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Dlx5-6-dependent GABAergic programs shape hypothalamic control of energy balance and insulin sensitivity

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Abstract

GABA-neurons are essential components of hypothalamic circuits that coordinate feeding, energy expenditure, glucose metabolism, and adaptive responses to peripheral metabolic signals. The homeodomain transcription factors Dlx5 and Dlx6 are well-established regulators of GABA-neurons development, but their contribution to the organization of metabolic control circuits remains incompletely understood. Here, we investigated the metabolic consequences of conditional Dlx5-6 deletion in VGAT-expressing GABA-neurons. Mice lacking Dlx5-6 in this lineage displayed reduced body weight and lean mass, decreased locomotor activity, and lower whole-body energy expenditure, together with altered substrate utilization. Despite this reduced metabolic expenditure, mutant mice showed improved insulin sensitivity, characterized by reduced glucose-stimulated insulin secretion and enhanced glucose lowering during insulin tolerance testing. In addition, Dlx5-6 deletion impaired the behavioral response to circulating metabolic hormones: ghrelin failed to stimulate food intake, and leptin failed to suppress refeeding after fasting. These physiological alterations were associated with developmental remodeling of hypothalamic AgRP/NPY circuitry, including an increased number of AgRP-expressing neurons at P21 and increased NPY-positive projections to selected hypothalamic and thalamic targets. In adult mice, ghrelin-induced neuronal activation was redistributed toward the median eminence, suggesting altered integration of peripheral hunger signals. Together, these findings identify Dlx5-6-dependent GABAergic programs as important determinants of hypothalamic circuit organization and systemic metabolic regulation. Rather than simply controlling food intake, Dlx5-6-dependent GABAergic mechanisms appear to shape the balance between energy conservation, nutrient partitioning, hormonal responsiveness, and glucose homeostasis

Introduction

The proper function of neural circuits depends on the activity of GABA-neuron, a remarkably diverse neuronal class exhibiting a broad anatomical, biochemical, and functional heterogeneity [1]. In fact, these neurons are essential for maintaining the balance of neural networks and shaping adaptive behaviors [2]. Defect in brain control of energy homeostasis is recognized as a culprit in the obesity pandemic and, while monogenic causes of obesity exist, metabolic regulation primarily arises from complex, multifactorial networks of molecular, cellular, and physiological interactions [3]. Central neuronal circuits integrate sensory and circulating signals to produce goal-directed behaviors through neuroendocrine and metabolic responses. The central nervous system (CNS) thereby maintains energy balance by modulating feeding behavior, adaptive thermogenesis, physical activity, and basal metabolic rate. Additionally, the CNS coordinates peripheral tissue activity to ensure the conversion, storage, and utilization of ingested nutrients, a process known as nutrient partitioning that is fundamental to energy balance. Excessive energy intake and dysregulated nutrient partitioning are critical drivers of obesity-related metabolic complications [4,5].

In CNS, the hypothalamus serves as the central neuroendocrine integrator, overseeing critical physiological processes such as thermoregulation, appetite control, sleep-wake cycles, and reproductive

functions [4,6,7].

Within the hypothalamus, GABA-neurons are distributed across key regions, including the preoptic area (POA), supramammillary nucleus (SMN), arcuate nucleus (ARC), and dorsomedial hypothalamus (DMH). In the ARC, they interact with other neuronal populations, including pro-opiomelanocortin (POMC) and agouti-related peptide (AgRP) neurons, to regulate energy homeostasis and adaptive responses [5,8,9]. It is important to mention that AgRP-neurons and 30–50% of POMC-neurons are themselves GABAergic [5,10].

Several key molecular and transcriptional events govern neuronal identity specification, including the Distal-less homeobox (*Dlx*) gene family, which encodes homeodomain transcription factors (TFs) that orchestrate key developmental processes, particularly within the GABAergic lineage [11,12]. During brain development, *Dlx5* and *Dlx6* are predominantly expressed in the subventricular zone (SVZ), migrating cortical neuroblasts, the rostral migratory stream (RMS), and the olfactory bulb (OB) [13]. In the adult brain, their expression persists in cortical, striatal, and hypothalamic GABAergic populations, as demonstrated by *Dlx5*^{lacZ} reporter mice and single-cell RNA sequencing (scRNAseq) analyses [13]. In the ARC, Dlx expression is mainly restricted to GABA-neurons that do not express either POMC or NPY/AgRP and to dopaminergic tyrosine hydroxylase (TH)-positive neurons [14,15]. However, the functional role of *Dlx5-6* in the adult CNS has remained unclear due to the perinatal lethality of *Dlx5-6*-null animals [16].

In addition to its transcription factor activity, Dlx5 participates in protein complexes with partners such as MAGED1 and NECDIN. Notably, *NECDIN* expression is linked to Prader-Willi syndrome, a neurodevelopmental disorder characterized by failure to thrive in infancy, followed by hyperphagia, obesity, and mental health complications [17–19].

Recent findings have demonstrated that *Dlx5-6*-dependent regulation in GABA-neurons influences behavior and healthspan and longevity. In fact, heterozygous and homozygous mutant mice exhibit reduced anxiety-like and obsessive-compulsive-like behaviors, decreased adipose tissue, and a 33% increase in lifespan compared to controls [13]. These findings highlight a pivotal role for *Dlx5-6* in integrating neuronal and metabolic signals.

In this study, we investigated the metabolic regulation of food intake and energy balance in *Vgat*$^{\Delta Dlx5-6}$ mice, where *Dlx5* and *Dlx6* are selectively inactivated in GABA-neurons. Our results reveal that GABAergic-specific ablation of *Dlx5-6* leads to decreased metabolic rate and thermogenic response, improved glucose metabolism, together with increased insulin sensitivity and impaired response to circulating hunger- and satiety-related signals. This metabolic phenotype was associated with an increased number of AgRP-neurons at postnatal day 21 (P21) and a plasticity in arcuate neural response to peripherally injected ghrelin. Collectively, these observations demonstrate that *Dlx5-6* in GABA-neurons play a critical role in the central control of energy balance and metabolic signal integration. They further suggest that *Dlx5-6* may act as a key transcriptional regulator of AgRP-neurons development and function.

Results

Genetic deletion of Dlx5-6 in GABA-neurons alters whole-body metabolism.

We assessed the role of Dlx5 and Dlx6 in GABA-neurons in the central control of energy homeostasis by using conditional knock-out mice. As previously described [13], Dlx5-6$^{flox/flox}$ mice, in which the homeodomain-encoding regions of both Dlx5 and Dlx6 are flanked by non-compatible lox sequences were crossed with *Slc32a1*$^{tm2(cre)Lowl}$ mice [20] (referred as to *Vgat*$^{cre/+}$ mice) in which an IRES-Cre recombinase cassette is inserted downstream of the stop codon of the endogenous vesicular GABA transporter *(Slc32a1* ,Vgat):) gene. This construct results in Dlx5-6 loss specifically in GABA-neurons (referred to as to *Vgat*$^{\Delta Dlx5-6}$ mice**).**

Previous work showed that with aging, *Vgat*$^{\Delta Dlx5-6}$ mice exhibit reduced body weight and adiposity compared to control littermates [13]. To determine whether these phenotypic traits emerge during aging and are associated with alteration in whole-body metabolism and metabolic efficiency, *Vgat*$^{\Delta Dlx5-6}$ mice were longitudinally monitored from 6 to 11 months of age and assessed for body composition, feeding behavior and energy metabolism. At both 6 and 11 months, deletion of Dlx5-6 in GABA-neurons resulted in decreased body weight and lean body mass, together with a non-significant trend toward reduced fat mass (Fig. 1A, **and Suppl. Figure 1A**). Food intake was comparable between Ctrl and *Vgat*$^{\Delta Dlx5-6}$ mice at 6 months of age. However, by 11 months, *Vgat*$^{\Delta Dlx5-6}$ mice consumed less food during the dark phase than control littermate (Fig. 1B, F **and Suppl. Figure 1B, F**). Calculated fatty acid oxidation revealed a decrease in lipid oxidation during the light phase in 6- but not 11-months-old *Vgat*$^{\Delta Dlx5-6}$ mice (Fig. 1C, G, **and Suppl. Figure 1C, G)**. Moreover, locomotor activity was significantly reduced at both ages in *Vgat*$^{\Delta Dlx5-6}$ mice compared to littermates (Fig. 1D, H, **and Suppl. Figure 1D, H).** At all ages *Vgat*$^{\Delta Dlx5-6}$ mice exhibited a profound decrease of energy expenditure compared to controls during both the light and dark phases (Fig. 1E, I, **and Suppl. Figure 1. E, I)** with an interaction with the lean body mass (LBM). This interaction increased with age in *Vgat*$^{\Delta Dlx5-6}$ mice and became significantly different from the Ctrl group (Fig. 1J **and Suppl. Figure 1J)** suggesting a change in intrinsic lean mass metabolic capability of peripheral tissue in *Vgat*$^{\Delta Dlx5-6}$ mice. Consistent with these observations, the temporal profile of fatty acid oxidation (FatOx) was altered in *Vgat*$^{\Delta Dlx5-6}$ mice, displaying both a reduced amplitude and an increased lipid oxidation between 00:00 am and 12:00 Zeitgebers time (Fig. 1C **and Supp** Fig. 1C). Collectively, these results indicate that inactivation of *Dlx5* and *Dlx6* in GABA-neurons profoundly remodels whole-body energy metabolism and substrate utilization.

Genetic deletion of Dlx5-6 in GABA-neurons improves insulin sensitivity and glucose tolerance.

We next investigated the consequences of Dlx5-6 loss in GABA-neurons onto glucose tolerance and insulin sensitivity in 9-months old mice. During the oral glucose tolerance test (OGTT) *Vgat*$^{\Delta Dlx5-6}$ mice tended to have a faster glucose clearance (not significant) than control mice (Fig. 2A). This was companied by a reduction in glucose-stimulated insulin secretion (Fig. 2B), while the glucose area under

the curve (AUC, 0–30 min) remained unchanged between genotypes (Fig. 2C). Consequently, the insulinogenic index (AUC_ins/AUC_glucose) was significantly decreased in *Vgat*$^{\Delta Dlx5-6}$ mice (Fig. 2D), indicating an overall enhanced insulin sensitivity. Consistent with this phenotype, *Vgat*$^{\Delta Dlx5-6}$ mice exhibited a markedly greater reduction in plasma glucose during the insulin tolerance test (ITT) than control mice, resulting in a significantly lower glucose AUC and supporting enhanced insulin responsiveness (Fig. 2E, F).

Genetic deletion of Dlx5-6 in GABA-neurons impairs responsiveness to ghrelin and leptin.

Energy balance is regulated by peripheral hormones and nutrients that signal to the hypothalamus and brainstem. In the hypothalamus GABA-neurons play a critical role in integrating these signals [2]. We therefore assessed feeding responses to peripherally administered ghrelin and leptin in both genotypes. As expected, ghrelin promoted food intake in control mice as compared with saline injection. In contrast, this orexigenic effect was absent in *Vgat*$^{\Delta Dlx5-6}$ mice (Fig. 3A). To examine the effect of leptin on food intake, 9-months old mice were fasted overnight and subsequently refed following saline or leptin administration. While leptin reduced food intake during the early refeeding phase in control mice, this effect was attenuated in *Vgat*$^{\Delta Dlx5-6}$ mice. Accordingly, leptin-treated VgatΔDlx5/6 mice consumed significantly more food than leptin-treated control mice 1 h after refeeding (Fig. 3B), suggesting impaired leptin sensitivity following *Dlx5-6* deletion. Interestingly, fasting induced a smaller reduction in body weight in *Vgat*$^{\Delta Dlx5-6}$ mice (-6.08% +/-0.67) compared with controls (-8.04% +/- 1.41) (Fig. 3C), suggesting altered mobilization of energy stores during nutrient deprivation. Additionally, we observed a lower brown adipose tissue (BAT) temperature in *Vgat*$^{\Delta Dlx5-6}$ mice (Fig. 3D), indicative of a diminished thermogenic response. The latter result suggests a strengthen protection of body energy store in *Vgat*$^{\Delta Dlx5-6}$ mice through both decreased metabolic rate, thermogenic response and fasting-induced mobilization of adipose stores.

Dlx5-6 in GABA-neurons regulate the development and circuit organization of AgRP-neurons.

Several TFs, including homeodomain TFs from the *Dlx* family, have been identified as critical components of hypothalamic gene regulatory networks [21–24] that define neuronal lineage specification, including AgRP-neurons. We therefore assessed AgRP-neurons density in young mice (P21) using *in situ* hybridization. We observed an increased number of AgRP-positive neurons in mice lacking *Dlx5-6* in GABA-neurons (Fig. 4A). This was accompanied by an increased density of NPY-positive projections from AgRP-neurons to the paraventricular thalamus (PVT), and the paraventricular hypothalamic nucleus (PVH), two major downstream targets of AgRP-neurons (Fig. 4A), suggesting enhanced AgRP circuit innervation at this developmental stage. However, in adult mice, immunohistochemical analysis of AgRP-containing fibers did not reveal significant differences in the mean intensity in the ARC of the hypothalamus or the lateral hypothalamus between genotypes (Fig. 4B), and overall spatial organization of AgRP projections appeared preserved. Finally, in order to gain insight of ARC neuronal response to circulating signals, we used c-Fos as a measure of neuronal activation after peripheral administration of

ghrelin. c-Fos detection revealed a different distribution of neural activation between the two genotypes (Fig. 4C). The quantification did not reveal any significant differences in neuronal activation of the ARC; however, it did show a significant increase in the median eminence (ME), suggesting a plastic change in hypothalamic ghrelin-responsive cells following loss of Dlx5-6 in GABA-neurons.

Deletion of Dlx5-6 in GABA-neurons and hypothalamic TH-neurons.

Beyond their role in AgRP-neuron development, TFs from the Dlx family have been implicated in the coordinated specification of both AgRP-neurons and growth hormone-releasing hormone (GHRH)-neurons in the hypothalamus [21]. Lee and colleagues have shown that deletion of *Dlx1-2* leads to reduced GHRH-neurons and increased AgRP-neurons at least at P28. A subset of GHRH-neurons in the ARC also expresses tyrosine hydroxylase (TH), the rate-limiting enzyme of dopamine biosynthesis, and has been identified as a direct target of ghrelin signaling [25]. We therefore examined whether loss of *Dlx5-6* in GABA-neurons affects TH-positive cells in the ARC. Our analysis revealed a trend toward a reduction in TH-positive cells in $Vgat^{\Delta Dlx5-6}$ mice, accompanied by a small yet significant decrease in TH-positive cell density (cells/mm^2, Fig. 4D).

Discussion

The Dlx family of homeodomain transcription factors are key molecular actors in regulatory gene network that controls fundamental aspects of GABA-neurons development. Among this family, Dlx5 and Dlx6 transcription factors gate GABA-neurons lineage and function in the adult brain [1,26], and play an essential role in the development of parvalbumin-expressing cortical interneurons [27]. GABA-neurons development and function are critically involved in brain functions, including metabolic control. In particular, hypothalamic GABA-neurons play a pivotal role in maintaining metabolic equilibrium and energy homeostasis by acting as central mediators between catabolic and anabolic pathways. These cells govern a wide array of metabolic processes, such as appetite regulation, nutrient processing, and metabolic rate [2,20]. Among these, hunger-activated hypothalamic AgRP-neurons are involved in adapting feeding and metabolic responses to changes in nutrient availability, they coordinate both acute orexigenic responses and orchestrate peripheral organ communication and whole-body nutrient partitioning [28–30]. In addition to the production and release of AgRP, a *bone fide* antagonist of melanocortin receptor signaling, and NPY, AgRP-neurons are GABAegic in nature [31] and orchestrate a variety of functions ranging from food cue integration, feeding, metabolic and reward-related output [30], by virtue of a coordinated temporal release and action these neuroactive components [32].

In this study, we provide metabolic and behavioral characterization of mice in which two transcription factors, Dlx5 and Dlx6, critically involved in GABAergic neural identity, have been selectively deleted. Abrogation of Dlx5-6-based transcriptional activity in GABA-neurons ($Vgat^{\Delta Dlx5-6}$ mice) resulted in decreased body weight and body fat content, decreased whole-body energy expenditure, and enhanced insulin sensitivity as well as improved glucose tolerance. These features were associated with impaired

behavioral and metabolic responses to peripherally injected ghrelin and leptin, and altered neural activity in the ARC to peripherally injected ghrelin. While Dlx5-6 are expressed throughout the brain, they show expression in key brain region involved in metabolic control including the hypothalamus [33,34] and targeted impairment of Dlx5-6 was associated with a reshaping of neural types in the ARC of the hypothalamus, AgRP-neuron quantity and projection density. These results suggest a critical role for Dlx5-6 transcriptional activity in mouse GABA-neurons, leading to a change in the hypothalamic control of energy homeostasis and integration of energy-related signals. Our data are in line with previous observations that depicted decreased body weight and adiposity in mice lacking Dlx5-6 in GABA-neurons [35].

Strikingly, the overall phenotype of *Vgat*$^{\Delta Dlx5-6}$ mice recapitulated the metabolic and neural phenotype observed in mice models with *Dlx1/2* invalidation in developing Arc cell lineage[21]. In this model, invalidation through *Nkx2.1-Cre*-driving Cre expression in several cell populations in the developing ARC [36] resulted in increased AgRP-neuron populations, increased NPY and AgRP expression, together with a dwarfism phenotype, reduced whole-body energy expenditure and glycemia in *Dlx1/2*$^{flox/flox}$*::Nkx2.1-Cre* mice[21]. In this study, Lee and colleagues also depicted a key role for Orthopedia (Otp), a homeodomain TF expressed in several hypothalamic nuclei [37] in Arc cell lineage establishment and proposed a model in which the Dlx1/2-Otp axis plays a role in the equilibrium between AgRP- and GHRH-neurons in the hypothalamus. Given the similarity between *Dlx1/2*$^{flox/flox}$*::Nkx2.1-Cre* and *Vgat*$^{\Delta Dlx5-6}$ mice, it is tempting to speculate that Dlx5-6 might serve as a common hub in the hypothalamic gene regulatory network that lies at ARC-neurons specification and regional identity. Indeed, consistent with published work identifying Dlx1/2 binding sites in the intergenic region of *Dlx5* and *Dlx6*, Lee and colleagues established *Dlx1* and *Dlx2* as positive transcriptional regulator of *Dlx5-6* [38]. However, while Dlx1 and Dlx2 were found to repress the transcriptional activity of the Otp promoter, Dlx5-6 failed to do so. This suggests that Dlx5-6 act downstream of the Dlx1/2-Otp axis in AgRP-neuron lineage establishment and possibly contribute as in defining the neuropeptide specification of AgRP-neurons. This hypothesis is supported by studies using single-cell multiomics to establish the hypothalamic gene regulatory network, which identified Dlx5-6 as key TF repressors of NPY expression in hypothalamic lineage [22,23]. Importantly, loss of Dlx 1/2 was shown to increase the number of AgRP neurons at an early stage while decreasing the number of TH/GHRH-neurons in the hypothalamus [21]. In that regard, genetic loss of *Dlx5-6* contrasts with *Dlx1/2* ablation by only moderately affecting TH/GHRH cell number while affecting AgRP-neuron development. Consistent with this, the dwarfism phenotype observed by Lee and colleagues in Dlx1/2 deficient mice aligns with an alteration of GHRH-neurons [21]. Although *Vgat*$^{\Delta Dlx5-6}$ mice displayed smaller body weight [35], no dwarfism phenotype was described, supporting the notion that *Dlx5-6* deletion might have a less drastic consequences on GHRH neurons development.

A central finding of this study is that Dlx5-6 deletion in GABA-neurons does not produce a simple increase or decrease in metabolic activity, but rather a coordinated reorganization of energy balance. The decreased body weight, energy expenditure and thermoregulation, together with enhanced insulin

sensitivity observed in *Vgat*$^{\Delta Dlx5-6}$ mice, suggest an increased inhibitory tone onto melanocortin-dependent signaling and a possible energy-salvaging state in *Vgat*$^{\Delta Dlx5-6}$ mice. Mutant mice displayed reduced body weight and lean mass, a reduced locomotor activity and lower whole-body energy expenditure. These observations indicate that the reduced body mass of *Vgat*$^{\Delta Dlx5-6}$ mice cannot be explained by increased energy expenditure or hyperactivity. Instead, the phenotype suggests a shift toward a lower-expenditure, energy-conserving state. This interpretation is further supported by the reduced loss of body weight after fasting and by the lower brown adipose tissue temperature observed in mutant mice. Thus, Dlx5-6-dependent GABAergic circuits appear to influence not only feeding behavior, but also the broader physiological balance between energy storage, energy expenditure, adaptive thermogenesis, and peripheral fuel use.

Despite reduced energy expenditure, *Vgat*$^{\Delta Dlx5-6}$ mice showed improved glucose handling and increased insulin sensitivity. During oral glucose tolerance testing, mutant mice exhibited decreased glucose-stimulated insulin secretion and a lower insulinogenic index. In addition, insulin tolerance testing revealed an enhanced ability to lower glucose levels following the administration of insulin in *Vgat*$^{\Delta Dlx5-6}$ mice. These results suggest that Dlx5-6 deletion in GABA-neurons modifies the central control of peripheral glucose metabolism, possibly by altering autonomic, neuroendocrine, or behavioral outputs that regulate tissue insulin responsiveness. Importantly, the improvement in insulin sensitivity occurs in the context of reduced energy expenditure, indicating that the metabolic phenotype cannot be reduced to a classical lean, high-energy-expenditure profile. Rather, Dlx5-6-dependent GABAergic programs may influence nutrient partitioning and peripheral metabolic efficiency through mechanisms that remain to be defined. Further, AgRP-neurons exert several actions via a melanocortin-independent mechanism [29,30,39] including actions restricted to GABA release, which could account for the overall phenotype observed in *Vgat*$^{\Delta Dlx5-6}$ mice and might be affected in adult mice as a result of Dlx5-6 deletion.

The impaired responses to ghrelin and leptin further indicate that Dlx5-6 deletion alters the integration of peripheral metabolic signals. In control mice, ghrelin increased food intake, whereas this orexigenic response was absent in VgatΔDlx5-6 mice. Similarly, leptin failed to produce the expected suppression of refeeding in mutant mice after fasting. These findings suggest that Dlx5-6-dependent GABAergic circuits are required for normal behavioral responsiveness to circulating hunger and satiety cues. The simultaneous impairment of ghrelin- and leptin-dependent responses is particularly informative, because it suggests a broader disruption of hypothalamic signal integration rather than a selective defect in a single hormonal pathway. This altered responsiveness may reflect changes in the development, connectivity, or functional state of hypothalamic GABA-neurons that normally participate in the interpretation of peripheral metabolic cues.

The AgRP/NPY system represents a likely downstream component of this altered metabolic regulation. At P21, mice lacking Dlx5-6 in GABA-neurons displayed an increased number of AgRP-expressing neurons in the ARC, together with increased NPY-positive projections to selected target regions. These developmental changes are consistent with previous work that have implicated Dlx-family transcription

factors in hypothalamic lineage specification. They also suggest that Dlx5-6-dependent GABAergic programs contribute to the organization of AgRP/NPY-related circuitry. However, the relationship between Dlx5-6 and AgRP neurons is likely to be indirect. Available expression data and litterature[14,15] indicate that Dlx5-6 is not primarily expressed in AgRP neurons themselves. Therefore, rather than acting as a terminal selector of AgRP identity in a strict cell-autonomous sense, Dlx5-6 may regulate the developmental environment, local inhibitory architecture, or upstream GABAergic populations that constrain AgRP neuron number, projection pattern, and functional responsiveness.

This interpretation is also supported by data obtained in adults. In adult mutant mice, AgRP fiber intensity in the ARC and lateral hypothalamus was not significantly altered, despite the developmental increase in AgRP-positive neurons and NPY projections observed at P21. Moreover, ghrelin-induced c-Fos activation was not significantly increased in the ARC itself, instead enhanced activity was observed in the median eminence. These findings suggest that the developmental remodeling of AgRP/NPY circuitry does not translate into a simple increase in adult AgRP output. Rather, Dlx5-6 deletion may produce a more subtle reconfiguration of hypothalamic access to peripheral signals, neuroendocrine interfaces, and downstream metabolic responses. This could explain why mutant mice show altered ghrelin and leptin responsiveness without developing overt hyperphagia.

While we primarily focused on the metabolic phenotype, deletion of Dlx5-6 in GABA-neurons has been shown to trigger various consequences. *Vgat*$^{\Delta Dlx5-6}$ mice have been shown to display increased anxiety and compulsivity-like behaviors [40], and Dlx5-6 is a direct molecular relay for the action of selective serotonin reuptake inhibitor (SSRI) antidepressants (*e.g.*, fluoxetine) onto parvalbumin-positive GABA-interneurons [41].

Given the intimate link between psychiatric disease and metabolic regulation, the concept of metabo-psychiatric alteration is being pushed forward, suggesting that metabolic control and cognitive function might be part of an intermingled regulator processes in psychiatric component of obesity [42] and, reciprocally, with the susceptibility for metabolic defect associated with psychiatric disease [43,44]. These observations echoes with studies underscoring the association between brain GABA homeostasis and metabolic defects, including genetic variants in the gene GAD2 encoding glutamic acid decarboxylase (GAD65) in humans with susceptibility to morbid obesity [45,46], while associations between prefrontal GABA concentration and cognitive performance are altered in human obesity [47]. Further, these data align with the known association of Dlx5 protein complex with Prader-Willi syndrome, whose etiopathology encompasses by hyperphagia, obesity, psychiatric disease and metabolic complication [17–19].

Finally, the metabolic phenotype described in *Vgat*$^{\Delta Dlx5-6}$ mice should be considered in light of previous observations [35] reporting increased lifespan and healthspan resulting from GABAergic-specific deletion of Dlx5-6. The decreased metabolic rate observed in *Vgat*$^{\Delta Dlx5-6}$ mice could play a protective role, ensuring metabolic fitness and increased healthspan. This hypothesis is in agreement with the general concept of metabolic control of lifespan [48]. While adverse environmental events and exposure to energy

dense food are known culprits of the obesity pandemic, it is also established that manipulation of energy metabolism is an efficient leverage point for interventions that extend healthy aging through the protection of inter-organ communication.

Given the central role of AgRP-neurons in coordinating inter-organ communication, feeding behavior and systemic metabolism, our findings raise the possibility that Dlx5-6-dependent establishment and maintenance of GABAergic neuronal identity influence susceptibility to metabolic, neuropsychiatric, and age-related disorders. More broadly, Dlx5-6 may represent a molecular link underlying the bidirectional relationship between obesity and brain/neuropsychiatric dysfunction.

Limitation of the study

In this work, we observed metabolic effects after deleting Dlx5-6 in GABA-neurons, suggesting a potential link with AgRP-neuron activity, which is critical for metabolic regulation. However, Dlx5-6 is not expressed in this specific subtype of neurons. Therefore, the precise identity of the Dlx5-6-expressing GABA-neurons responsible for the metabolic effects remains unclear. Further work is needed to decipher the genetic identity of neuronal subpopulations in the ARC. In addition, our study does not cover sexual dimorphism that could arise from Dlx5-6 deletion.

Methods

Animal studies

All animal protocols were approved by the Animal Care Committee of the University of Paris Cité (APAFIS # 2018031911161453). *Slc32a1*$^{tm2(cre)Lowl}$ knock-in mice (*Vgat*$^{cre/+}$ mice) were obtained from Jackson Laboratories through Charles River, France. Conditional deletion of the DNA-binding domains of both Dlx5 and 6 in GABA-neurons was carried out by first breeding *Vgat*$^{cre/+}$ mice with *Dlx5-6*$^{flox/flox}$ mice to obtain *Vgat*$^{cre/+}$;*Dlx5-6*$^{flox/+}$ (*Vgat*$^{\Delta Dlx5-6/+}$) [13]. These mice were then bred with *Dlx5-6*$^{flox/+}$ mice to obtain *Vgat*$^{cre/+}$;*Dlx5-6*$^{flox/flox}$ (Vgat$^{\Delta Dlx5-6}$) and *Vgat*$^{cre/+}$ (Ctrl) littermates to be compared. Mice were housed at constant temperature (23 ± 2°C) and submitted to a 12/12h light/dark cycle. All mice had access to regular chow diet (Safe, Augy, France) and water *ad libitum*. Body weight was measured every week.

We confirm that all methods reported in this manuscript are in accordance with ARRIVE guidelines (https://arriveguidelines.org).

Metabolic efficiency analysis

All mice were monitored for metabolic efficiency (Labmaster, TSE Systems GmbH, Bad Homburg, Germany). After an initial period of acclimation in the calorimetry cages of at least two days, food and water intake, whole energy expenditure (EE), oxygen consumption and carbon dioxide production, respiratory quotient (RQ=VCO2/VO2, where V is volume) and locomotor activity were recorded as

previously described [49]. Additionally, fatty acid oxidation was calculated as described by Bruss and colleagues [50] and as previously reported [51]. Before and after indirect calorimetry assessment, body mass composition was analyzed using an Echo Medical system EchoMRI (Whole Body Composition Analyzers, EchoMRI, Houston, USA).

Infrared temperature measurements

Thermogenesis was visualized using a high-resolution infrared camera (FLIR E8; FLIR Systems, Portland, OR, USA). The temperature (°C) was measured at the level of the brown adipose tissue (BAT, interscapular regions), lower back and a reference region in the environment of the mouse. Infrared thermography images were analyzed using the FLIR TOOLS software.

Oral glucose tolerance test

Mice were fasted for four hours. A small tail cut incision was performed to take blood samples and measure glycaemia with a glucometer (A. Menarini Diagnostics, France). The mice received a glucose oral gavage (3 g/kg). Blood glucose levels were measured after 0, 5, 10, 15, 30, 60 and 120 minutes and blood samples were taken at 0, 15, 30, and 90 minutes after receiving the glucose oral gavage. Plasma samples were further processed for insulin (mouse ultrasensitive insulin ELISA kit, ALPCO, Salem, USA). Insulinogenic index (AUC of plasma insulin during OGTT/ AUC of glycaemia during OGTT) was calculated as previously described (Singh, 2010).

Insulin tolerance test

Mice were fasted for four hours. A small tail cut incision was performed to take blood samples and measure glycaemia with a glucometer (A. Menarini Diagnostics, France). The mice received an insulin i.p. injection (0.5 U/kg). Blood glucose levels were measured after 0, 5, 10, 15, 30, 60 and 120 minutes.

Ghrelin and Leptin sensitivity

To assess the response to ghrelin, mice were habituated to individual cages for 4 hours. The cage bedding was changed, and food was removed. Mice were intraperitoneally injected with ghrelin (500µg/kg) 30 min before placing 2 pellets of food which have been carefully weighed. The amount of food eaten was measured 2hrs and 4hrs following the injection. To test leptin sensitivity, mice are fasted overnight. The cage bedding was changed, and food was removed at 5pm until the next day at 10 am. Body weight was measured before and after the fasting period and 4 hrs after refeeding. 30 min before placing 2 pellets of food which have been carefully weighed, animals received an i.p. injection of leptin (150/µg/kg). Food was weighed again 4 hrs later.

Brain tissue Immunofluorescence

Mice were euthanized with pentobarbital (500 mg/kg, Dolethal, Vetoquinol, France) and transcardially perfused with 0.1 M sodium phosphate buffer (PBS, pH 7.5) followed by 4% paraformaldehyde in

phosphate buffer (0.1 M, pH 7.2). Brains were removed and post-fixed overnight in 4% paraformaldehyde. Afterwards, the brains were transferred to 30% sucrose in PBS for 2 days for cryoprotection. Next, 30 µm brain sections were cut in a freezing cryostat (Leica, Wetzlar, Germany) and further processed for immunofluorescence. Free-floating brain sections were incubated at 4°C overnight with the following primary antibodies: rabbit anti-cFos (1:1000; Cell Signaling Technology #2250) or rabbit anti–AgRP (1:500; Abcam, #ab254558), and mouse anti-TH (1:500 or 1:1000; Millipore, #MAB318). The next day, sections were rinsed in Tris-buffered saline (TBS, 0.25M Tris and 0.5M NaCl, pH 7.5) and incubated for 2 hours with secondary antibodies (1:1000, Thermo fisher Scientific, MA, USA) conjugated with fluorescent dyes: goat anti-chicken Alexa 488, donkey anti-rabbit Alexa 594, donkey anti-mouse Alexa 488 and donkey anti-rabbit Alexa 647. After rinsing, the sections were mounted and coverslipped with DAPI (Vectashield, Burlingade, California, USA) and examined with a confocal laser scanning microscope (Zeiss LSM 510, Oberkochen, Germany) and AxioVision 3.0 imaging software. Quantification were performed using Fiji (https://fiji.sc/)[52]

In situ hybridization and immunohistochemistry on P21 mice

P21 mice were killed by an overdose of pentobarbital (50 mg/kg i.p.). They were perfused with 4% paraformaldehyde in phosphate buffer. Brains were dissected and postfixed overnight at 4°C. Brains were cryoprotected overnight in 30% sucrose in PBS at 4°C before sectioning at 60 µm with a freezing microtome. Immunocytochemistry was performed on free-floating sections. They were incubated overnight at 4°C in rabbit polyclonal antibodies anti-NPY (1:2000, AbCam 30914 Biotechnology). After several rinses, sections were incubated with HRP-bound anti-rabbit antibodies (1:400, ThermoFisher) for 2H at room temperature before diaminobenzidine reaction. Sections were mounted on glass slides with Eukitt. *In situ* hybridization was performed on free-floating frozen sections as described previously [53]. NBT/BCIP was used as blue substrate for revelation. AgRP probe was synthetized from a 500 bp template amplified by PCR from brain cDNA. *In situ* hybridization an immunohistochemistry pictures were captured with an optical microscope and quantified with Fiji. “Cell counter” was used for in situ analysis and the integrated density of NPY labelling was quantified as described in [54].

Statistical analyses

Compiled data are reported and represented as mean ± standard error (SEM), with single data points plotted. Data were statistically analyzed with GraphPad Prism 9. Normal distribution was tested with Shapiro-Wilk test. When n was > 7 and normality test passed, data were analyzed with Student’s t test, one-way ANOVA, two-way ANOVA or repeated-measures ANOVA as applicable, and Holm-Sidak’s post-hoc tests for two-by-two comparisons. Otherwise, non-parametric Mann-Whitney test was used. All tests were two-tailed. Significance was considered as $p < 0.05$.

Declarations

DECLARATION OF INTEREST

"The authors declare no competing interests"

Author Contribution

Conceptualization: R.H, C.M, S.L, N. N-M, G.L Methodology: R.H, J.C, R.D, A.A, S.L Validation, C.M, S.L . Formal analysis: R.H, C.M, S.L. Investigation: R.H, A.A, J.C, N.M, S.Y, R.D, C.M, S.L. Resources: S.L., C.M. Data Curation: R.H, A.A, J.C, R.D, C.M, S.L. Writing - Original Draft: C.M, S.L. Writing - Review & Editing: G.G, C.M, S.L. Visualization: R.H, C.M, S.L Supervision: C.M, S.L. Project administration: S.L, C.M. Funding acquisition: S.L, C.M, G.L

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Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information

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Figures

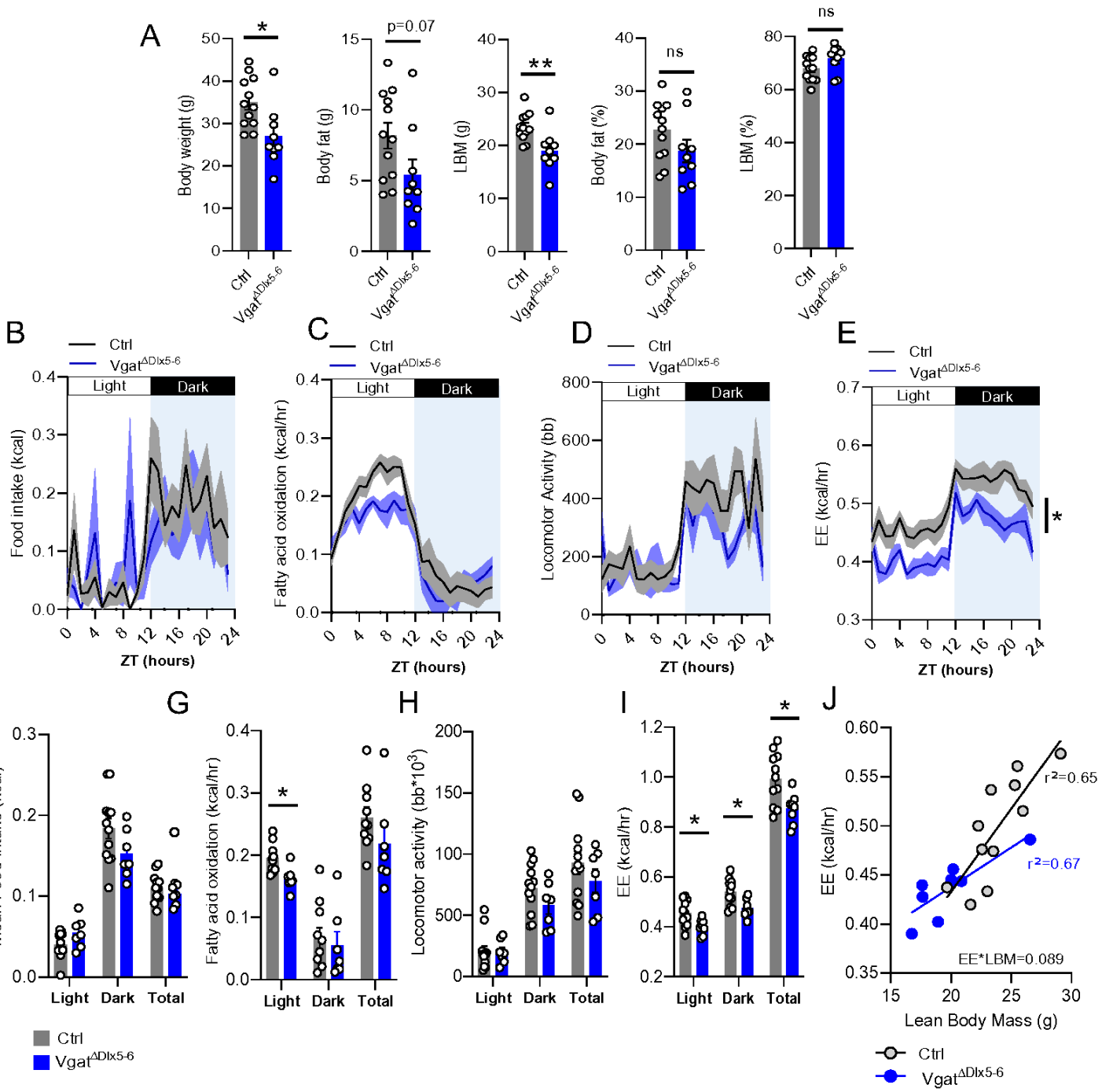


Figure 1

Deletion of *Dlx5-6* in GABA-neurons leads to metabolic changes.

(**A**) Body weight and body composition, body fat, lean body mass (LBM) in 6 months old Ctrl and (grey) and *Vgat*$^{\Delta Dlx5\text{-}6}$ (blue) mice. (**B, F**) Averaged food intake, (**C, G**) Fatty acid oxidation, (**D, H**) locomotor activity and (**E, I**) total energy expenditure, (**J**) Simple linear regression with lean body mass (LBM) as covariate, ANCOVA group effect: p=0.089. n = 8-11 mice each group. Data represent means ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.

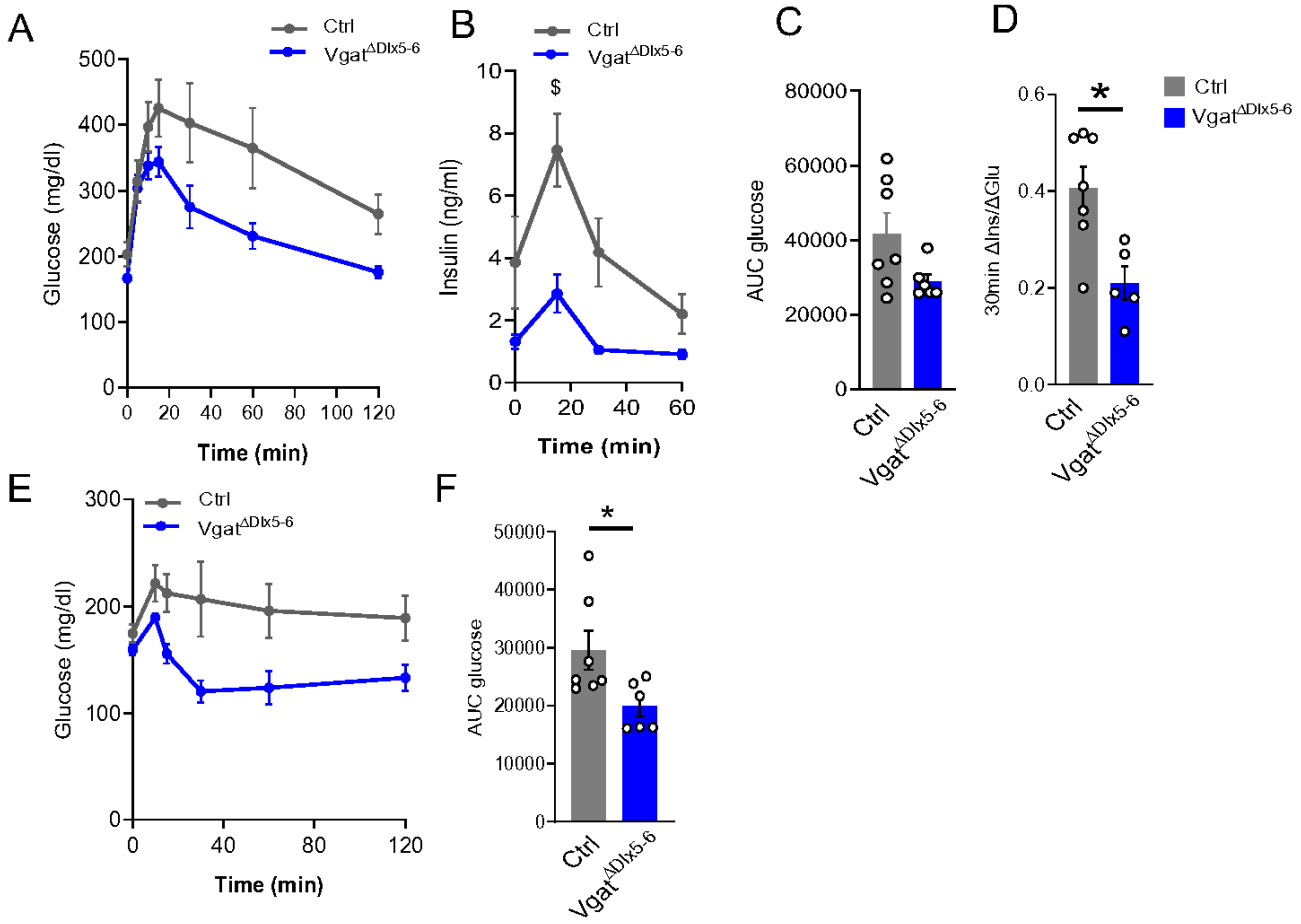


Figure 2

Deletion of *Dlx5-6* in GABA-neurons leads to improved glucose metabolism and insulin sensitivity.

(**A**) Oral glucose tolerance test (OGTT; 2way ANOVA not significant interaction p=0.1232) and (**B**) plasma insulin change during the OGTT in Ctrl and *Vgat*$^{\Delta Dlx5\text{-}6}$ (blue) mice (2way ANOVA : genotype x time interaction *p=0.0419; Bonferroni's multiple comparisons test at 15 min $ p=0.0282), (**C**) area under the

curve for glucose, (AUC; Mann Whitney test p=0.1375) (**D**) insulinogenic index during OGTT (Mann Whitney test *p=0.0101), (**E**) Blood glucose during an insulin tolerance test (0.5U/kg) (2way ANOVA not significant genotype x time interaction p=0.1463) and (**F**) AUC glucose during ITT (Mann Whitney test *p=0.035). Data represent means ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001). n = 6-7 mice each group.

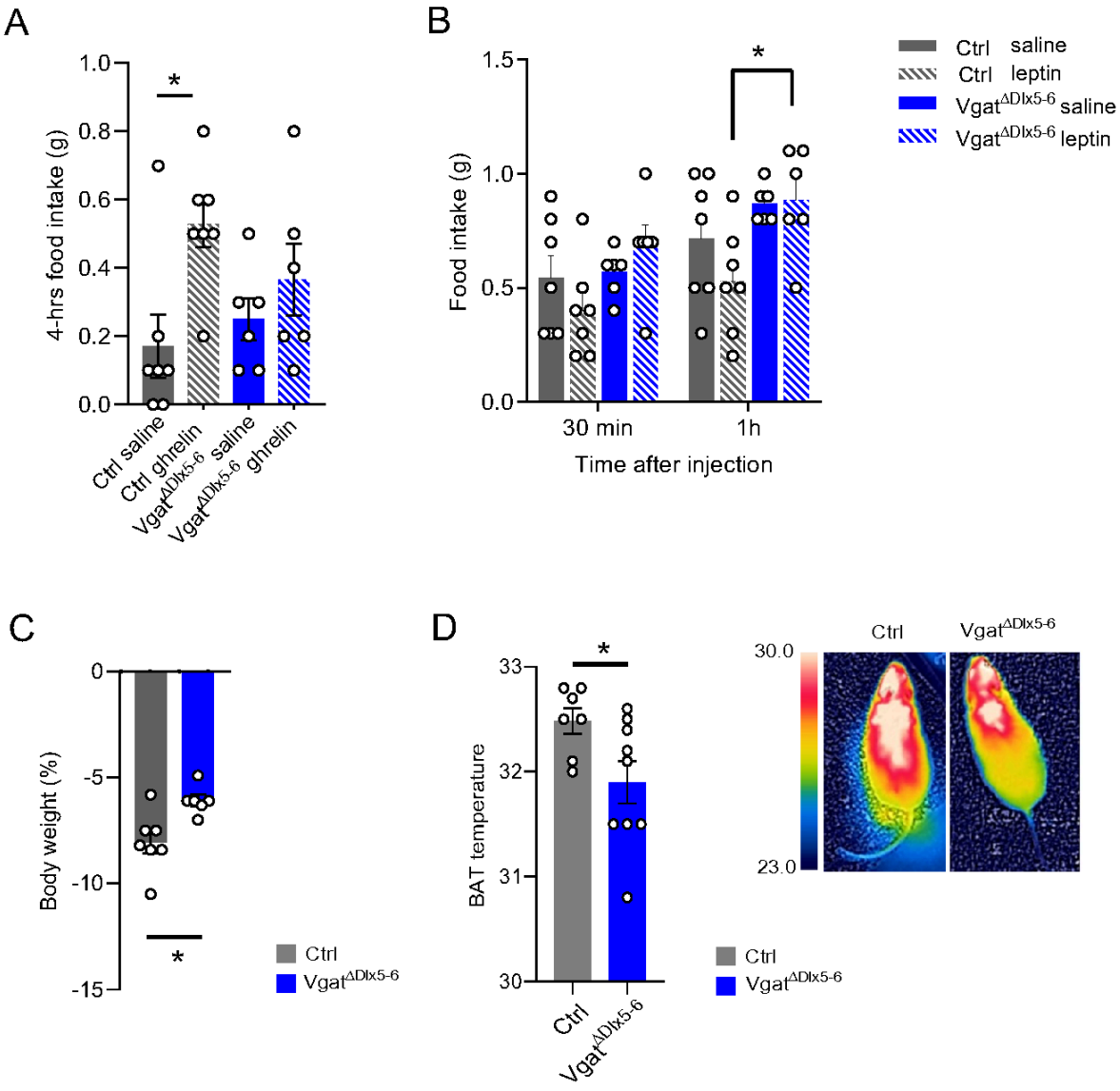


Figure 3

Deletion of *Dlx5-6* in GABA-neurons abolishes response to circulating energy-related signals.

(**A**) 4-hours food intake in response to an i.p. injection of ghrelin (500 mg/kg) in Ctrl (n=7; grey) and *Vgat*$^{\Delta Dlx5\text{-}6}$ mice (n=6; blue) Kruskal-Wallis test *p=0.0247 Dunn's multiple comparisons test Ctrl saline

vs. Ctrl ghrelin *p=0.0175. (**B**) Cumulative food intake in response to an i.p. injection of leptin (150 µg/kg) in Ctrl (n=7; grey) and *Vgat*$^{\Delta Dlx5\text{-}6}$ male mice (n=6; blue). 2way ANOVA interaction treatment x group p=0.0219 Bonferroni's multiple comparisons test Ctrl leptin *vs*. *Vgat*$^{\Delta Dlx5\text{-}6}$ leptin p=0.0309. (**C**) Body weight change (in % of initial BM) after overnight fasting (Mann Whitney test *p=0.0181). (**D**) Brown adipose tissue (BAT) temperature after an overnight fast in Ctrl (grey) and *Vgat*$^{\Delta Dlx5\text{-}6}$ (blue) mice (Mann Whitney test *p=0.0448). Representative infrared thermal image with the corresponding color bar. The region corresponding to the interscapular brown adipose tissue depots (BAT) where temperature was monitored is circled. n = 7-9 mice each group. Data represent means ± SEM. $*p < 0.05$.

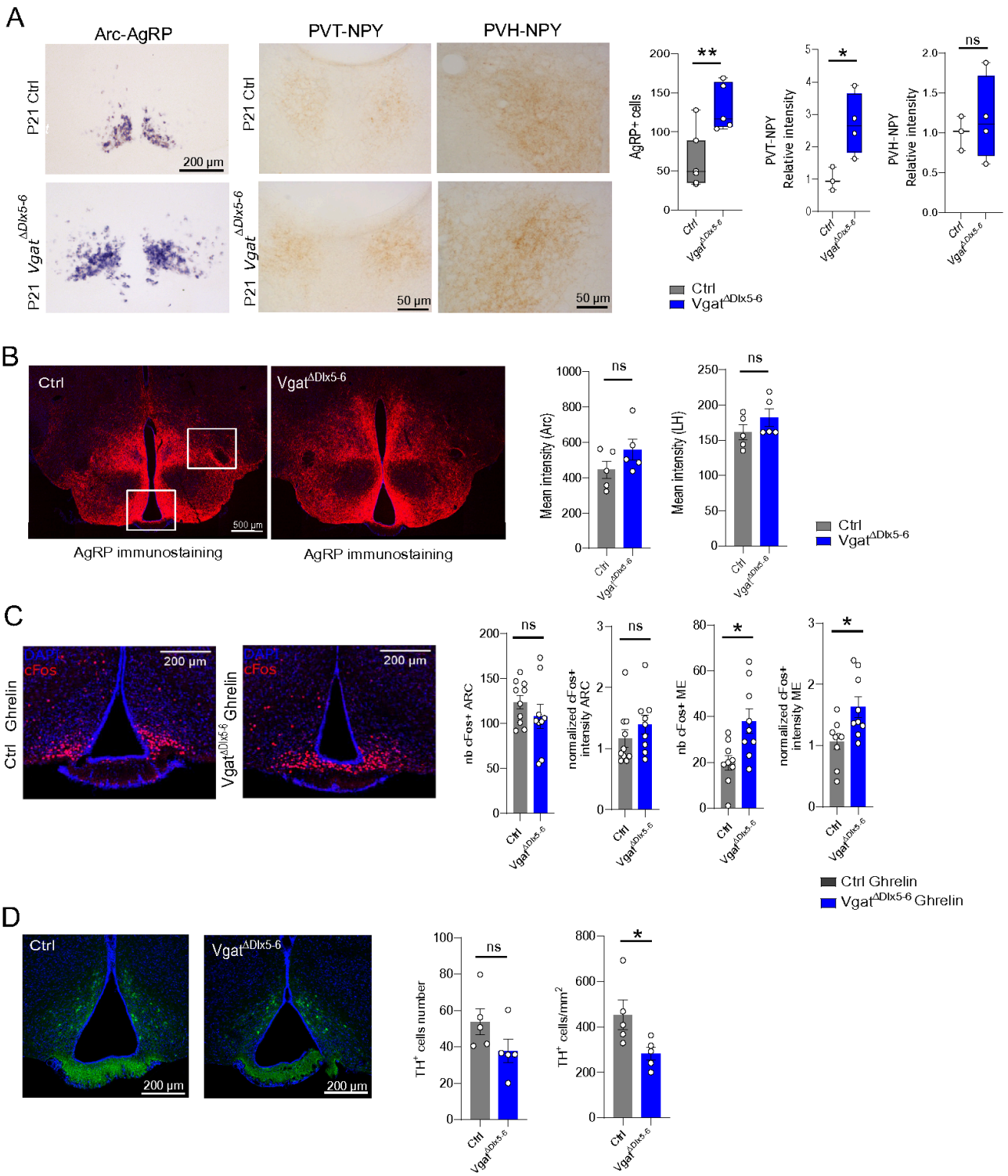


Figure 4

Deletion of *Dlx5-6* in GABA-neurons leads to modified AgRP neurons number, projections and arcuate neural response.

(**A**) *In situ* hybridization performed on P21 Ctrl (n=7) or *Vgat*$^{\Delta Dlx5\text{-}6}$ (n=5) mice revealed a significant 2.5x increase in the number of AgRP-positive cells in the arcuate nucleus in the absence of Dlx5-6. NPY

immunohistochemistry revealed an increased in the density of arcuate nucleus-NPY positive axonal projections in the PVT, but not in the PVH. ARC: Arcuate nucleus, PVH: paraventricular hypothalamus, PVT: paraventricular thalamus, *: Mann-Whitney, p<0.05. (**B**) representative photomicrograph for AgRP immunohistochemistry in Ctrl (n=5) or *Vgat*$^{\Delta Dlx5\text{-}6}$ (n=5) mice in the hypothalamic region and quantification of fiber intensity in the Arc and lateral hypothalamus. (**C**) c-fos activation 2 hours after an i.p. injection of ghrelin (500 mg/kg) in Ctrl (n=7; grey) and *Vgat*$^{\Delta Dlx5\text{-}6}$ male mice (n=6; blue), Mann Whitney test Ctrl ghrelin vs. *Vgat*$^{\Delta Dlx5\text{-}6}$ ghrelin ARC : number of cFos+ p=0.4589, normalized cFos+ intensity p=0.1564; ME :number of cFos+ *p=0.0174, normalized cFos+ intensity *p=0.0188. (**D**) Tyrosine hydroxylase (TH) staining in Ctrl (n=5; grey) and *Vgat*$^{\Delta Dlx5\text{-}6}$ mice (n=5; blue). Data represent means ± SEM. Mann Whitney test TH+ Number p =0.0952; TH+ density ∗p = 0.0159.

Supplementary Files

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