akt inhibitor VIII (Sigma). Cells were maintained in 150 µl of this medium per well for 30 days and fed with half-volume media changes (75 µl) every 2 days. Owing to evaporation, the wells nearest to the edge of the plate were excluded from analysis. On D30, cell aggregates were washed three times with KSR medium (Wataya et al., 2008) and gradually switched to maturation medium with half-volume medium changes every 2 days. Maturation medium consisted of Neurobasal-A (Life Technologies), 2 mM glutamax-I (Life Technologies), 1× N2 supplement (Life Technologies), 1× B27 supplement (Life Technologies), 0.075% sodium bicarbonate (Life Technologies), 200 nM ascorbic acid (Sigma), 1 µM dibutyryl cyclic AMP (dbcAMP) (Sigma) and 10 ng/ml each of GDNF, BDNF and CNTF (R&D Systems). Cell aggregates were maintained as free-floating aggregates until analysis on D90, or were transferred to monolayers of cortical glia (see above) as either whole cell aggregates or as dissociated cells. Cell aggregates were dissociated by enzymatic digestion in papain and DNAseI (Worthington) for 20 min at 37°C, followed by mechanical trituration, passage through a 40 µm cell strainer, pelleting and plating at a density of 10-100,000/cm^2 in maturation medium containing 10 µM Y27632 (Sigma).

Directed differentiation of hPSCs to hypothalamic neurons

HPSCs were dissociated to single cells and plated on matrigel in mTeSR1 containing 10 µM Y27632 at a density of 70,000 cells/cm^2. The following day (D0), mTeSR1 was replaced with KSR medium (Chambers et al., 2009) containing 10 µM SB431542 (DNSK International), 100 nM LDN193189 (DNSK International) and 2 µM XAV939 (Calbiochem). KSR medium was gradually replaced with N2 medium every other day from D4-D8, as previously described (Chambers et al., 2009). To ventralize the resulting putative prosencephalic progenitors, 1 µM purmorphamine (EMD Millipore) and 1 µM SAG (DNSK International) were added from D2 to D8. On D30, cultures enzymatically dissociated and re-plated onto a monolayer of cortical astrocytes (Di Giorgio et al., 2007) at a density of 0.5-5×10^5 cells/cm^2 in maturation medium as described above. Cells were maintained under these conditions by half-volume media changes every other day.

Quantitative RT-PCR

Total RNA was purified using the RNAeasy kit (Qiagen) after treatment with DNAse (Qiagen). RNA (500 ng) was used to synthesize cDNA with SuperScriptIII (Life Technologies). Quantitative RT-PCR was then performed using SYBR green (Promega) and the iCycler system (Bio-Rad). Quantitative levels for all genes were normalized to endogenous *GAPDH* expression. Standard curves were run using cDNA generated from human whole brain and hypothalamic RNA (Ambion) to validate primers. Primer sequences are given in supplementary material Table S3.

Histology

Juvenile and adult mouse brains were dissected from C57BL/6 (Jax) or *Il2rg*$^{-/-}$*Rag2*$^{-/-}$ (Taconic) mice that were deeply anesthetized with tribromoethanol and transcardially perfused with 0.9% saline followed by 4% PFA. Brains were postfixed overnight in 4% PFA, washed with PBS and sectioned on a vibratome (Leica) at 50 µm and collected in PBS with 0.1% sodium azide. Free-floating cell aggregates from differentiated mouse and human pluripotent stem cells were fixed for 1 hour in freshly made 4% PFA on ice, cryoprotected overnight at 4°C with 30% sucrose in PBS and embedded in OCT (Fisher). Adherent cultured cells were fixed with freshly made 4% PFA on ice for 30 min. Embedded tissue was sectioned at 20 µm on a cryostat (Leica) and collected on Superfrost Plus slides (VWR) that were then stored at −80°C. Vibratome-sectioned material was immunostained as free-floating sections that were then mounted onto Superfrost Plus slides (VWR).

Immunohistochemistry and imaging

Tissue and cultured cells were incubated in primary antibodies (supplementary material Table S2) overnight at 4°C in blocking medium (10% normal donkey serum and 0.1% Triton X-100 in PBS). After washing with PBS+0.1% Triton X-100, secondary incubation was performed in blocking medium for 2 h at room temperature. Secondary antibodies were generated in donkey or goat and were coupled to Alexa Fluor 488, 555, 594 or 647 (Life Technologies). All antibodies used to characterize human *in vitro*-derived hypothalamic neurons were previously verified to give specific staining. Omission of primary antibodies abolished staining.

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Fluorescently stained sections were counterstained with 10 µg/ml Hoechst 33342 dye (Life Technologies) and preserved with Poly Aqua/Mount (PolySciences). Epifluorescent and bright-field images were captured on an inverted Olympus epifluorescence microscope or Zeiss Axiomax epifluorescence microscope. Confocal images were taken on an Olympus FV1000MPE multiphoton laser scanning microscope. Images were processed for brightness and contrast using ImageJ and Adobe Photoshop CS (Adobe), and assembled into figures using Adobe Illustrator (Adobe).

Quantification and morphological analysis

Cell quantification and morphological analysis was performed on visual fields or images taken at 200× magnification (20× objective) on a Zeiss Axiomax microscope. The efficiency of cell type production was determined counting cells immunopositive for a gene product of interest and normalized to Hoechst-positive nuclei. For morphological analysis, the arbors of immunostained neurons were imaged and images combined as montages for tracing in Adobe Illustrator. For analysis of neurite length, the linear distance spanned by the primary neurite (tip of neurite to cell body) was measured and converted to µm.

Xenotransplantation of *in vitro*-derived human hypothalamic neurons

Cultures containing neuropeptide-immunopositive hypothalamic neurons derived from hESCs were treated with 1 µM mitomycin C, enzymatically dissociated with TrypLE Express (Life Technologies) and DNAseI (Worthington), pelleted and resuspended in maturation medium (see above) containing 10 µM Y27632 at a density of 3-4×10^5 cells/µl. Cells were kept on ice until immediately before unilateral transplantation into neonatal C57Bl/6 or immunocompromised (*Il2rg*$^{-/-}$*Rag2*$^{-/-}$) mice using a beveled pulled glass micropipette. Mice were injected with 300 nl of cell suspension delivered as six boluses of 50 nl spaced at 100 µm intervals along the needle tract. Pups were returned to their mothers immediately following transplantation with no observable deficit in survival or behavior. Mice were sacrificed for histology by transcardial perfusion.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

F.T.M., A.F.S. and K.E. conceived the project. F.T.M. performed all experiments and wrote the manuscript. A.F.S. and K.E. assisted with experimental design, data interpretation and manuscript preparation. T.W., Y.S., A.M. and L.S. provided access to protocols, gave advice on hypothalamic differentiation and provided comments on the manuscript.

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Supplementary material

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