Specification of select hypothalamic circuits and innate behaviors by the embryonic patterning gene *Dbx1*

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SUMMARY

The hypothalamus integrates information required for the production of a variety of innate behaviors such as feeding, mating, aggression and predator avoidance. Despite an extensive knowledge of hypothalamic function, how embryonic genetic programs specify circuits that regulate these behaviors remains unknown. Here, we find that in the hypothalamus the developmentally regulated homeodomain-containing transcription factor *Dbx1* is required for the generation of specific subclasses of neurons within the lateral hypothalamic area/zona incerta (LH) and the arcuate (Arc) nucleus. Consistent with this specific developmental role, *Dbx1* hypothalamic-specific conditional-knockout mice display attenuated responses to predator odor

and feeding stressors but do not display deficits in other innate behaviors such as mating or conspecific aggression. Thus, activity of a single developmentally regulated gene, *Dbx1,* is a shared requirement for the specification of hypothalamic nuclei governing a subset of innate behaviors.

INTRODUCTION

Seminal studies over the past twenty years, first in the spinal cord and hindbrain, then later in the forebrain, have revealed that neuronal subclass identity is established via the combinatorial actions of transcription factors expressed in ventricular and subventricular zone neural progenitors (Briscoe et al., 2000; Dasen and Jessell, 2009; Flames and Hobart, 2009; Hébert and Fishell, 2008; Shirasaki and Pfaff, 2002; Wonders and Anderson, 2006). Programs of migration and connectivity are then carried out as development proceeds, ultimately resulting in a fully formed and functional nervous system. While the developmental logic for specification of a variety of neuronal populations throughout the neuraxis has now been largely identified, how embryonic transcriptional programs are linked to the emergence of complex behaviors remains unknown.

Innate behaviors, defined as those that manifest without prior training, are controlled via the coordinated actions of hypothalamic nuclei. Depending on the context, different hypothalamic nuclei are activated and function to integrate biologically relevant information to achieve appropriate behavioral outputs (Gross and Canteras, 2012; Sokolowski and Corbin, 2012). For example, the arcuate (Arc) nucleus and the lateral hypothalamic area/ zona incerta (LH) are dedicated to controlling feeding through the coordinated actions of orexigenic (feeding-promoting) and anorexigenic (feeding-inhibiting) neurons (Sohn et al., 2013; Sternson et al., 2013; Yeo and Heisler, 2012; Zeltser et al., 2012). In addition, neurons in the Arc and LH have been implicated in the regulation of stress responses, such as in response to food deprivation and predator odor (Maniam and Morris, 2012; Canteras et al., 1997; Beijamini and Guimarães, 2006). These nuclei send direct projections to the paraventricular nucleus (PVN), which acts as the hypothalamic gate of the hypothalamic-pituitary-adrenal (HPA) axis (Maniam and Morris, 2012; Atasoy et al., 2012; Betley et al., 2013; Sohn et al., 2013; Sternson et al., 2013). Other hypothalamic nuclei, such as the ventral medial hypothalamus (VMH) and premammillary nucleus (PMN) perform overlapping and non-overlapping functions with the Arc and LH in not only regulating different types of responses to innate stressors such as predator odor, but also conspecific aggressive, mating and maternal care behaviors (Canteras et al., 1997; Lin et al., 2011; Silva et al., 2013; Takahashi, 2014; Yang et al., 2013). Despite an understanding of these aspects of hypothalamic control of behavior, the link between developmental mechanisms specifying neuronal identity and manifestation of innate behaviors remains unexplored.

To explore the relationship between genetic mechanisms governing neuronal specification and regulation of hypothalamic-driven innate behaviors, we focused on the developmentally regulated transcription factor *Dbx1*. *Dbx1* is widely expressed in hypothalamic progenitors (Alvarez-Bolado et al., 2012; Causeret et al. 2011; Flames et al., 2007; Hirata et al., 2009; Lu et al., 1992; Shoji et al., 1996) and is required for specification of neuronal subgroups in

the spinal cord, midbrain and hindbrain (Bouvier et al., 2010; Gray et al., 2010; Inamata and Shirasaki, 2014; Pierani et al., 2001). Employing a combination of approaches, we find that *Dbx1* functions in a highly selective manner in hypothalamic development acting at three interrelated levels. First, at the developmental level, via regulation of a select set of embryonic patterning genes, *Dbx1* function is restricted to specification of orexigenic neurons in the Arc and LH. Second, at the circuit level, *Dbx1* is required for the ability of the Arc and LH, and subsequently the HPA axis, to mount appropriate physiological responses to fasting and predator odor. Third, this specificity of *Dbx1* function in hypothalamic development and circuit function translates to select behavioral deficits in responses to innate feeding and predator-odor stressors. Thus, our data reveal that *Dbx1*-dependent transcriptional control is a common developmental mechanism for specification of functionally related neurons in two distinct hypothalamic nuclei and links embryonic patterning to the manifestation of innate behaviors.

RESULTS

Hypothalamic-specific conditional deletion of *Dbx1*

In the embryonic ventral diencephalon, *Dbx1* is expressed from approximately embryonic day (E) 9.5 to E13.5 from the rostral preoptic domain to the caudal mammillary domain (Alvarez-Bolado et al., 2012; Causeret et al., 2011; Flames et al., 2007; Hirata et al., 2009; Figure 1B.i–G.i). $Dbx1^{-/-}$ knockout mice do not survive beyond birth (Bouvier et al., 2010; Pierani et al., 2001; Gray et al., 2010), precluding assessment of the long-term consequences of *Dbx1* loss-of-function. To overcome this limitation, we generated a *Dbx1* conditional-knockout allele ($Dbx1^{flox/flox}$) (Figure 1A), which were crossed to previously generated $Nkx2.1^{Cre}$ mice (Xu et al., 2008) to generate hypothalamic-specific conditional-knockout mice. The resulting conditional-knockout progeny ($Nkx2.1^{cre};Dbx1^{c/-}$ mice, herein referred to as $Dbx1^{cKO}$ mice) were viable, and for all experimental analyses $Nkx2.1^{Cre};Dbx1^{c/+}$ mice produced from the same cross served as controls (herein referred to as Ctrl).

In the E13.5 $Dbx1^{cKO}$ forebrain, *Dbx1* expression was maintained in progenitor zones that contribute to the dorsal diencephalon (Figure 1D.i-ii), amygdala and septum (Figure S1A.i–C.ii). In the ventral diencephalon, loss of *Dbx1* expression was observed across multiple domains including the preoptic, anterior, posterior tuberal and mammillary regions (Figure 1B.i–G.ii), areas previously established to generate the postnatal POA, AH, PVN, Arc, LH and part of the VMH and PMN nuclei (Alvarez-Bolado et al., 2012; Caqueret et al., 2006; Kurrasch et al., 2007; Shimogori et al., 2010; Yee et al., 2009). Partial recombination was observed in the anterior tuberal and posterior mammillary regions. Thus, $Nkx2.1^{Cre}$-driven recombination resulted in loss of *Dbx1* function across the majority of the hypothalamic primordium and not in the telencephalon.

To assess the consequences of conditional loss of *Dbx1* function on embryonic patterning and postnatal gene expression in an unbiased manner, we performed four separate microarray screens of mRNA isolated from microdissected $Dbx1^{cKO}$ and Ctrl, male and female embryonic hypothalamic primordium and postnatal hypothalamus (Figure 1H–I). From this analysis, we identified cohorts of genes potentially misregulated in $Dbx1^{cKO}$ mice at both embryonic and postnatal stages (Table S1). We refined the candidate genes for

subsequent validation based on three criteria: 1) known or hypothesized role in neural development or hypothalamic function, 2) known expression in the developing or postnatal hypothalamus based on either the published literature or gene expression atlases (e.g., Allen Brain Atlas) and 3) significant fold-changes 1.5 in the microarray data.

Altered patterning in the embryonic *Dbx1^{cKO}* and *Dbx1^{−/−}* hypothalamus

We next performed *in situ* hybridization (*ISH*) (or immunohistochemistry when antibodies were available) on 3 *Dbx1^{cKO}* and Ctrl males and females at four different ages: E13.5, E17.5, postnatal day (P) 21 and P90 (Table S2). Four of the validated genes expressed in the Arc or LH – the orexigenic neuromodulators agouti related protein (*Agrp*), neuropeptide Y (*Npy*), hypocretin/orexin (*Hcrt*) and pro-melanin concentrating hormone (*Pmch*) – are highly implicated in stress responses, feeding and arousal (Nahon 2006; Berridge et al., 2010; Pankevich et al., 2010; Maniam and Morris, 2012; Williams and Elmquist, 2012; Domingos et al., 2013). The other validated genes included *Nkx2.4*, which encodes a developmentally regulated transcription factor expressed in the ventral diencephalon with no known function (Small et al., 2000); *Ctnnb1*, encoding a mediator of the canonical Wnt signaling pathway (Valenta et al., 2012); and *Dbx1*, the targeted gene. In addition to these markers, we assayed known markers that define hypothalamic patterning (Table S2). For embryonic analyses, we conducted our assays using both *Dbx1^{cKO}* and *Dbx1^{−/−}* embryos, which importantly provided two genotypes for validating gene expression changes assigned to *Dbx1* function.

At E13.5, we observed a significant decrease in *Npy* expression in *Dbx1* mutant embryos in the primordial Arc, which was combined with significant decrease in expression of the homeodomain-containing transcriptional regulator *Bsx* (Figure 2B–C.iv), a gene previously shown to be required for generation of the Npy+/Agrp+ population (Sakkou et al., 2007). In contrast, we observed no changes in the expression of the anorexigenic marker *Pomc* in *Dbx1^{cKO}* and *Dbx1^{−/−}* embryos (Figure 2D–D.iv). Analysis of *Dbx1^{−/−}* embryos importantly confirmed that the spared *Pomc* expression at embryonic *Dbx1^{cKO}* stages was not due to remnant *Dbx1* expression in *Dbx1^{cKO}* mice.

In the LH, we observed a dramatic decrease in *Pmch* expression in the LH (Figure 2F–F.iv). In addition, *Hcrt* expression, which along with *Pmch* marks the two major LH output populations, was also significantly decreased during embryogenesis (Figure 2G–G.iv). This corresponded with a significant decrease in expression of *Lhx9* (Figure 2H–H.iv), a LIM-homeodomain containing transcription factor required for the specification of Hcrt+ neurons (Dalal et al., 2013). In the primordial LH, using previously generated *BAT-GAL* mice as a readout of Wnt-signaling (Maretto et al., 2003), we also observed increased numbers of Wnt-responsive cells in *Dbx1* mutant embryos (Figure 2I–I.iv). This increase was combined with an expansion of *Nkx2.4* expression (Figure 2J–J.iv). Together, these data reveal that *Dbx1* is required for repression of *Nkx2.4* expression and over-production of Wnt-responsive cells in the LH. In the E15.5 LH, some of these Wnt-responsive cells express Calbindin, a marker of inhibitory interneurons (Figure S2B–F). The number of co-labeled cells, as well as the number of Calbindin+ neurons, was significantly increased in the embryonic *Dbx1^{−/−}* LH (Figure 2K–K.ii; Figure S2). This increase in Calbindin+ neurons persisted in the postnatal *Dbx1^{cKO}* LH (Figure 3I–I.ii). Thus, *Dbx1* both positively and negatively regulates

the expression of patterning genes and markers of select Arc and LH neuronal subpopulations in the hypothalamic primordium (Figure 2L).

Despite the widespread expression of *Dbx1* in a broad portion of the embryonic ventral diencephalon, patterning of other hypothalamic nuclei including the PVN, VMH and PMN were surprisingly unaffected in both *Dbx1^{cKO}* and *Dbx1$^{-/-}$* embryos (Figure S3). Thus, *Dbx1* function appears to be restricted to specification of orexigenic neurons and generation of proper numbers of Calbindin+ neurons.

Changes in expression of orexigenic neuromodulators in postnatal *Dbx1^{cKO}* mice

To determine if the select embryonic changes mentioned above resulted in alterations in neuronal populations in the postnatal Arc and LH, we assessed expression of a series of genes that mark these populations. As the hypothalamus contains many sexually dimorphic neuronal populations (Manoli et al., 2013; Simerly 2002), we quantified neuronal changes in both sexes. In the Arc, orexigenic neurons are characterized by their co-expression of *Agrp* and *Npy*, whereas anorexigenic neurons are characterized by their expression of *Pomc* and *Cart* (Broberger, 1999; Horvath et al., 1997; Ovejso et al., 2001); both populations are *Dbx1*-derived (data not shown). In both male and female postnatal *Dbx1^{cKO}* mice, we observed a significant decrease in expression of *Agrp* and *Npy* (Figure 3B–C.ii). This corresponded with a decrease in the level of Agrp detected in Agrp+ projection fields (Figure S4A.i–G). In contrast, we observed no changes in expression of *Pomc* mRNA (Figure 3D–D.ii), or Pomc or Cart protein (Figure S4H–I.ii) in the *Dbx1^{cKO}* Arc. Moreover, expression of tyrosine hydroxylase (TH), which marks dopaminergic neurons in the Arc (Chan-Palay et al., 1984), was also unchanged in *Dbx1^{cKO}* mice (Figure S4J–J.ii).

Differential expression of the neuropeptides Pmch and Hcrt define the two major neuronal output populations in the LH (Burdakov et al., 2005b). At P21 and P90, we observed a significant decrease in the expression of *Pmch* mRNA (Figure 3F–Fii) and Pmch protein (Figure S4K–K.ii) in both male and female *Dbx1^{cKO}* mice. Pmch neurons also co-express Nesfatin and Cart (Croizier et al., 2010; Elias et al., 2001; Fort et al., 2008), which were also decreased in *Dbx1^{cKO}* LH (Figure S4L–M.ii); populations which are also *Dbx1*-derived (data not shown). Surprisingly, however, the decrease in *Hcrt* and *Lhx9* observed in the embryonic LH (Figure 2G–H.iv) was not observed in the postnatal LH of *Dbx1^{cKO}* mice (Figure 3G–H.ii), indicating that postnatal expression of these genes are *Dbx1*-independent. Collectively, these data show that in the LH and Arc, *Dbx1* is selectively required for the postnatal expression of most orexigenic, but not anorexigenic or dopaminergic, neuromodulators.

To explore whether loss of *Dbx1* function affected postnatal gene expression in the PVN, VMH and PMN, we assessed expression of *Avp, Sim1, Oxt, Fezf1, Nr5a1* and *Lef1*, markers that define these nuclei (Caqueret et al., 2006; Goshu et al., 2004; Shimogori 2010). Comporting with the lack of changes in embryonic patterning of these nuclei (Figure S3), the expression of each of these markers was unchanged in postnatal *Dbx1^{cKO}* mice (Figure S5). This finding further illustrates the restricted function of *Dbx1* in specification of neuronal subsets in the Arc and LH.

Altered hypothalamic circuit function in *Dbx1^cKO* mice

Given the observed alterations in embryonic expression of *Lhx9*, *Hcrt*, *Pmch* and Calbindin (Figures 2 and S2) and in postnatal expression of Pmch, Nesfatin, Cart and Calbindin (Figures 3 and S4) in *Dbx1* mutants, we hypothesized that function of LH would be disrupted. Unique to Pmch+ and Hcrt+ neurons in the LH are their evoked action potentials in response to glucose. Pmch+ neurons are electrophysiologically silent at low physiological levels of extracellular glucose but generate action potentials at higher levels. In contrast, Hcrt+ neurons respond to low glucose but are silent at increased concentrations (Burdakov and Alexopoulos, 2005; Burdakov et al, 2005a; Burdakov et al, 2005b). To investigate the function of these two neuronal populations, we performed 60-grid multiple electrode array (MEA) extracellular field recordings of the LH in acute brain slices from postnatal *Dbx1^cKO* and Ctrl mice bathed in low- and high- glucose medium (Figure 4A–B). Compared with Ctrl brain slices, slices from *Dbx1^cKO* brains displayed ~85% lower average spike density in low-glucose aCSF (0.2mM). Similarly, when the superfusion medium was switched to high-glucose aCSF (5.0 mM), the average spike frequency in *Dbx1^cKO* mice was significantly lower (by ~50%) than in Ctrl mice (Figure 4C–H). These data demonstrate that glucose-sensitive LH neuron function is impaired in *Dbx1^cKO* mice. Despite this dysfunction, glucose tolerance, a process primarily mediated by the pancreas (Efendi et al., 1976), was unaffected in *Dbx1^cKO* mice (Figure 4I). This functional defect in the ability of the *Dbx1^cKO* LH to respond to high glucose levels is likely due to reduced Pmch+ neuron number. In addition, despite recovery of expression of *Lhx9* and *Hcrt* in postnatal *Dbx1^cKO* mice, the mutant LH also fails to properly respond to low glucose.

The Arc and the LH form a circuit with each other (Larsen et al., 1994; Peyron et al., 1998; Atasoy et al., 2012; Betley et al., 2013 and Figure S6A–E.ii) and with the PVN, which initiates corticosterone (Cort) release in response to stress via the HPA axis (Takahashi, 2014 and Figure 5A). The Arc-LH-PVN circuit regulates homeostatic responses to a variety of stressors as well as regulation of food intake (Maniam and Morris 2012; Canteras et al., 1997; Berridge et al., 2010). In addition, Agrp neurons are activated during fasting (Wagner et al., 2004; Wu et al., 2014), and the LH is activated in response to predator-induced stress (Canteras et al., 1997; Beijamini and Guimarães, 2006). Because our above findings revealed deficits in specification and function of Arc and LH neuronal subpopulations (Figures 2–4, S2, S4), we next wanted to examine if these alterations resulted in deficits in Arc, LH and PVN responsiveness to various stressors: innate predator stress (exposure to rat odor) and a food deprivation stressor (fasting). After a single exposure to the rat odor, Ctrl females, but not Ctrl males, displayed the expected increase in plasma levels of Cort (Figure 5B–C, white bars). Interestingly, *Dbx1^cKO* females had an attenuated Cort response to this innate stressor that is unchanged from the response to benign bedding. (Figure 5C, gray bars). Thus, *Dbx1^cKO* females did not display the typical physiological response to this stimulus. To determine whether neurons in the Arc-LH-PVN circuit are activated normally in the presence of both predator odor and fasting stressors, we next assessed c-Fos expression. After exposure to predator odor, female Ctrl animals displayed the expected significant increase in the number of c-Fos+ cells in the Arc, LH and PVN (Canteras et al., 1997; Beijamini and Guimarães, 2006). Strikingly, the number of c-Fos+ cells in these regions in *Dbx1^cKO* females did not increase (Figure 5D–I.i). To further probe the activation

of the Arc-LH-PVN circuit in another stress paradigm, we assessed stress-feeding responsiveness in which animals were fasted for 12 hr and then analyzed for c-Fos expression. Similar to the lack of neuronal activation in response to predator odor, *Dbx1^cKO* mice also displayed a lack of increase in numbers of c-Fos+ cells in all three regions after fasting (Figure S6F–N.i). Together, these data reveal deficits in the level of neuronal activation within the Arc, LH and PVN in *Dbx1^cKO* mice in response to two different stressors and links misspecification of subpopulations of Arc and LH neurons to circuit dysfunction.

Dbx1 is required for innate stress behavioral responses

As our above data revealed, Arc and LH neuronal populations and circuits that are essential for feeding and stress responses were altered in *Dbx1^cKO* mice. To determine the behavioral effect of these alterations, we next conducted a comprehensive battery of assays to examine the status of energy homeostasis and predator avoidance responses in *Dbx1^cKO* mice. First, we observed a subtle decrease in body weight in male *Dbx1^cKO* mice after the weaning period (P>30), a phenotype not observed in females (Figure 6A–B). This sexually dimorphic weight decrease correlated with increased activity (Figure 6C–D) rather than changes in food intake or metabolism as measured by our assays (Figure 6G and S7E–G). No changes in these measures were detected in female *Dbx1^cKO* mice (Figure 6E–F, I and S7H–J). Therefore, despite causing a similar decrease in orexigenic neurons in both sexes, loss of *Dbx1* function only affects male activity, representing a potential contributor to lower body weight. Interestingly, however, female *Dbx1^cKO* mice ate less than Ctrl after fasting or restricted-feeding paradigms (Figure 6J, L), and a decrease in body weight was detected in both males and females after restricted-feeding or high-fat diet paradigms (Figure 6M–Q). Together these findings indicate that although *Dbx1* functions to specify the same neuronal subgroups in both sexes, there is a sexually dimorphic difference in weight/activity and stress-related feeding behaviors.

To examine behavioral responses to predator-induced stress responses, we next exposed *Dbx1^cKO* and Ctrl mice to rat odor. Responses to this strong innate stressor are typically characterized by stereotyped fear behaviors that begin with an initial risk assessment (cautious approach), followed by escape responses including climbing (Apfelbach et al., 2005; Blanchard et al., 2005; Martinez et al., 2008; Silva et al., 2013). Because of the female-specific stress response (*i.e.*, Cort release) in this paradigm (Figure 5B–C), we focused our behavioral assessments on female mice. Ctrl female mice presented with rat odor displayed a significant increase in escape behaviors compared to presentation with a benign odor (Figure 7A–B). In striking contrast, *Dbx1^cKO* female mice responded equivalently to rat odor and benign stimulus; these females displayed significantly fewer and shorter escape behaviors compared to Ctrl (Figure 7A–B; Supplemental Movie). Similarly, when exposed to rat odor, *Dbx1^cKO* females did not display the normal increased duration of risk assessments (Figure 7C). Instead, *Dbx1^cKO* females displayed more casual chemoinvestigative behavior with a significantly longer duration of sniffing the rat odor compared to Ctrl females (Figure 7D). No differences were observed in the open field assay, suggesting that baseline stress was unaffected in *Dbx1^cKO* mice (Figure 7E–F). In summary,

these data show that loss of *Dbx1* strikingly correlates with a lack of appropriate behavioral responses to a strong stressor stimulus.

The hypothalamus also regulates a number of other innate behaviors such as mating, male conspecific aggression, maternal aggression, pup retrieval and territorial urine marking (Yang and Shah, 2014; Stowers et al., 2013; Sokolowski and Corbin, 2012; Dulac and Wagner, 2006). Although *Dbx1* was deleted from the VMH and PMN progenitor zones (Figures S1), nuclei that are involved in these behaviors (Gross and Canteras, 2012; Sokolowski and Corbin, 2012), we found that they were not altered (Figure S8). These data also importantly indicate that *Dbx1^{cKO}* mice do not lack the ability to sense and process specific modalities of chemosensory information. This is further supported by the ability of *Dbx1^{cKO}* mice to find hidden food in a basic olfaction test (Figure S8G, O). Furthermore, the narrow range of observed behavioral phenotypes – namely, those involved in stress responses – is consistent with the restricted function of *Dbx1* in specification of subsets of Arc and LH neurons.

DISCUSSION

A central goal in neuroscience is to understand the mechanisms by which information encoded during embryogenesis is translated into building circuitry that mediates the vast array of behaviors displayed by complex organisms. Here, we used a multidisciplinary approach combining conditional gene deletion, gene expression analyses, electrophysiology and animal behavior to demonstrate that a single developmentally regulated transcription factor, *Dbx1*, specifies a restricted subset of neurons forming hypothalamic circuits that selectively control innate physiological and behavioral responses to stress. Collectively, our results suggest a model in which *Dbx1*-dependent processes link developmental genetic control of hypothalamic development with circuit function and innate behaviors (Figure 8).

Dbx1 specifies orexigenic neurons

While the hypothalamus is less well studied than other regions of the neuraxis such as the spinal cord and cerebral cortex, a series of relatively recent genetic loss- and gain-of-function studies have identified some of the genes critical for development of diverse sets of hypothalamic neuronal subpopulations. Collectively, these studies have revealed that in a manner similar to those seen in other regions of the nervous system, both developmentally regulated extrinsic (e.g. Shh, BMPs, Wnts) and intrinsic (e.g. bHLH and homeodomain-containing transcription factors such as *Lhx2*, *Rax*, *Nkx2.1*, *Mash1* and *Otp*) factors are essential for either regional patterning of the hypothalamus or specification of neuronal subpopulations (Caqueret et al., 2006; McNay et al., 2006; Lu et al., 2013; Peng et al., 2012; Shimogori et al, 2010; Szabó et al., 2009; Wang et al., 2012; Wolf and Ryu, 2013; reviewed in Hoch et al., 2009; Kaji and Nonogaki, 2013; Lee and Blackshaw, 2014; Salvatierra et al., 2014). Here, we find that *Dbx1* is required for the specification of orexigenic Npy+/Agrp+ neurons in the Arc and Pmch+ and Hcrt+ output neurons in the LH. This selective function for *Dbx1* in the hypothalamus is reminiscent of its role in the specification of select subsets of neurons in the spinal cord, hindbrain and midbrain. In each of these areas, *Dbx1* is required for the expression of cohorts of specific effector genes, which work together in a

region-specific manner to direct the formation of either V0 spinal cord interneurons, hindbrain Pre-Bötzinger complex neurons, or midbrain commissural neurons (Bouvier et al., 2010; Gray et al., 2010; Inamata and Shirasaki, 2014; Pierani et al., 2001). Our results further suggest a molecular mechanism by which *Dbx1* may act to specify orexigenic hypothalamic neuronal subgroups in the Arc and LH via regulation of select embryonic effector genes (*i.e.*, *Bsx*, *Nkx2.4* and *Lhx9*).

The Arc complex contains molecularly and functionally distinct neuronal populations operating within the feeding and stress neural circuitry (Maniam and Morris, 2012; Atasoy et al., 2012; Betley et al., 2013; Sohn et al., 2013; Sternson et al., 2013). Previous studies have revealed that development of the Npy+/Agrp+ neurons requires the action of the homeodomain-containing gene *Bsx* (Sakkou et al., 2007). We find a reduction of *Bsx* expression in *Dbx1* mutant embryos that correlates with the reduction in *Npy/Agrp* expression. This reveals a genetic interaction between *Dbx1* and *Bsx* as part of a combinatorial transcriptional cascade specifying Arc orexigenic neurons. However, whether this is direct transcriptional control remains unknown, and will prove interesting to explore. In contrast, we find that development of the anorexigenic Pomc+ and TH+ neuronal populations is *Dbx1*-independent.

In the LH, the two major projection neuron populations are the Pmch+ and Hcrt+ neurons. With regard to the Pmch+ population, we find a dramatic reduction of Pmch, Cart and Nesfatin expression in the postnatal $Dbx1^{cKO}$ LH. Since Cart and Nesfatin are co-expressed with Pmch (Croizier et al., 2010; Elias et al., 2001; Fort et al., 2008), the most parsimonious explanation is that this population is almost completely absent in the postnatal LH. Moreover, this loss correlates with an expansion of the Calbindin+ population in the *Dbx1* mutant LH. These changes are also linked to an expansion of embryonic expression of the homeodomain-containing gene *Nkx2.4* and an increase in the number of Wnt-responsive cells that co-label with Calbindin. This suggests that *Dbx1* is required for the specification of the progenitor pool that generates the Pmch+ population possibly via repression of *Nkx2.4*. This positive and negative regulation by *Dbx1* is similar to its function in the spinal cord where it acts to positively regulate V0 genetic programs and to repress genetic programs required for the development of V1 neurons derived from the adjacent progenitor domain (Pierani et al., 2001).

Although we have yet to understand the full cohort of genes that are directly and indirectly regulated by *Dbx1*, our data show that *Dbx1* is required high in the hierarchy of genes that control development of orexigenic neuronal subpopulations (Figure 8).

Dbx1 is required for stress-feeding circuit function

Altered specification of the Agrp+/Npy+, Pmch+ and Hcrt+ neurons in the Arc and LH would be expected to lead to a dysfunctional circuit. Here we demonstrate by a number of criteria that *Dbx1* is required for proper circuit function in the postnatal hypothalamus. Unlike most other neurons, a known function of Pmch+ and Hcrt+ neurons is to respond to high and low glucose levels, respectively (Burdakov et al, 2005a; Burdakov et al, 2005b). A notable decrease in the electrophysiological response to high glucose was observed in the $Dbx1^{cKO}$ LH, demonstrating an impaired function of the Pmch+ population, most likely due

to the reduced Pmch+ population. Likewise, the postnatal *Dbx1^cKO* LH also fails to respond properly to low glucose; therefore, the Hcrt+ population remains electrophysiologically dysfunctional despite the postnatal recovery of *Hcrt* and *Lhx9* expression.

The LH, Arc and paraventricular nucleus (PVN) form a circuit that regulates normal responses to a variety of stressors. In response to stressors, including predator stress and fasting, the PVN stimulates peripheral Cort release as part of the hypothalamic-pituitary-adrenal (HPA) axis (Herman et al., 1996; Takahashi, 2014). LH neuronal activation is also part of the hypothalamic response to predator-odor stressors (Canteras et al., 1997; Beijamini and Guimarães, 2006), and the LH sends projections directly to the PVN (Larsen et al., 1994; Figure S6A–E.ii). Moreover, Pmch signaling in the LH acts as an anxiogen to facilitate the stress response, including Cort release (Borowsky et al., 2002; Smith et al., 2006). Thus in *Dbx1* mutants, misspecification and dysfunction of the Pmch+ and Hcrt+ populations, both of which comprise projection neurons, can manifest as an impairment in HPA axis activation. The PVN is also a major projection target of Npy+/Agrp+ neurons (Atasoy et al., 2012; Betley et al., 2013; and Figure S4C.i), which we show to be under *Dbx1* regulatory control. In *Dbx1^cKO* mice, the reduced Npy+/Agrp+ population produces fewer Agrp+ projections to all known targets, including LH and PVN. Moreover, previous studies have revealed links between stressors, Agrp expression and HPA axis activation (Maniam and Morris, 2012; Vieau et al., 2007). In addition to revealing a function for *Dbx1* in specification of Arc and LH populations and in producing normal responses to glucose, we demonstrate that c-Fos activation in the Arc, LH and PVN of *Dbx1^cKO* mice is impaired in response to predator odor and fasting, two well characterized stressors that engage the HPA axis. Correlating with this finding is the lack of normal Cort release in *Dbx1^cKO* mice after exposure to the predator odor stressor. Together, these data suggest that *Dbx1* is required for normal development and function of the HPA axis and provides an important link between the molecular and circuit deficits.

In addition to the PVN, two other major nuclei regulating predator-stress responses are the VMH and PMN (Gross and Canteras, 2012; Sokolowski and Corbin, 2013). Although we cannot rule out subtle defects in neuronal specification within the PVN, VMH, or PMN, gross patterning of these nuclei as assessed by multiple markers appears unaffected in *Dbx1* mutants. Most significantly, we find normal expression of *Nr5a1*, a marker of neurons that comprise the VMH innate fear circuit (Silva et al., 2013). This is consistent with the notion that the deficit in the stress-feeding circuit arises from defects in the LH and Arc. Thus, the observed decreases in Agrp+ projections to the PVN, combined with defects in LH function, provide the most direct explanation for the altered physiological responses to food deprivation and predator odor stressors (Figure 8). Despite being transiently expressed only during the progenitor stage within the hypothalamic primordium, *Dbx1* appears to set in motion a genetic program whose disruption carries significant consequences for later circuit function. However, whether this is through direct consequence of mis-specification of orexigenic neurons or by other secondary compensatory mechanisms is still unknown.

Dbx1 is required for stress-feeding behaviors

Considering the known roles of the Npy+/Agrp+, Pmch+ and Hcrt+ populations in regulating feeding behavior, we anticipated a decrease in feeding in *Dbx1^{cKO}* mutants. However, under normal feeding conditions, we observed a subtle decrease in weight only in male mutants. This decrease was not correlated to changes in food intake or metabolism, but instead was associated with hyperactivity. However, the precise cause of the decrease in weight remains unclear and may be due to a combination of activity and subtle metabolic changes undetected in our assays. Nevertheless, the relatively mild decrease in body weight observed in male *Dbx1^{cKO}* mice is generally consistent with studies uncovering differences in the severity of phenotypes between embryonic versus postnatal manipulations of feeding systems. For example, developmental ablation of Agrp+/Npy+ or Pmch+ neurons or embryonic deletion of the genes encoding *Agrp, Npy*, or *Pmch* has mild to no effect on adult feeding (Luquet et al., 2005; Shimada et al., 1998; Erickson et al., 1996a; Erickson et al., 1996b; Qian et al., 2002; Palmiter et al., 1998). In contrast, adult neuronal ablation leads to dramatic effects on feeding (Luquet et al., 2005; Whiddon and Palmiter, 2013; Gropp et al., 2005).

Interestingly, in response to restricted access to food or fasting, we observed a deficit in food consumption in female *Dbx1^{cKO}* mice. This suggests that the *Dbx1*-dependent developmental defect in neuronal specification manifests as a feeding deficit only under stress-related feeding conditions, such as limited food sources, and in a sexually dimorphic manner. The apparent critical role for *Dbx1* in specific stress pathways is most strongly supported by the striking finding of a profound deficit in predator odor avoidance in *Dbx1^{cKO}* mice. This dramatically correlates with a lack of elevated plasma levels of Cort, suggesting that the *Dbx1^{cKO}* hypothalamus is deficient in its ability to process aversive information imparted by predator odor. These select behavioral deficits are consistent with the above mentioned molecular and circuit deficits and suggests a model which links *Dbx1*-dependent deficits at molecular, circuit and behavioral levels (Figure 8).

In summary, although the precise mechanistic function of *Dbx1* in executing hypothalamic developmental programs remains to be elucidated, our findings reveal a common requirement for *Dbx1* in establishing circuits that regulate innate responses to select stressors. This appears to occur via requirement for this transcription factor in specification of hypothalamic neuronal subpopulations critical for normal HPA axis function.

EXPERIMENTAL PROCEDURES

Animals

Conditional-knockout mice (*Dbx1^{cKO}*: *Nkx2.1Cre$^{+/−}$;Dbx1$^{c/−}$*) and controls (Ctrl: *Nkx2.1Cre$^{+/−}$; Dbx1$^{c/+}$*) were obtained by crossing *Nkx2.1Cre$^{+/−}$; Dbx1$^{+/−}$* males with *Dbx1$^{flox/flox}$* females. *Dbx1* knockout mice (KO: *Dbx1$^{−/−}$*) and controls (WT/Het: *Dbx1$^{+/+}$* and *Dbx1$^{+/−}$*) were obtained by crossing male and female *Dbx1$^{+/−}$* mice. See Supplemental Experimental Procedures for more details.

Gene Expression Profiling

RNA was isolated from E13.5 hypothalamic primordium or 3–4 month old hypothalamic tissue and analyzed using Illumina® (San Diego, CA) Gene Expression BeadChip Array technology. See Supplemental Experimental Procedures for more details.

Histology

We performed *ISH* and IHC on serial coronal sections spanning the rostro-caudal extent of the hypothalamus. All comparable sections (e.g. Ctrl vs. *Dbx1^{cKO}*) were on the same slide and treated/imaged under the same conditions. See Supplemental Experimental Procedures for more details.

Multiple Electrode Array Electrophysiology

300 μM P17–20 brain slices (bregma –2.06 to –2.30 mm) were transfered to a 60 channel MEA. The chamber was maintained at 30°C under continuous perfusion (2 ml/min) of oxygenated aCSF for 1 hr prior to data acquisition. A 15-min recording of spontaneous spiking activity of each slice was acquired. The superfusion solution was then switched to a high-glucose (5.0 mM) aCSF solution, and a 1 hr recording was obtained. See Supplemental Experimental Procedures for more details.

Glucose Challenge

Tail vein blood was used to determine blood glucose levels (BG) with a glucometer (Accu-Chek Compact Plus, Roche Diagnostics, IN). BG was determined at time 0 before an ip injection of glucose (2 mg/kg) and then 15, 30, 45 and 120 min afterwards. Mice were then fasted 12 hr and BG was taken again.

Behavior Assays

All non-feeding behavior assays were video-recorded and scored independently by two investigators blind to genotype using the Scorevideo program for MatLab (Wu et al., 2009). Unless otherwise stated, all animals were group-housed by sex after weaning and then singly housed and habituated to the behavioral assay 3 days prior to experiment, which took place >1 hr after the beginning of the dark cycle. See Supplemental Experimental Procedures for more details.

ELISA

Serum from blood was collected 1 hr after first exposure to bedding (benign or rat). Samples were run in duplicate using corticosterone ELISA kits (Abcam ab108821) per manufacturer's recommendations. See Supplemental Experimental Procedures for more details.

Statistical evaluation

Quantitation of data was performed blind to relevant variables, including genotype and exposure group. Using GraphPad Prism 6 statistical software, a Repeated Measure or One-way ANOVA followed by Tukey–Kramer multiple comparison test was used for analysis of experiments involving three groups or more (Figures 5B, C, G, H, I; 6A, B; 7; S6I, J, K, L,

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M, N), and an unpaired *t*-test with Welch's correction was used for analysis of experiments involving two groups (Figures 2–4; 5G.i, H.i, I.i; 6D, F, H, J, K–P; S2–S5; S6I.i, J.i, K.i, L.i, M.i, N.i; S7–S8).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- *Dbx1* is widely expressed in the hypothalamic primordium

- *Dbx1* specifies select neuronal subpopulations in the Arc and LH hypothalamic nuclei

- Pmch-, Hcrt-, Npy- and Agrp-expressing orexigenic neurons require *Dbx1* function

- *Dbx1* regulates innate stress responses but not other innate behaviors
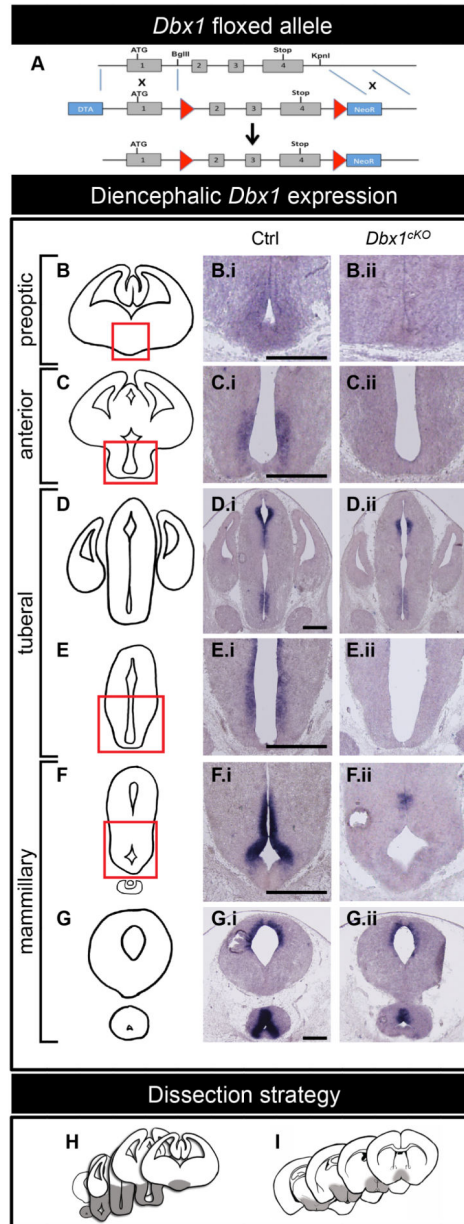
Figure 1. *Dbx1^cKO* targeting construct and embryonic *Dbx1* expression

(A) Schematic of *Dbx1* conditional targeting approach. LoxP sites flank exons 2, 3 and 4 of the *Dbx1* gene. The DNA-binding homeodomain is encoded by exons 3 and 4.

(B–G) Schematic of rostral (top) to caudal (bottom) coronal views of the embryonic forebrain. *Dbx1* expression at E13.5 is shown in Ctrl (B.i–G.i) and *Dbx1^cKO* (B.ii–G.ii) embryos in serial coronal sections. Complete loss of *Dbx1* expression is observed in the preoptic (B.ii), anterior (C.ii), posterior tuberal (E.ii) and anterior mammillary (F.ii) regions, with partial loss of expression in the anterior tuberal and sparing of the dorsal diencephalon (D.ii) and posterior mammillary regions (G.ii).

(H–I) Regions of the E13.5 embryonic (H) and P90 (I) diencephalon (shaded grey) dissected for RNA extraction and microarray analyses. The scale bar represents 500 μm.
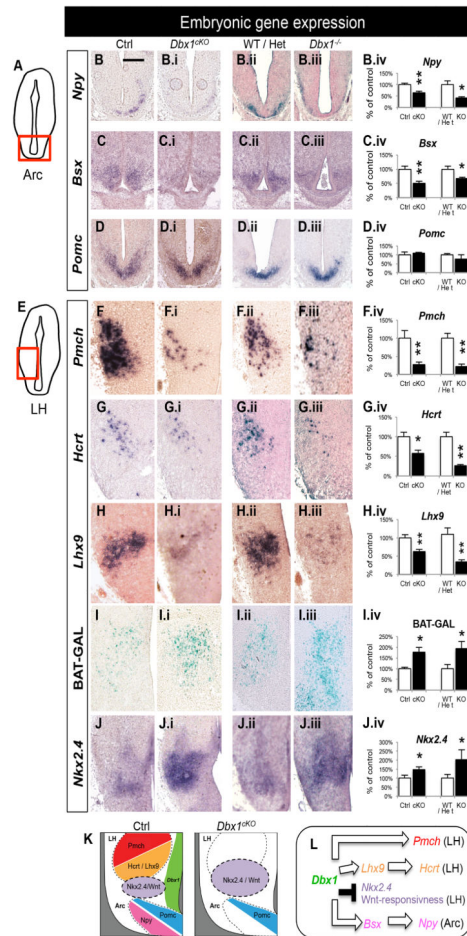
Figure 2. Embryonic gene expression changes in *Dbx1^cKO^* and *Dbx1^−/−^* mice

Schematic of a coronal view of the E13.5 brain at the level of the Arc (A) and LH (E) show the regions analyzed in (B–D.iv) and (F–K.ii), respectively.

(A–D) In the Arc, significant decreases in *Npy* (B–B.iv) and *Bsx* (C–C.iv) expression are observed in both *Dbx1^cKO^* and *Dbx1^−/−^* embryos at E13.5 and E17.5, respectively. No changes in expression of *Pomc* are observed in either *Dbx1^cKO^* or *Dbx1^−/−^* embryos at E13.5 (D–D.iv).

(E–K) In the LH, a significant decrease in *Pmch* (F–F.iv), *Hcrt* (G–G.iv) and *Lhx9* (H–H.iv) expression is observed in both *Dbx1^cKO^* and *Dbx1^−/−^* embryos at E13.5 or E17.5, respectively. Significant increases in numbers of Wnt-responding cells as revealed by LacZ staining in E17.5 *Dbx1^cKO^;BAT-GAL^+/−^* and *Dbx1^−/−^;BAT-GAL^+/−^* embryos (I–I.iv), expansion of *Nkx2.4* expression domain (D–D.iv) in *Dbx1^cKO^* and *Dbx1^−/−^* embryos at E13.5, and increases in Calbindin+ cells in the E15.5 *Dbx1^−/−^;BAT-GAL^+/−^* embryos (K–K.ii) were observed.

(L) Summary diagram of the changes in embryonic gene expression in *Dbx1* mutants.

Mean ± SEM; n = 3 – 22 per experimental group; *, p < 0.05; **, p < 0.01
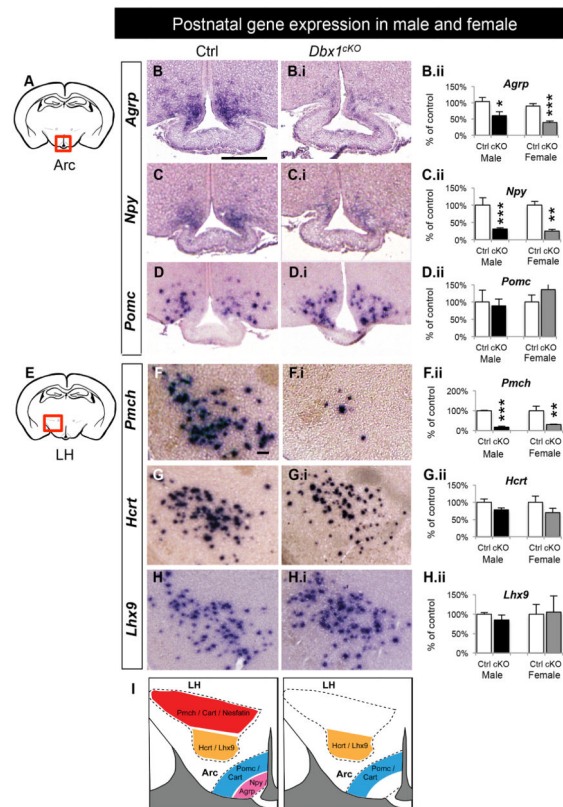
The scale bar represents 500 μm.

Figure 3. Postnatal decreases in orexigenic gene expression in *Dbx1^cKO* males and females

Schematic of a coronal view of the postnatal brain at the level of the Arc (A) and LH (E) show regions analyzed at P90 in (B–D.ii) and (F–I.ii), respectively.

(A–D) In the Arc of both male and female *Dbx1^cKO* brains, a significant decrease in expression of *Agrp* (B–B.ii) and *Npy* (C–C.ii) is observed with no change in expression of *Pomc* (D–D.ii).

(E–I) In the LH of both male and female *Dbx1^cKO* brains, a significant decrease in expression of *Pmch* (B–B.ii), with no changes in expression of *Hcrt* and *Lhx9* and increases in Calbindin+ cells (I–I.ii) is observed.

(J) Summary diagram of the changes in postnatal gene expression in *Dbx1^cKO* mice.

Mean ± SEM; n = 4 – 17 per sex, per experimental group; * $p < 0.05$; ** $p < 0.01$, *** $p < 0.0001$
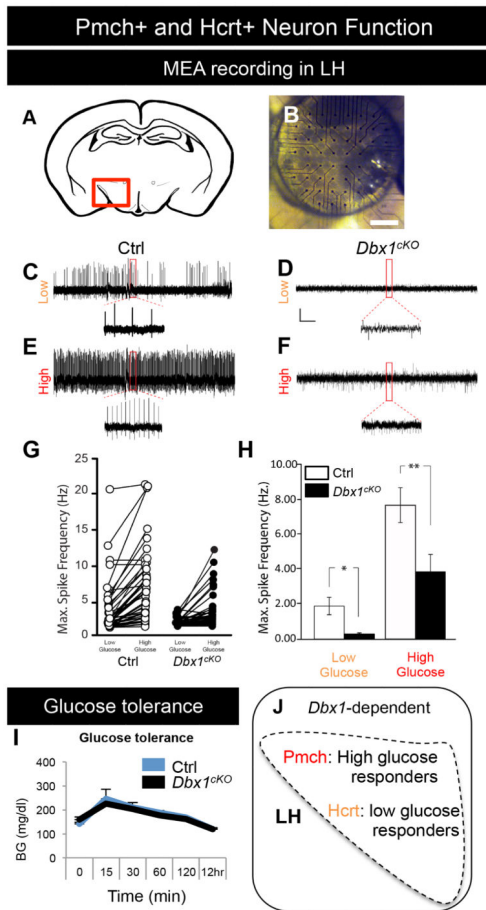
The scale bars represent 250 μm.

Figure 4. Altered function of LH neurons in postnatal *Dbx1^cKO* males

(A) Schematic indicating location of the placement of the 60-channel multiple electrode array (MEA) at the region of the LH (red square). (B) Image of the MEA on the hypothalamus of a coronal brain.

(C–F) Prototypical traces from extracellular recordings of glucose-responsive units (neurons) from the LH of Ctrls (C, E) and *Dbx1^cKO* (D, F) male mice at P17 – P20. Traces show the response of the units in low (0.2 mM; C–D) and high (5.0 mM; E–F) glucose aCSF.

(G) Maximum spike frequency of glucose responsive units obtained during a 15 min recording in 0.2 mM glucose and subsequent 15 min recording in 5.0 mM glucose.

(H) Averages of the maximum spike frequency of glucose-responsive units shown in panel G.

(I) Glucose tolerance was unaffected in adult *Dbx1^cKO* males after glucose injection (2g/kg bw) or a 12 hr fast.

(J) Diagram showing the known function of high glucose sensing (Pmch+) and low glucose sensing (Hcrt+) neurons in the LH.

Mean ± SEM; n = 3 – 5 per experimental group; *, p < 0.05; **, p < 0.01
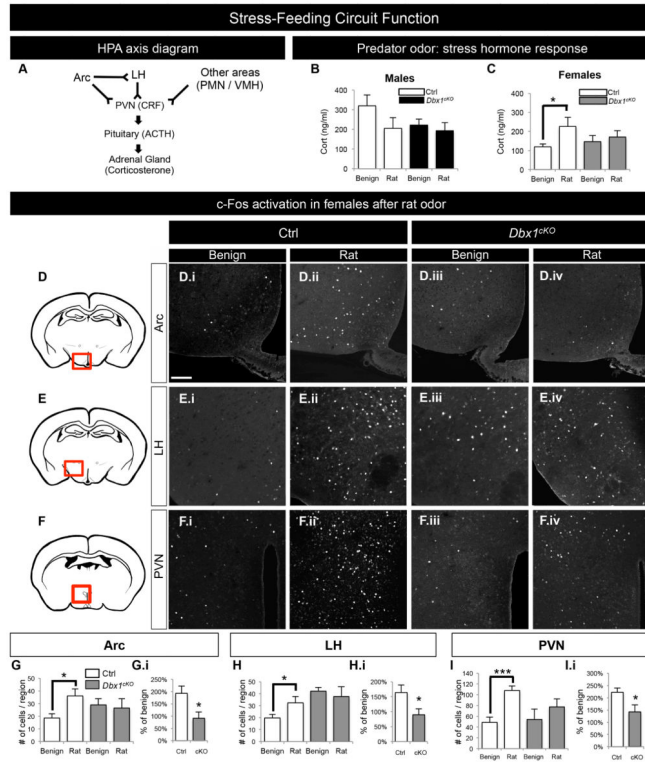
Scale bar represents 200 μm.

Figure 5. Altered function of LH and Arc circuit in postnatal *Dbx1^cKO* males and females

(A) Diagram of the hypothalamic-pituitary-adrenal (HPA) axis, with neurons in the Arc and LH projecting to the PVN forming the stress-feeding circuit. During a stressful event, the PVN stimulates the release of CRF, triggering the release of ACTH from the pituitary, which causes the release of corticosterone from the adrenal glands (Herman et al., 1996).

(B) Male plasma corticosterone (Cort) levels in Ctrl mice exposed to benign odors (clean bedding; white bars) are not significantly changed compared to Ctrl mice exposed to rat odor (bedding from a rat cage; white bars). Male *Dbx1^cKO* mice also display no changes in Cort response (black bars).

(C) Female Cort levels in Ctrl mice (white bars) are significantly increased in the presence of rat odor as compared to benign odor. In contrast, in female *Dbx1^cKO* mice (gray bars) Cort levels do not increase in the presence of rat odor compared to benign odor.

(D–F) Schematic of a coronal view of the postnatal brain at the level of the Arc (D), LH (E) and PVN (F) with a red box indicating corresponding areas of IHC images.

(D.i–F.iv) Representative images of c-Fos expression in the Arc, LH and PVN 1 hr after exposure to benign (D.i–F.i and D.iii–F.iii) or rat odors (D.ii–F.ii and D.iv–F.iv) in Ctrl (D.i–F.ii) and *Dbx1^cKO* (D.iii–F.iv) mice.

(G–I.i) Significant increases in numbers of c-Fos+ cells in Ctrl females exposed to rat odor compared to benign odor are observed in the Arc (G), LH (H) and PVN (I) (white bars), with no change in numbers of c-Fos+ cells in *Dbx1^cKO* females (gray bars). Compared to Ctrl, the fold change in c-Fos+ cells after exposure to rat odor is significantly lower in *Dbx1^cKO* female Arc (G.i), LH (H.i) and PVN (I.i).

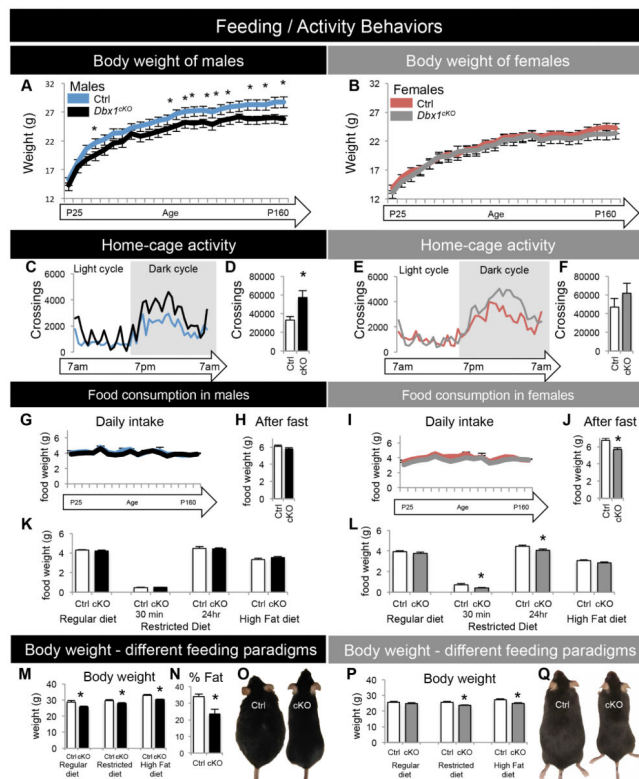Mean ± SEM; n= 3–11, *p value < 0.05, **p<0.01, ***p<0.001

Figure 6. Body weight, activity and food consumption in *Dbx1^cKO* males and females
(A–B) Body weight (in grams) of mice on a regular chow diet was assessed from P25 until
P160. A significant decrease in body weight of *Dbx1^cKO* males (A), but not females (B), is
observed at later postnatal ages.

(C–F) Home-cage activity was assessed in P30 mice over a 24 hr period in a metabolic
chamber in P30 mice given a regular chow diet. A significant increase in the number of
horizontal line crossings is observed in *Dbx1^cKO* males (D), but not females (F).

(G, I) Daily food consumption of mice on a regular chow diet was assessed from P25 until
P160, with no significant changes observed in either sex.

(H, J) After a 12 hr fast, food consumption was recorded for 24 hr. A significant decrease in
the amount of food consumed during the 24 hr re-feeding period is observed in *Dbx1^cKO*
females (J), but not males (H).

(K, L) Food consumption was recorded during different feeding paradigms (regular,
restricted, or high-fat diet) after a 24 hr (and 30 min with restricted diet) re-feeding period.
Dbx1^cKO females ate significantly less than Ctrl while on a restricted diet (L) with no
changes in food consumption while on a regular or high-fat diet. In contrast, *Dbx1^cKO* males
showed no changes in feeding under any condition (K).

(M, P) Body weight was recorded daily during different feeding paradigms (regular,
restricted, or high-fat diet). (M) Body weight in *Dbx1^cKO* males is significantly lower
compared to Ctrl males under all feeding paradigms tested. (P) Significant decreases in body
weights of *Dbx1^cKO* females were only observed after restricted or high fat feeding
paradigms.

(N) Significant decrease in body fat composition in *Dbx1^cKO^* males after 7 weeks on a high fat diet. Body weight and fat composition was matched in each subject to give a % fat for each subject (n = 5 mice per group). (O, Q) Images of Ctrl and *Dbx1^cKO^* mice after 7 weeks on a high-fat diet.

Mean ± SEM; unless stated otherwise n = 9 – 15 per experimental group; *, p < 0.05
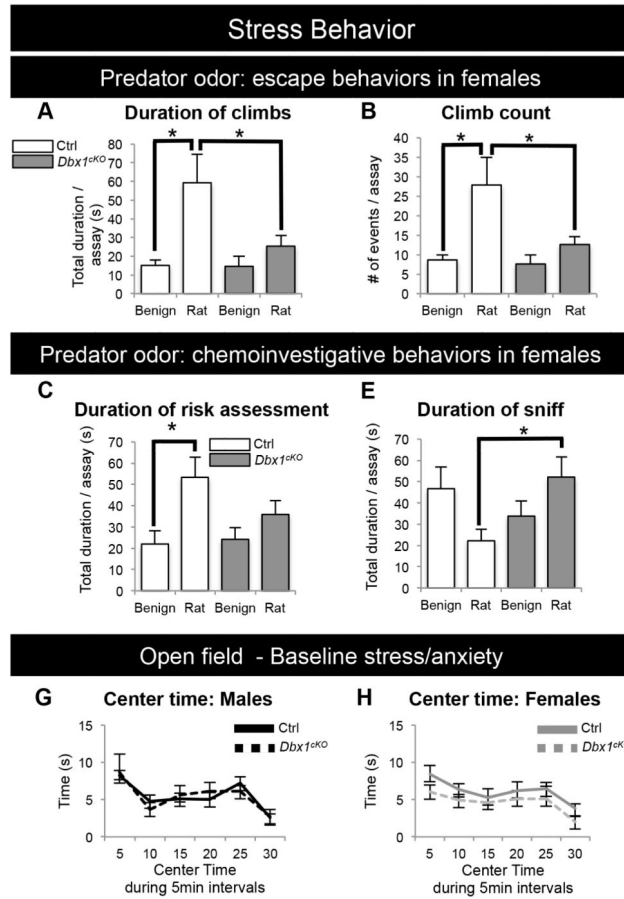
Figure 7. Altered innate stress behaviors of *Dbx1^cKO^* females

(A, B) Female Ctrl mice spend significantly more time climbing (A) and have increased number of climbs (B) during a 15 min exposure to rat odor compared to benign odor (white bars). In contrast, female *Dbx1^cKO^* mice display no significant difference in the amount of time climbing (A) or number of climbs (B) when exposed to rat odor compared to benign odors (gray bars).

(C–D) Female Ctrl mice (white bars) in the presence of rat odor compared to benign odor spend more time engaged in cautious chemoinvestigative (risk assessment) behaviors (C) and less time engaged in casual chemoinvestigation (sniffing) (D). In contrast, female *Dbx1^cKO^* mice (gray bars) in the presence of rat odor have no change in the time spent risk assessing (C) and spend significantly more time sniffing the rat odor compared to Ctrl mice (D).

(E–F) Baseline stress levels as assessed by time spent in the center of an open field are unchanged in both male and female *Dbx1^cKO^* mice compared to Ctrls.

Mean ± SEM; n = 11 – 15 per experimental group, *, p value < 0.05. See also Supplemental Movie.
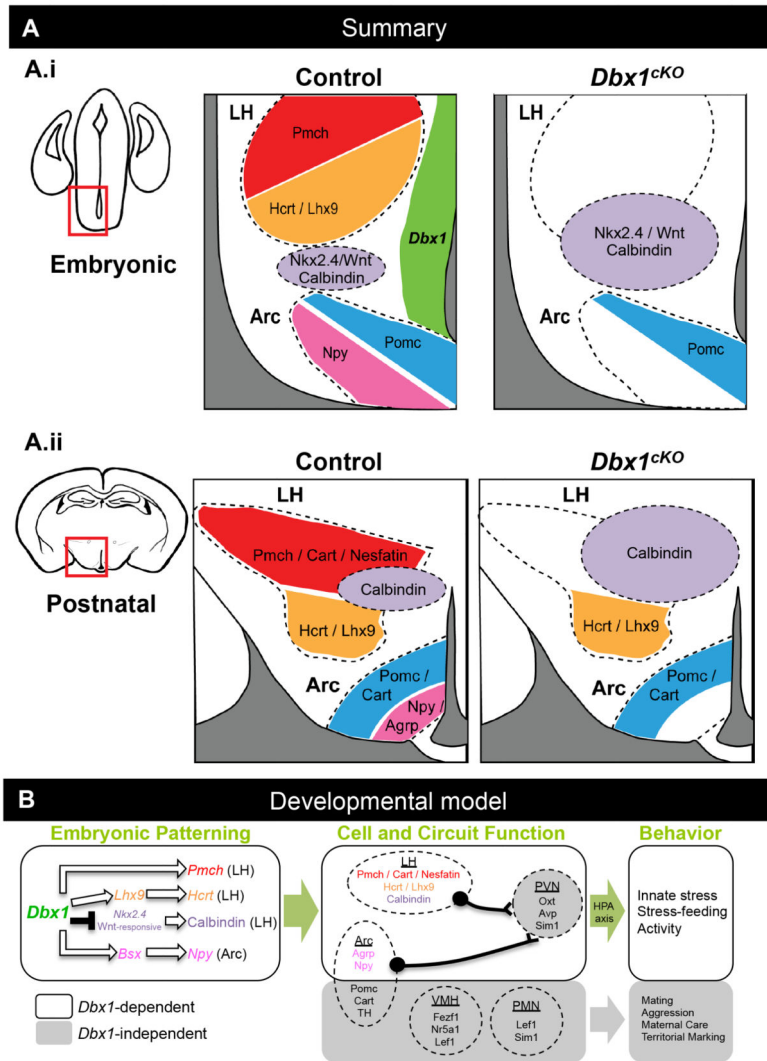
Figure 8. Summary and Model

(A) Summary of findings in *Dbx1* mutant embryonic and postnatal brains. Changes in gene expression are schematically shown at embryonic and (A.i) and postnatal stages (A.ii). *Dbx1* expression is shown in the embryonic VZ (A.i. green). (B) Proposed model of *Dbx1*-dependent and *Dbx1*-independent specification of hypothalamic neurons, nuclei and behaviors (B.i) *Dbx1* appears to function via repression of *Nkx2.4* and positive genetic interaction with *Lhx9* and *Bsx* to specify orexigenic neuropeptide (*Pmch*, *Hcrt*, *Npy*) expressing neurons. *Dbx1* also negatively regulates the number of Wnt-responsive cells, either directly or via an intermediary such as *Nkx2.4,* resulting in regulation of the appropriate number of Calbindin+ neurons. White arrows indicate putative genetic interactions, but do not detail direct or indirect mechanisms. (B.ii) Defects in the specification of LH and Arc in *Dbx1cKO* are correlated with alterations in postnatal orexigenic peptide expression, neuronal function, and stress-feeding circuit function after fasting and predator odor exposure (white areas). *Dbx1*-independent pathways (grey areas) presumably act in parallel to specify anorexigenic peptide (Pomc/Cart) expressing neurons

in the Arc, as well as other genes and innate behaviors associated with PVN, PMN and VMH hypothalamic nuclei. (B.iii) Behaviors altered in the adult $Dbx1^{cKO}$ mice are shown in white area while behaviors unaltered in adult $Dbx1^{cKO}$ mice are termed $Dbx1$-independent (grey area). Large green arrows indicate presumed link between $Dbx1$-dependent embryonic patterning, neuron and circuit function and behavior.