Hindbrain catecholaminergic inputs to the paraventricular thalamus scale feeding and metabolic efficiency in stress-related contexts

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25 **Abstract**

26

27 **Aim:** The regulation of food intake and energy balance relies on the integration of
28 exteroceptive and interoceptive signals monitoring nutritional, metabolic and
29 emotional states. This study aims at unraveling the role of catecholaminergic (CA)
30 inputs to the paraventricular thalamus (PVT) in scaling feeding and metabolic
31 efficiency.

32 **Methods:** To tackle this question, a region-specific retrograde disruption of PVT CA
33 inputs was performed. Behavioral tests and *in vivo* calorimetric technologies were
34 mobilized to study the adaptive strategies underlying feeding, metabolic efficiency
35 and nutrient partitioning. Moreover, immunofluorescence investigations were
36 conducted to identify the sources of TH-neurons projecting to the PVT and to reveal
37 the molecular pattern of neuronal activation (cFos) in the PVT as well as in
38 homeostasis-related regions of the hypothalamus.

39 **Results:** First, we show that PVT CA inputs mainly arise from the hindbrain, notably
40 the locus coeruleus (LC) and the nucleus tractus solitarius (NTS). Second, we reveal
41 that PVT CA inputs contribute to feeding strategies and metabolic efficiency in
42 environmental, behavioral, physiological and metabolic stress-like contexts. Third, we
43 show that PVT CA inputs contribute in modulating the activity of PVT as well as
44 lateral (LH) and dorsomedial (DMH) hypothalamic neurons.

45 **Conclusion:** This study, by assessing the key role of PVT CA inputs in scaling
46 homeostatic and allostatic regulations of feeding patterns, reveals the integrative and
47 converging hindbrain→PVT paths that contribute to whole-body metabolic
48 adaptations in stress-like contexts.

1 Introduction

50

51 In mammals, the regulation of food intake intricately relies on the orchestration of
52 several signals mirroring the dynamic integration of interoceptive and exteroceptive
53 (environment) states ¹. Indeed, emotional states (stress, anxiety, motivation), by
54 modulating the activity of central neuronal hubs, thoroughly scale the regulation of
55 food intake and the establishment of feeding habits ². This is particularly evident in
56 stress-related contexts where emotional states compete with the homeostatic
57 regulation of feeding ^{3,4}, thereby leading to feed-forward allostatic adaptations
58 (*stability through changes*) which may culminate in psychiatric and metabolic
59 disorders. These observations support the idea that emotional and homeostatic
60 states may share similar, although not identical, neuronal networks ¹. Nonetheless,
61 the systems underlying the functional connection between these states remain poorly
62 understood.

63 Emerging evidence strongly suggests that the paraventricular nucleus of the
64 thalamus (PVT), a dorsal midline thalamic relay, may represent a functional *hot-spot*
65 where interoceptive and exteroceptive stimuli may converge to orchestrate the
66 selection of adaptive and appropriate behavioral responses aimed at regulating
67 homeostatic and cognitive processes ⁵. Given their elaborated connectivity ⁶, PVT
68 excitatory (glutamate) neurons are well positioned to serve as functional integrators
69 of orexigenic and anorexigenic stimuli ^{7,8}, glucose fluctuations ^{9,10}, learning and
70 memory processes ^{11,12} as well as emotional states ^{13–15}. This plethora of PVT-
71 related brain functions highly relies on different afferent projections which, by carrying
72 distinct neurochemical information, synergistically scale the activity of PVT neurons
73 and their downstream connected regions. Among the different afferents, the PVT
74 also harbors a dense plexus of catecholaminergic (CA) fibers mainly arising from the
75 hindbrain ^{16–19} and only few scattered CA fibers from the hypothalamus ²⁰. In
76 addition, recent reports have indicated that brain CA (norepinephrine, NE, and/or
77 dopamine, DA), by modulating different homeostatic functions (*i.e.* wakefulness,
78 arousal, glucoprivation-induced food seeking), may represent key neuromodulators
79 of the PVT ^{16–18}. Moreover, brain CA are also important contributors to the regulation
80 of stress responses ^{21,22} which ultimately impact, directly and/or indirectly, on feeding
81 patterns and behaviors ^{23,24}.

82 Indeed, the PVT has already been involved in food-seeking behaviors mostly
 83 associated to positive (motivation, reward, reinforcement) or negative valance^{10,25–29}
 84 as well as in stress and emotional arousal³⁰. However, whether and how the PVT
 85 and its afferent CA inputs may contribute to the regulation of food intake and
 86 metabolic efficiency in stress-related contexts remain to be fully established.
 87 In order to dissect the contribution of PVT CA inputs in the regulation of food intake,
 88 we chronically and permanently suppressed local CA inputs by microinjecting the
 89 neurotoxin 6-OHDA into the PVT and performed several experiments aimed at
 90 revealing the adaptive strategies associated to the regulation of feeding and energy
 91 balance. Here, we demonstrate that CA inputs to the PVT exerted a modulatory role
 92 on food intake and metabolic efficiency specifically in stress-related contexts since no
 93 major alterations were detected in basal conditions. Notably, we found that 6-
 94 OHDA^{PVT}-lesioned mice, following both exteroceptive (environmental) and
 95 interoceptive (body energy) stressors, showed enhanced food intake and metabolic
 96 efficiency.
 97 Altogether, our results reveal a novel homeostatic/allostatic mechanism by which
 98 stressors impinge on the regulatory circuits underlying feeding behaviors, metabolic
 99 efficiency and nutrient partitioning to scale stress-associated adaptive responses.

2 Results

2.1 Catecholaminergic inputs modulate the activity of PVT neurons

To study whether catecholaminergic (TH-positive) inputs to the PVT participate/contribute to the regulation of food intake, we decided to ablate catecholamine (CA) fibers projecting to the PVT by locally microinjecting 6-OHDA (**Figure 1A**), a neurotoxin reuptaken by DAT- and/or NET-expressing terminals. Since the PVT extends throughout the midline thalamic axis, we decided to mainly focus on the mid-posterior PVT as this region has been shown to be involved in stress and stress-induced hypophagia^{13,14,31}. Indeed, 6-OHDA was able to strongly reduce TH immunostaining in the PVT (**Figure 1B**), with few remaining terminals most likely corresponding to DAT/NET-negative TH fibers (terminals releasing adrenaline and/or devoid of monoamine transporters).

We therefore examined the regional sources of our 6-OHDA-induced degeneration of TH afferents. We focused on noradrenergic TH-positive projecting neurons since the PVT do not receive dopaminergic inputs from the midbrain (SNc and VTA)²⁰ and it harbors only minor scattered DAT-fibers³². Immunofluorescence analysis revealed a significant reduction of TH-expressing neurons in the nucleus tractus solitarius (NTS) and the locus coeruleus (LC), in line with the presence of *Slc6a2* (NET)-catecholaminergic neurons in these nuclei^{19,33} and the sensitivity of these neurons to 6-OHDA^{34,35}. However, we observed a more robust reduction of PVT-projecting TH-neurons in the NTS (-34.2%) compared to the LC (-15.1%) (**Figure 1C, D**). No differences were observed in the A1 area of the hindbrain (**Figure 1C, D**) as well as in the hypothalamus (**Suppl. Figure 1**).

Then we investigated whether the reduction in local TH afferents was followed by the modulation of PVT neurons activity. Using cFos as a molecular proxy of neuronal activity, we observed a significant increase in cFos-positive neurons in the PVT of 6-OHDA^{PVT} compared to Sham^{PVT} mice (**Figure 1E, F**). This set of results suggests that CA inputs may serve as modulators of PVT neurons activity.

2.2 Catecholaminergic inputs to the PVT contribute to novelty-induced hypophagia

Following 3-4 weeks from 6-OHDA microinjections, no major differences in body weight were detected in 6-OHDA^{PVT} compared to Sham^{PVT} mice (group-housed

animals, **Figure 2A**). In order to study the role of PVT catecholaminergic inputs in the regulation of feeding patterns, 6-OHDA^{PVT} and Sham^{PVT} mice were single-housed (**Figure 2B**). Environmental and social isolation, as triggered by single housing, represent behavioral/environmental stress-like allostatic stimuli^{36,37} which can trigger a transient reduction of food intake^{38,39}. Interestingly, we observed that 6-OHDA^{PVT} mice were less sensitive to single housing-induced hypophagia compared to Sham^{PVT} mice (**Figure 2C**). This phenotype prompted us to investigate whether catecholaminergic inputs to the PVT were important in driving feeding patterns and metabolic adaptations to exposure to other behavioral/environmental challenges. After 2 weeks of single-housing habituation, we analyzed metabolic efficiency in 6-OHDA^{PVT} and Sham^{PVT} mice exposed to a novel environment during two consecutive days (**Figure 2D**). While Sham^{PVT} mice transiently (Day 1) showed a reduction in food intake during the dark period (**Figure 2E**), 6-OHDA^{PVT} mice were again less sensitive to novelty-induced hypophagia. No differences in food intake were measured during Day 2 between the two groups, indicating a rapid restoration of homeostatic regulations associated to environmental habitation (**Figure 2E, E¹**). This phenotype was not associated to changes in locomotor activity as indicated by the similar patterns of exploration (Day 1, novelty) and habituation (Day 2) (**Figure 2F, F¹**). We also measured key whole-body metabolic parameters such as the respiratory exchange ratio (RER, indicative of the energy substrates used, RER≈1 for carbohydrates, RER≈0.7 for lipids), fatty acid oxidation (FAO) and energy expenditure (EE) during the exposure to the novel environment (Day 1). Compared to Sham^{PVT} mice, 6-OHDA^{PVT} animals showed increased RER (**Figure 2G, G¹**) and decreased FAO (**Figure 2H, H¹**), mirroring the changes in food intake and indicating a shift of energy substrates (carbohydrates and lipids) use during exposure to a novel environment. However, these adaptations did not impact on energy expenditure (**Figure 2I, I¹**), suggesting that nutrient partitioning⁴⁰, rather than energy balance, was affected by the loss of hindbrainTH→PVT fibers. One may wonder whether the absence of novelty-induced hypophagia in 6-OHDA^{PVT} mice may be associated to enhanced perception and/or reward value of food. To investigate this aspect, Sham^{PVT} and 6-OHDA^{PVT} mice were intermittently (1h/day) exposed to high-fat high-sugar (HFHS) diet during two consecutive days. No differences in HFHS food intake were observed between groups (**Suppl. Figure 2**), suggesting intact food palatability, perception and preference. These results suggest

that hindbrainTH→PVT fibers represent an important node for the integration of homeostatic regulations.

Exposure to novel environments can lead to the occurrence of anxiogenic traits and novelty-triggered thermogenic adaptations⁴¹ which may impact on and therefore confound the mechanisms underlying feeding strategies. Thus, we performed an open field test (OF, **Figure 3A**) to evaluate both anxiety and thermogenic adaptations. We observed no significant differences in anxiety-like parameters between Sham^{PVT} and 6-OHDA^{PVT} animals (**Figure 3B-F**). Moreover, both groups showed similar thermogenic enhancements in the brown adipose tissue (BAT), the lower back and the tail (**Figure 3G-J**). These results indicate that PVT-projecting TH-afferents modulate feeding patterns and metabolic efficiency independently from affective (anxiety) and thermogenic adaptations.

Feeding patterns and metabolic efficiency highly depend on circadian rhythms and strong functional interactions exist between circadian rhythms, feeding and energy balance⁴². Moreover, the PVT, which is bidirectionally connected with the suprachiasmatic nucleus (SCN)^{43,44}, has been pointed as a contributor to circadian cycles and its activity varies depending on active/inactive phases^{6,43}. Thus, we decided to investigate whether PVT catecholaminergic inputs participated to the adaptive metabolic strategies occurring during circadian challenges by inverting the light/dark cycle (**Figure 4A**). First, before inverting the light/dark cycle, we confirmed (**Figure 2**) that exposure to the new environment was associated to reduced novelty-induced hypophagia in 6-OHDA^{PVT} compared to Sham^{PVT} mice (**Suppl. Figure 3A**). Then, after habituation, light/dark cycles were inverted. When measuring food intake, we observed that both experimental groups rapidly shifted and adapted toward the new light/dark schedule (average of first 2 days of standard and inverted cycles) (**Figure 4B, B¹**). In fact, despite the adaptive shift (**Figure 4B, B¹**), no differences were observed in the cumulative food intake (**Figure 4C**). Moreover, we also detected a similar pattern of circadian adaptation in the EE profile of both groups (**Figure 4D, D¹**), with an increased EE in the 7h-19h inverted period (iDark) and a decreased EE in the 19h-7h inverted period (iLight) (**Figure 4E, E¹**). Interestingly, we found a significant difference in FAO and RER. In particular, while Sham^{PVT} animals rapidly adapted to the inverted light/dark cycle (**Figure 4F**), 6-OHDA^{PVT} mice did not show significant changes in FAO during the 7h-19h inverted period (iDark) (**Figure 4F¹, G**), whereas both groups showed increased FAO during the 19h-7h inverted

period (iLight) (**Figure 4G¹**). In line with this shift in energy substrates partitioning, measurements of RER indicated an impaired adjustment of metabolic efficiency during the iDark period (7h-19h) in 6-OHDA^{PVT} mice (**Figure 4H, H¹, I, I¹**). These results suggest that hindbrain CA inputs to the PVT, although not involved in the adaptation of food intake to circadian challenges, are important in allowing the adjustments of metabolic efficiency and the partitioning of energy substrates.

2.3 Catecholaminergic inputs to the PVT contribute to feeding under physiological and metabolic challenges

Next, we wondered whether CA inputs to the PVT guided feeding following physiological and metabolic stressors. First, to mimic a conflict between hunger and environmental stress, we decided to study food intake in overnight fasted mice in a novelty-induced hypophagia test. After an overnight fasting, both groups showed a similar reduction in body weight and plasma glucose levels (**Suppl. Figure 4A, B**). Then mice underwent the novelty-suppressed feeding (NSF) test (**Figure 5A**). Sham^{PVT} and 6-OHDA^{PVT} mice showed similar latency to eat (**Figure 5B**), with a significant difference in food intake which was higher in 6-OHDA^{PVT} mice (**Figure 5C, D**). The NSF test (latency to eat) is mainly used to assess anxiety- and depressive-like phenotypes. Therefore, our results suggest that the increased food intake observed following PVT catecholaminergic ablation may not be confounded by potential alterations triggered by anxiety. This is in line with our above-mentioned observations using the open field test (**Figure 3A-F**).

Then, we used an acute restraint (immobilization) paradigm which is known to alter metabolism and food intake⁴⁵⁻⁴⁷. Sham^{PVT} and 6-OHDA^{PVT} mice underwent a 30 min acute restraint and food intake was measured during the dark period. Although both groups showed a significant reduction in food intake (**Figure 5E**), stress-induced hypophagia was significantly more pronounced in Sham^{PVT} compared to 6-OHDA^{PVT} mice (**Figure 5E**).

To further investigate the role of PVT CA inputs in scaling feeding, we used metabolic stressors to modulate food intake. First, Sham^{PVT} and 6-OHDA^{PVT} mice were administered with 2-deoxy-d-glucose (2-DG) which, in virtue of its glucoprivic effects (neuroglucopenia), elicits food consumption as well as the typical glucose counterregulatory response^{48,49}. Moreover, PVT neurons are highly sensitive to glucoprivation¹⁰. In this conditions, 6-OHDA^{PVT} mice consumed more chow food than

Sham^{PVT} mice (**Figure 6A**), even though the magnitude of the glucose counterregulatory response was similar between groups (**Figure 6B**). Of note, this food-related phenotype was not associated to altered glucose dynamics (glucose rise and clearance) as assessed by both the oral glucose tolerance test (OGTT, **Figure 6C**) and the insulin tolerance test (ITT, **Figure 6D**).

Second, we performed a fasting-refeeding test to mimic a negative energy balance (food deprivation). In line with the NSF test (**Figure 5C, D**), after an overnight fasting and a similar loss of body weight (**Suppl. Figure 4C**), both experimental groups showed an enhanced food intake with 6-OHDA^{PVT} mice consuming more food than Sham^{PVT} mice (**Figure 6E**). Third, we also decided to measure drinking and food intake in overnight water-deprived animals. Both groups showed again a similar decrease in body weight (**Suppl. Figure 4D**). After deprivation, mice were exposed to water. While no differences were observed in drinking behavior (**Figure 6F**), 6-OHDA^{PVT} mice again consumed more food than Sham^{PVT} mice (**Figure 6G**). These results suggest that hindbrainTH→PVT inputs scale food intake also following physiological and metabolic stressors.

In order to assess whether orexigenic signals without metabolic challenges also required an intact PVT catecholaminergic transmission, we administered ghrelin in fed mice. As shown in **Figure 6H**, ghrelin similarly induced an increase in food intake in both groups, thereby indicating that canonical orexinergic circuits are not altered.

2.4 Reduced catecholaminergic transmission in the PVT promotes the activation of hypothalamic regions

The above-mentioned results point to hindbrainTH→PVT afferents as major actors in scaling food intake and metabolic efficiency. Since these homeostatic functions highly depend on the hypothalamus, classically described as the master regulator of energy balance^{50,51}, we decided to study whether 6-OHDA^{PVT} mice were characterized by an altered basal activity (cFos-positive neurons) of key hypothalamic regions such as the dorsomedial hypothalamus (DMH), the ventromedial hypothalamus (VMH), the lateral hypothalamus (LH) and the arcuate nucleus (Arc).

Interestingly, in 6-OHDA^{PVT} mice we observed an increase in cFos-positive neurons specifically in the DMH and LH (**Figure 7A-C**) compared to Sham^{PVT} mice, whereas no differences were detected in the VMH and Arc (**Figure 7A, D, E**). These results

270 suggest that the increased activity of PVT neurons following catecholamines
271 depletion (**Figure 1**) may impact, directly and/or indirectly, on the regulatory activity
272 of the hypothalamus which may ultimately result in the modulation of food intake and
273 metabolic efficiency.

3 Discussion

The regulation of food intake represents one of the most complex biological functions. Pivotal for adaptation and survival, the regulatory processes underlying feeding are constantly shaped by signals reflecting/sensing physiological adjustments. In virtue of their heterogeneity (exteroceptive vs interoceptive sources), stress-like allostatic stimuli are indeed powerful modulators of food intake and feeding habits.

In this study, we report that hindbrain catecholaminergic (putative noradrenergic) inputs to the PVT play a key role in modulating food intake and metabolic efficiency in stress-related contexts. In fact, permanent ablation of TH afferents to the PVT resulted in enhanced food intake, adjusted metabolic efficiency and nutrient partitioning whenever environmental, behavioral, physiological and/or metabolic stressors were introduced as dependent variables of feeding behaviors. In particular, 6-OHDA^{PVT} mice were resistant or less sensitive to environmental and behavioral stress-induced hypophagia and showed enhanced feeding patterns following physiological and metabolic challenges. The different nature of stressors used in this study highlights the highly conserved role of CA inputs to the PVT in readily scaling feeding and metabolic adaptations. However, beyond the impact on feeding and metabolic efficiency, it is important to mention that stressors-elicited homeostatic adaptations such as energy expenditure and thermogenesis did not depend on PVT CA inputs, suggesting a functional tropism of hindbrainTH→PVT circuits toward food consumption. Therefore, these results indicate that the PVT, a key node of the limbic circuitry¹³, contributes to the elaboration of food-related decisions and strategies by integrating, among others, also catecholaminergic information. In addition, our results provide new evidence for the existence of distinct but converging hindbrain CA inputs (NTSTH- and LCTH-neurons) capable of gating food-related homeostatic adaptations under stress-like allostatic stimuli.

Surprisingly, we found that the NTS represented one of the major sources of TH-positive projections to the PVT. In fact, local microinjection of 6-OHDA resulted in a significant reduction of TH-expressing neurons in the NTS and to a less extent in the LC, sparing CA neurons in the medulla (A1 area) and the hypothalamus, the latter known to send only minor scattered projections to the PVT¹⁸. Although to our knowledge no other studies have functionally assessed this NTSTH→PVT connection,

the impact of 6-OHDA^{PVT} on NTSTH-neurons is in line with the presence of a dense plexus of PVT-reaching TH fibers when fluorescent recombinant markers are microinjected in the NTS of *Th-Cre* animals⁵². This evidence is of paramount importance since NTS and LC catecholaminergic neurons, by converging onto the PVT, may synergistically modulate feeding patterns and metabolic efficiency in stressogenic contexts. In fact, NTSTH- and LCTH-neurons are well-known to modulate food intake and stress/novelty, respectively^{53–55}.

Recent studies have shown that activation of CA-releasing NTS^{DBH/TH}-neurons may result in a reduction^{53,56} as well as in an increase^{52,56} of food intake depending on CA cell types and/or projection sites (parabrachial nucleus vs arcuate nucleus). Although not directly assessed in this study, according to our results NTSTH→PVT projecting neurons may serve as anorexigenic stimuli since their ablation enhances food intake under stress-related contexts. Indeed, it may be argued that the use of local microinjections of 6-OHDA may result in the degeneration of LCTH- and NTSTH-neurons projecting to the PVT but eventually to also other brain regions. However, loss of hindbrainTH neurons projecting to the medial hypothalamus resulted in a loss of glucoprivation-induced feeding^{57,58}, while in our study we show an increased food consumption under glucoprivic conditions when hindbrainTH→PVT projections were ablated. Moreover, opto-activation of NTSTH→Arc projections leads to an increase in food intake⁵², whereas in our case enhanced food intake was elicited by the absence of hindbrainTH→PVT projections. These effects may substantiate the functional selectivity of hindbrainTH→PVT projections in the adaptive responses of feeding, metabolic efficiency and nutrient partitioning to stress-related contexts. Indeed, future investigations using projection-specific optogenetics and/or chemogenetics may definitely help in dissecting out the distinct and, most importantly, the synergistic roles of NTSTH→PVT and LCTH→PVT transmissions in guiding the tight interaction between food intake and stress-like allostatic stimuli.

Moreover, we observed that deletion of hindbrainTH→PVT inputs led to an increase in PVT-neurons activity (cFos), suggesting that catecholamines may act as negative modulators onto PVT-neurons. At first, this is surprising and counterintuitive since *ex vivo* bath-applications of DA (precursor of NE) or NE, which can be synaptically co-released from CA terminals^{59,60}, lead to disinhibition (DA,¹⁶) or activation (NE,¹⁸) of PVT neurons. However, it should be mentioned that mid-posterior-PVT neurons express DA D2 and D3 receptors^{16,61,62} as well as NE receptors such as the $\alpha 1$, $\alpha 2$,

$\beta 1$ and $\beta 2$ receptors^{63–65}. Indeed, (*i*) how this variety of G-protein-coupled receptors (G_i -, G_s -, G_q - and β -arrestin-coupled receptors) mechanistically contribute to the overall modulation of PVT neurons and (*ii*) whether 6-OHDA-induced TH deletion reorganizes the expression of the above-mentioned CA receptors in the PVT require future in-depth investigations. Although our results cannot distinguish between the functional roles of distinct catecholamines onto their associated multiple receptors located onto PVT neurons, it is worth to mention that the PVT does not receive pure DA fibers from the midbrain (SNc and VTA)^{20,66} and that direct 6-OHDA-induced LCTH-neurons loss was associated to an increased expression of G_q -coupled $\alpha 1$ receptor in the thalamus⁶⁷ which may explain, at least in part, the increase in PVT cFos-neurons.

The enhanced activation of PVT-neurons in 6-OHDA^{PVT} mice and the associated feeding behaviors are in line with reports showing that stressors as well as hunger are able to activate PVT-neurons^{16,68,69}. In addition, our results are also in line with a recent report showing that activation of PVT-neurons by oxytocin was efficient in suppressing stress-induced hypophagia³¹. However, it is worth to mention that satietogenic signals are also able to activate PVT neurons⁸, therefore indicating that PVT excitatory (glutamate) neurons may be actually segregated into several cell types with distinct neurochemical, cellular and functional features. This is already supported by the existence of at least two neuronal populations (galanin- and D2R-positive neurons,⁶²) and, as already suggested by the presence of several neuropeptides in PVT-neurons⁷⁰, it is not hazardous to hypothesize that future cell type-specific transcriptomic analyses will reveal new sub-families and clusters.

The homeostatic processes underlying food intake, energy balance and metabolic efficiency strongly depend on the activity of the hypothalamus^{50,51}. We observed that depletion of TH afferents to the PVT resulted not only in the activation of PVT neurons but also in the concomitant activation of hypothalamic regions, notably the lateral (LH) and the dorsomedial (DMH) hypothalamus. Indeed, activation of LH and DMH cell types has been shown to promote feeding^{71–74} even following anxiogenic environmental cues⁷⁵. Although we cannot rule out yet whether and how the adaptive activation of PVT-neurons following TH deletion may be responsible for the direct activation of LH and DMH regions, it is interesting to note that PVT excitatory (glutamate) neurons also project to the hypothalamus^{29,76}, therefore potentially

modulating feeding and energy homeostasis. Indeed, while the existence of a hypothalamus→PVT→accumbal circuit seems critical for behavioral adaptations^{7,26,29,77–80}, our results combined to previous and recent literature^{8,16,17,76,81} also suggest a hindbrain→PVT→hypothalamus path that may regulate homeostatic functions requiring the integration of exteroceptive and interoceptive signals.

In conclusion, the PVT has been classically positioned as a functional node of the limbic circuit¹³. Only recently the hypothesis of the PVT as a homeostatic relay has been proposed⁵. Altogether, our results support the working hypothesis according to which the PVT, through its strong afferent connections with NTSTH- and LCTH-neurons, may represent a functional interface between homeostatic and emotional states, thereby leading to allostatic adaptations. This study, besides highlighting the existence of a dual hindbrain-to-thalamus connection, (i) provides new evidence to better understand the dynamic processes underlying the regulation of food intake and energy metabolism, and (ii) may serve as starting step to explore the causal-relationship between psychiatric (stress) and metabolic (anorexia, obesity, binge eating) disorders.

4 Materials and methods

4.1 Ethics statement and animals

All experiments were approved by the Animal Care Committee of the Université de Paris and carried out following the 2010/63/EU directive. 8-14 weeks old male C57Bl/6J mice (Janvier, Le Genest St Isle, France) were used and housed in a room maintained at 22 +/-1 °C, with a light period from 7h00 to 19h00. Regular chow diet (3.24 kcal/g, reference SAFE[®] A04, Augy, France) and water were provided *ad libitum* unless otherwise stated.

4.2 Stereotaxic microinjections of 6-OHDA

Mice were anaesthetized with isoflurane (3.5% for induction, 1.5% for maintenance), administered with Buprécare[®] (buprenorphine 0.3 mg) and Ketofen[®] (ketoprofen 100 mg), and placed on a stereotactic frame (Model 940, David Kopf Instruments). 6-OHDA-HCl (Sigma-Aldrich, #H4381) was dissolved in a saline (NaCl 0.9% w/v) solution containing 0.02% of ascorbic acid at a final concentration of 6 µg/µl. Animals were randomly assigned to either 6-OHDA or vehicle microinjections. A volume of 0.35 µl of 6-OHDA or vehicle (0.02% ascorbic acid) was injected into the PVT (L= 0.0; AP= -1.46; V= -2.8, mm) at an infusion rate of 0.05 µl/min. The injection needle was carefully removed after waiting 5 minutes at the injection site. Animals were allowed to recover for 3-4 weeks before any experimental evaluation.

4.3 Indirect calorimetry and metabolic efficiency analysis

Indirect calorimetry was performed as previously described⁸². Mice were monitored for whole energy expenditure (EE), O₂ consumption and CO₂ production, respiratory exchange rate (RER=VCO₂/VO₂, where V is a volume), fatty acid oxidation (FAO), and locomotor activity using calorimetric cages with bedding, food and water (Labmaster, TSE Systems GmbH, Bad Homburg, Germany). Ratio of gases was determined through an indirect open circuit calorimeter^{83,84}. This system monitors O₂ and CO₂ concentration by volume at the inlet ports of a tide cage through which a known flow of air is being ventilated (0.4 L/min) and compared regularly to a reference empty cage. For optimal analysis, the flow rate was adjusted according to the animal body weights to set the differential in the composition of the expired gases

between 0.4-0.9% (Labmaster, TSE Systems GmbH, Bad Homburg, Germany). The flow was previously calibrated with O₂ and CO₂ mixture of known concentrations (Air Liquide, S.A. France). O₂ consumption and CO₂ production were recorded every 15 min for each animal during the entire experiment. Whole energy expenditure (EE) was calculated using the Weir equation for respiratory gas exchange measurements. Food consumption was measured as the instrument combines a set of highly sensitive feeding sensors for automated online measurements. Mice had access to food and water *ad libitum*. To allow measurement of every ambulatory movement, each cage was embedded in a frame with an infrared light beam-based activity monitoring system with online measurement at 100 Hz. The sensors for gases and detection of movements operated efficiently in both light and dark phases, allowing continuous recording. When required, the inversion of circadian light/dark cycles was programmed using the Labmaster software.

Body mass composition was analyzed using an Echo Medical systems' EchoMRI (Whole Body Composition Analyzers, EchoMRI, Houston, USA), according to manufacturer's instructions. Readings of body composition were given within 1 min. Data analysis was performed on Excel XP using extracted raw values of VO₂ consumed (expressed in ml/h), VCO₂ production (expressed in ml/h), and energy expenditure (kcal/h).

4.4 Novelty-suppressed feeding (NSF)

After an overnight fasting, mice were placed in a cage (40×40×40 cm) with a single regular chow pellet in the center. The latency (time in seconds) to eat was scored. To measure food intake, food consumption was evaluated 60 minutes after the beginning of the test.

4.5 Open field (OF)

Spontaneous exploratory behavior was monitored in an open field (40×40×40 cm, BIOSEB) for 20 min, video-tracked and analyzed using the SMART software (BIOSEB). The open field was wiped with 70% ethanol between sessions.

4.6 Infrared temperature measurements

Thermogenesis was visualized using a high-resolution infrared camera (FLIR E8; FLIR Systems, Portland, OR, USA). To measure the temperature (°C) of the brown

adipose tissue (BAT, interscapular regions), lower back and tail, images were captured before and after the open field (OF) test. Infrared thermography images were analyzed using the FLIR TOOLS software.

4.7 Food intake following food or water deprivation

In two distinct experiments we measured food intake following either an overnight fasting (food deprivation) or water deprivation.

Overnight fasting. Mice were first weighted in the morning following an overnight fasting to ensure the loss of body weight. Then, they were exposed to pre-weighted chow pellets. Food intake was measured at the following time points: 30 min, 1h, 2h, 3h, 4h.

Overnight water deprivation. Mice were first weighted in the morning following an overnight water deprivation to ensure the loss of body weight. Then, they were exposed to a calibrated drinking bottle and pre-weighted chow pellets. Water consumption and food intake were measured at the following time points: 30 min, 1h, 2h, 3h, 4h.

4.8 Food intake induced by 2-DG and ghrelin

Mice were handled and injected with vehicle during 3 consecutive days before drugs administration. After this habituation period, they were administered with ghrelin (#1465, Tocris, 0.5 mg/kg, i.p.) or 2-DG (#14325, Cayman Chemical, 500 mg/kg, i.p.) and exposed to chow pellets 30 min after the injections. Food intake was measured during 3 hours. For 2-DG treated mice, blood glucose (counterregulatory response) was measured from the vein tail using a glucometer (Menarini Diagnostics, Rungis, France) at 0 and 30 min.

4.9 Glucose dynamics

Oral glucose tolerance test (OGTT). Animals were fasted 5 hours before receiving a bolus of glucose solution (2 g/kg) by gavage. Blood glucose (hyperglycemia) was measured from the vein tail using a glucometer (Menarini Diagnostics, Rungis, France) at 0, 5, 10, 15, 30, 60, 90, and 120 min.

Insulin tolerance test (ITT). Animals were fasted 5 hours before receiving an injection of insulin (0.5 U/kg, Novo Nordisk, i.p.). Blood glucose (hypoglycemia) was measured from the vein tail at 0, 5, 10, 15, 20, 30, 60, 90, and 120 min.

4.10 Tissue preparation and immunofluorescence

Animals were injected with pentobarbital (500 mg/kg, i.p., Sanofi-Aventis, France). Once anaesthetized, they were transcardially perfused with cold (4°C) PFA 4% for 5 minutes. Brains were collected, put overnight in PFA 4% and then stored in PBS (4°C). 40 µm-thick sections were sliced with a vibratome (Leica VT1000S, France) and stored in a cryoprotective solution at -20 °C until immunofluorescence investigations. Immunofluorescence on brain slices was performed as previously described^{85,86}.

Briefly, sections were processed as it follows. Day 1: free-floating sections were rinsed in Tris-buffered saline (TBS; 0.25 M Tris and 0.5 M NaCl, pH 7.5), incubated for 5 min in TBS containing 3% H₂O₂ and 10% methanol, and then rinsed three times for 10 min each in TBS. After 15 min incubation in 0.2% Triton X-100 in TBS, sections were rinsed three times in TBS again. Slices were then incubated 48 hrs at 4°C with the following primary antibodies: rabbit anti-cFos (1:1000, Synaptic Systems, #226 003) or rabbit anti-TH (1:1000, Millipore, #AB153). Sections were rinsed three times for 10 min in TBS and incubated for 60 min with secondary donkey anti-rabbit Cy3 AffiniPure (1:1000, Jackson ImmunoResearch, #711-165-152). Sections were rinsed for 10 min twice in TBS, stained with DAPI (10 min) and rinsed in TB (0.25 M Tris) before mounting.

Acquisitions were performed with a confocal microscope (Zeiss LSM 510). The objective (10X) and the pinhole setting remained unchanged during the acquisition of a series for all images. Depending on the extension of the region of interest, either single or mosaic acquisitions were conducted. Quantification of immunoreactive cells (cFos- or TH-positive neurons) was performed using the cell counter plugin of the ImageJ software taking as standard reference a fixed threshold of fluorescence. For cell counting, three (TH) or two (cFos) rostro-caudal levels for each brain region were used. Quantifications of immunopositive neurons were averaged between hemispheres and then summed for consecutive brain slices.

4.11 Statistics

All data are presented as mean ± SEM. Statistical tests were performed with Prism 7 (GraphPad Software, La Jolla, CA, USA). Detailed statistical analyses are listed in the **Suppl. Table 1**. Normality was assessed by the D'Agostino-Pearson test.

Depending on the experimental design, data were analyzed using either Student t-test (paired or unpaired) with equal variances, One-way ANOVA or Two-way ANOVA. The significance threshold was automatically set at $p < 0.05$. ANOVA analyses were followed by Bonferroni *post hoc* test for specific comparisons only when overall ANOVA revealed a significant difference (at least $p < 0.05$).

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Author Contributions

C.D. performed and analyzed most of the experiments. G.L. performed immunofluorescence studies. J.C. performed surgeries. S.L. provided critical feedback. G.G. conceived and supervised the whole project, and wrote the manuscript with contribution from all coauthors.

Competing interests

The authors declare no competing interests.

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Figure legends

Figure 1: Retrograde ablation of hindbrain catecholaminergic inputs contribute to PVT activity. (A) Drawing represents the microinjection of 6-OHDA or Vehicle (Veh) in the mid-posterior PVT. (B) Immunofluorescence detection of tyrosine hydroxylase (TH) within the PVT in Sham^{PVT} and 6-OHDA^{PVT} mice. Scale bar: 150 μ m. (C) Immunofluorescence detection of TH in PVT-projecting hindbrain regions [nucleus tractus solitarius (NTS), area A1 and locus coeruleus (LC)] in Sham^{PVT} and 6-OHDA^{PVT} mice. Scale bars: 150 μ m. (D) Quantification of TH-positive neurons in the NTS, A1 and LC. Statistics: * $p < 0.05$, ** $p < 0.01$, 6-OHDA^{PVT} vs Sham^{PVT} mice. (E) Immunofluorescence detection of cFos-positive neurons in the mid-posterior PVT (mPVT and pPVT). Scale bars: 150 μ m. (F) Quantification of cFos-positive neurons in the mid-posterior PVT. Statistics: *** $p < 0.001$, 6-OHDA^{PVT} vs Sham^{PVT} mice. For statistical details see **Suppl. Table 1**.

Figure 2: Catecholaminergic inputs to the PVT participate to novelty-induced hypophagia. (A) Body weight of Sham^{PVT} and 6-OHDA^{PVT} mice following 3-4 weeks from 6-OHDA or Veh microinjection in the PVT. (B) Experimental design indicating the transition from grouped to singled housing. (C) Food intake (g/day and kcal/day) during the first three days of isolation (D1 to D3). Statistics: * $p < 0.05$, ** $p < 0.01$, 6-OHDA^{PVT} vs Sham^{PVT} mice. (D) Investigation of food intake and metabolic efficiency using metabolic cages during two consecutive exposures to a novel environment. (E) Food intake during the dark phase (spontaneous eating) in Sham^{PVT} and 6-OHDA^{PVT} mice during two consecutive exposures to a novel environment. (E¹) Cumulative food intake. Statistics: *** $p < 0.01$, Sham^{D2} vs Sham^{D1}, ## $p < 0.01$, 6-OHDA^{D1} vs Sham^{D1} groups. (F) Spontaneous locomotor activity (beam breaks, bb) during the dark phase in Sham^{PVT} and 6-OHDA^{PVT} mice during two consecutive exposures to a novel environment. (F¹) Cumulative locomotor activity. Statistics: ** $p < 0.01$, Sham^{D2} vs Sham^{D1}; ### $p < 0.001$, 6-OHDA^{D2} vs 6-OHDA^{D1} groups. Note that both experimental groups showed similar degrees of habituation (reduced locomotor activity during the dark period). Measurements of the respiratory exchange ratio (RER, G), fatty acid oxidation (FAO, H), and energy expenditure (EE, I) in Sham^{PVT} and 6-OHDA^{PVT} mice during the first exposure (D1) to a novel environment. (G¹⁻¹) Averaged RER, FAO

and EE during the dark phase. Statistics: ** $p < 0.01$, 6-OHDA^{D1} vs Sham^{D1}. For statistical details see **Suppl. Table 1**.

Figure 3: Deletion of PVT catecholaminergic inputs does not alter novelty-induced anxiety and thermogenesis. (A) Drawing represents the open field (OF) test. (B-F) Parameters measured during a 20 min OF test: total distance, number of entries in the center, % of time in the center, mean exploration visits to the center, distance in the center. (G) Infrared thermographic images of animals after the OF test (20 min). (H-J) Temperature (°C) of the brown adipose tissue (BAT), lower back and tail in Sham^{PVT} and 6-OHDA^{PVT} mice before and after the OF test. Statistics: *** $p < 0.001$, After vs Before OF test (Sham^{PVT} mice); ### $p < 0.001$, After vs Before OF test (6-OHDA^{PVT} mice). No differences were detected between Sham^{PVT} and 6-OHDA^{PVT} mice. For statistical details see **Suppl. Table 1**.

Figure 4: Catecholaminergic inputs to the PVT contribute to the circadian adaptation of metabolic efficiency. (A) Drawing indicates the experimental procedure used to study feeding and metabolic adaptations during an inverted cycle (transition from Light-to-Dark to Dark-to-Light). (B, B¹) Food intake in Sham^{PVT} (B) and 6-OHDA^{PVT} (B¹) mice during the standard and inverted cycles. Note how the temporal dynamics of feeding change during the inverted cycle. (C) Cumulative food intake in Sham^{PVT} and 6-OHDA^{PVT} mice during the standard and inverted cycles. (D, D¹) Temporal dynamics of EE adaptations in Sham^{PVT} (D) and 6-OHDA^{PVT} (D¹) mice during the standard and inverted cycles. (E, E¹) Averaged EE in Sham^{PVT} and 6-OHDA^{PVT} mice according to matched inverted phases (7h-19h, E, and 19h-7h, E¹). Statistics: *** $p < 0.001$, Inverted vs Standard cycle (Sham^{PVT} mice); ### $p < 0.001$, Inverted vs Standard cycle (6-OHDA^{PVT} mice). (F, F¹) Temporal dynamics of FAO adaptations in Sham^{PVT} (F) and 6-OHDA^{PVT} (F¹) mice during the standard and inverted cycles. (G, G¹) Averaged FAO in Sham^{PVT} and 6-OHDA^{PVT} mice according to matched inverted phases (7h-19h, G, and 19h-7h, G¹). Statistics: *** $p < 0.001$, ** $p < 0.01$, Inverted vs Standard cycle (Sham^{PVT} mice); # $p < 0.01$, Inverted vs Standard cycle (6-OHDA^{PVT} mice). (H, H¹) Temporal dynamics of RER adaptations in Sham^{PVT} (H) and 6-OHDA^{PVT} (H¹) mice during the standard and inverted cycles. (I, I¹) Averaged RER in Sham^{PVT} and 6-OHDA^{PVT} mice according to matched inverted

phases (7h-19h, I, and 19h-7h, I¹). Statistics: *** $p < 0.001$, ** $p < 0.01$, Inverted vs Standard cycle (Sham^{PVT} mice); ## $p < 0.01$, Inverted vs Standard cycle (6-OHDA^{PVT} mice). For statistical details see **Suppl. Table 1**.

Figure 5: Catecholaminergic inputs to the PVT scale feeding following physiological stressors. (A) Drawing represents the novelty-suppressed feeding (NSF) test in overnight fasted Sham^{PVT} and 6-OHDA^{PVT} mice. (B) Latency to eat (first bite) during the NSF test. (C, D) Food intake, total kcal (C) and normalized kcal/BW (D), during the NSF test. Statistics: ** $p < 0.01$, 6-OHDA^{PVT} vs Sham^{PVT} mice. (E) Food intake in Sham^{PVT} and 6-OHDA^{PVT} mice after a restraint stress (immobilization). NoR: No Restraint (control conditions). R: Restraint. Statistics: *** $p < 0.001$, Restraint vs No Restraint (Sham^{PVT}); ## $p < 0.01$, Restraint vs No Restraint (6-OHDA^{PVT}); \$\$\$ $p < 0.001$, 6-OHDA^{PVT} vs Sham^{PVT} mice (Restraint). For statistical details see **Suppl. Table 1**.

Figure 6: Catecholaminergic inputs to the PVT scale feeding following metabolic stressors. (A) Food intake in Sham^{PVT} and 6-OHDA^{PVT} mice administered with 2-DG (500 mg/kg, i.p., neuroglucopenia-induced feeding). (B) Glucose variation (%) following administration of 2-DG (0 vs 30 min post administration). Note: neuroglucopenia-induced hyperglycemia is the typical readout of the glucose counterregulatory response. (C, D) Glucose dynamics during the oral glucose tolerance test (C) and the insulin tolerance test (D). No differences were observed between groups. (E) Food intake in overnight fasted Sham^{PVT} and 6-OHDA^{PVT} mice (refeeding). (F) Water intake in overnight water-deprived Sham^{PVT} and 6-OHDA^{PVT} mice. (G) Food intake in overnight water-deprived Sham^{PVT} and 6-OHDA^{PVT} mice. (H) Food intake in Sham^{PVT} and 6-OHDA^{PVT} mice administered with ghrelin (0.5 mg/kg, i.p., orexigenic response). Statistics: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, 6-OHDA^{PVT} vs Sham^{PVT} mice. For statistical details see **Suppl. Table 1**.

Figure 7: Deletion of PVT catecholaminergic inputs increases cFos expression in the hypothalamus. (A) Immunofluorescence detection of cFos in hypothalamic regions of Sham^{PVT} and 6-OHDA^{PVT} mice, notably the dorsomedial hypothalamus (DMH), the lateral hypothalamus (LH), the ventromedial hypothalamus (VMH) and the arcuate nucleus (Arc). Regions of interest are delineated by white dotted lines.

889 Insets represent higher magnifications of DMH and LH regions. Scale bars: 500 μ m.
890 Quantification of cFos-positive neurons in the DMH (**B**), LH (**C**), VMH (**D**) and Arc (**E**)
891 regions. Statistics: ** $p < 0.01$, 6-OHDA^{PVT} vs Sham^{PVT} mice. For statistical details see
892 **Suppl. Table 1**.

893
894 **Suppl. Figure 1: Detection of TH-positive neurons in the hypothalamus**
895 **following PVT 6-OHDA microinjections.** Immunofluorescence detection of cFos
896 (red) and DAPI (blue) in the hypothalamus of Sham^{PVT} and 6-OHDA^{PVT} mice. Scale
897 bars: 500 μ m.

898
899 **Suppl. Figure 2: Deletion of PVT catecholaminergic inputs does not alter**
900 **palatability for HFHS diet.** (A) Food intake in Sham^{PVT} and 6-OHDA^{PVT} mice
901 following time-locked feeding (1h) of high-fat high-sugar (HFHS) diet during two
902 consecutive days. Statistics: ** $p < 0.01$, Day2 vs Day1 (Sham^{PVT} mice); ## $p < 0.01$,
903 Day2 vs Day1 (6-OHDA^{PVT} mice). No differences between experimental groups. For
904 statistical details see **Suppl. Table 1**.

905
906 **Suppl. Figure 3: Confirmation of sensitivity to novelty-induced hypophagia in**
907 **Sham^{PVT} and 6-OHDA^{PVT} mice before the inverted cycle.** (A) Cumulative food
908 intake during the dark phase (spontaneous eating) in Sham^{PVT} and 6-OHDA^{PVT} mice
909 during the first exposure (D1) to a novel environment (calorimetric chambers).
910 Statistics: *** $p < 0.001$, 6-OHDA^{PVT} vs Sham^{PVT} mice. For statistical details see
911 **Suppl. Table 1**.

912
913 **Suppl. Figure 4: Effect of fasting and water deprivation on Sham^{PVT} and 6-**
914 **OHDA^{PVT} mice.** (A) Loss of body weight and (B) glucose variations in overnight
915 fasted animals used for the novelty-suppressed feeding (NSF) test. (C) Loss of body
916 weight in overnight fasted animals before the refeeding schedule. (D) Loss of body
917 weight in overnight water-deprived animals before having access to water and chow
918 pellets. No differences between experimental groups. For statistical details see
919 **Suppl. Table 1**.

Figure 1

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Figure 2

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