Deletion of p22phox-dependent oxidative stress in the hypothalamus protects against obesity by modulating β3-adrenergic mechanisms

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Introduction

The central nervous system (CNS) is critical for the control of body weight. In addition to modulating feeding behavior, the brain is involved in the control of energy expenditure through regulation of basal metabolism, adaptive thermogenesis, and locomotor activity. In this regard, a hypothalamic network including the arcuate nucleus, ventromedial and lateral hypothalamus, and paraventricular nucleus (PVN) is crucial for regulating feeding behavior and thermogenesis (1–3).

Several studies have implicated the PVN in the autonomic control of energy expenditure by modulating sympathetic outflow to brown adipose tissue (BAT) (4, 5). BAT transforms energy from substrates into heat by free fatty acid oxidation (6), a crucial function for thermoregulation during cold stress and regulation of body weight. Alterations in BAT function may be involved in diet-induced obesity (DIO) (7). Previous reports suggested that a population of neurons within the PVN activate BAT thermogenesis via the sympathetic nervous system (4). In contrast, a more recent study suggests that PVN neurons send signals to reduce BAT sympathetic nerve activity (8). Thus, while the involvement of the PVN in regulating BAT activity has been demonstrated, it remains unclear whether alterations in PVN control of BAT activity contribute to DIO.

White adipose tissue (WAT) is metabolically less active than BAT and is specialized for storing chemical energy. Sympathetic stimulation of WAT induces breakdown of triacylglycerol via hormone-sensitive lipase (9). Recent studies show that increased norepinephrine in WAT induces expression of uncoupling protein 1 (Ucp1) and increases metabolic activity (10). The increased Ucp1 expression in WAT is termed browning (10). Importantly, browning of WAT has antiobesity effects (11, 12), and the PVN is involved in regulating sympathetic outflow to WAT (13, 14). Furthermore, bilateral lesioning of the PVN reduces sympathetic signaling and diminishes lipid mobilization (15). The involvement of the PVN in browning...
has yet to be determined. Collectively, these findings demonstrate a role of the PVN in energy homeostasis and suggest that changes in this brain region might influence adiposity and obesity.

Chronic oxidative stress contributes to pathophysiological conditions such as atherosclerosis and hypertension (16, 17). Further, excess generation of reactive oxygen species (ROS) is an underlying mechanism of metabolic disorders (18), and oxidative stress in adipose tissue has a central role in the pathogenesis of metabolic syndrome and type 2 diabetes (19). Chronic overnutrition contributes to hypothalamic inflammation and endoplasmic reticulum (ER) stress, both of which are linked to oxidative stress (20–22). NADPH oxidases are major sources of ROS in a variety of chronic diseases such as hypertension (23). The catalytic subunits of NADPH oxidases are Nox proteins (Nox1, Nox2 [gp91phox], Nox3, and Nox4), which, in rodents, require the membrane protein p22 phox to be active (17). Thus, alterations in p22 phox regulate cellular NADPH oxidase activity and ROS production (23–25). Interestingly, a recent study linked vascular p22phox-mediated oxidative stress to accelerated weight gain during high-fat diet (HFD) (26).

While these and other investigations have focused on oxidative stress in peripheral tissues during obesity, recent evidence has suggested that NADPH oxidase–dependent oxidative stress in the CNS is involved in the regulation of body weight and adipose tissue metabolism (22). A recent study showed that DIO increased hypothalamic expression of NADPH oxidase, a major source of ROS (27). However, a causal link between increased hypothalamic NADPH oxidase and obesity has not been investigated.

We provide evidence that deletion of p22phox and inhibition of NADPH oxidase–derived oxidative stress selectively in the PVN increases BAT thermogenesis and browning of subcutaneous WAT via a β3-adrenoceptor–dependent pathway during DIO. This results in increased energy expenditure and less adiposity and weight gain during HFD, independent of changes in food intake or locomotor activity. Our findings establish a causal link between oxidative stress in the PVN and obesity, and advance our understanding of the neural regions and metabolic mechanisms involved.

Results

Selective deletion of p22phox in the PVN blunts ROS production during DIO. AdCre was injected bilaterally into the PVN of p22phox−/− mice to eliminate this critical NADPH oxidase subunit selectively from the PVN. Control p22phox+/− animals were injected with AdLacZ. Following 7–10 days of recovery, mice were fed HFD or remained on normal chow (NC) for 10 weeks. At the end of this period, we verified that AdCre reduced p22phox expression in the PVN. Quantitative real-time PCR from micropunches of the PVN showed...
that whereas DIO increased PVN p22phox mRNA expression in control vector–treated animals, targeting of AdCre to this brain area prevented the HFD-induced increase in p22phox in the PVN (Figure 1A).

We found no change in p22 phox expression in the arcuate nucleus or cerebral cortex (CTX) after AdCre injection in the PVN, demonstrating the selectivity of AdCre for the PVN (Figure 1A).

To verify that deletion of p22phox in the PVN leads to functional inactivation of NADPH oxidase, ROS were measured with dihydroethidium (DHE) staining (Figure 1, B and C). Ten weeks of HFD resulted in a significant increase in DHE fluorescence in the PVN (Figure 1, B and C). AdCre injection into the PVN blunted the increase in DHE fluorescence during HFD (Figure 1, B and C). During NC, AdCre injection did not alter DHE fluorescence in the PVN (Figure 1, B and C).

Deletion of p22phox protects against weight gain in DIO mice. Next, we studied the physiological effects of p22phox deletion in the PVN during DIO in AdLacZ- or AdCre-treated p22phox mice during 10 weeks of normal chow (NC) or diet-induced obesity (DIO). Weekly food intake in AdLacZ or AdCre mice during 10 weeks of NC or DIO (n = 7–17). (D–G) Food intake (D), feeding frequency (E), average meal size (F), and average meal length (G) measured by indirect calorimetry in AdLacZ- or AdCre-injected p22phox mice over 24 hours (n = 4–8). (H) Spontaneous locomotor activity during the light and dark cycles in AdLacZ and AdCre mice after 10 weeks of NC or high-fat diet (n = 4–8). The gray area indicates the dark cycle between 6 pm and 6 am. *P < 0.05 vs. NC; **P < 0.05 vs. AdLacZ DIO; 2-way ANOVA followed by a Sidak multiple comparison post-hoc test. All data are the mean ± SEM.

Deletion of p22phox in the PVN protects against weight gain in DIO mice. Next, we studied the physiological effects of p22phox deletion in the PVN during DIO in AdLacZ- or AdCre-injected p22phox mice. Removal of p22phox from the PVN abolished the body weight gain in response to HFD compared with mice treated with AdLacZ (Figure 2A). Injection of AdCre in the PVN did not alter body weight in mice fed NC (Figure 2A). Food intake increased transiently during HFD but did not differ between mice with PVN injection of AdLacZ versus AdCre (Figure 1B). Importantly, prevention of weight gain in AdCre mice was not accounted for by differences in caloric or food intake between AdLacZ- and AdCre-injected mice (Figure 2, B and C).
Food intake monitored during indirect calorimetry at the end of the 10-week period also demonstrated that selective ablation of \( p22^{phox} \) from the PVN did not influence food intake, feeding frequency, average meal size or meal length (Figure 2, D–G). Together, these findings demonstrate that the observed effect of ablation of \( p22^{phox} \) in the PVN on body weight in DIO mice is independent of food and caloric intake.

We also tested the possibility that the prevention of weight gain during DIO was due to increased locomotor activity. Spontaneous locomotor activity as measured by laser beam breaks in an indirect calorimetry system did not differ between AdLacZ and AdCre PVN-targeted animals during either the light or dark cycles (Figure 2H). Thus, the blunting of weight gain in AdCre-treated \( p22^{phox/fl} \) mice was also independent of locomotor activity.

\( p22^{phox} \) deletion in the PVN blunts WAT accumulation and augments iBAT during DIO. Given the finding that ablation of \( p22^{phox} \) from the PVN prevented HFD-induced increases in body weight (Figure 2A), we also examined regional adipose tissue depots following HFD and NC feeding. As expected, AdLacZ-treated
DIO mice exhibited significant increases in various WAT masses, including subcutaneous (sc), perigonadal (g), and perirenal (r) WAT after 10 weeks of HFD (Figure 3, A–C). Deletion of \( p22^{phox} \) in the PVN caused a significant attenuation in HFD-induced increases in mass of these WAT depots (Figure 3, A–C). As seen in representative histological images of scWAT in Figure 3D, adipocytes were enlarged after 10 weeks of HFD in control vector–treated mice. In HFD-fed mice lacking \( p22^{phox} \) in the PVN, cells were similar in size to those of NC-fed controls.

Since BAT is the major site of adaptive thermogenesis and is thus crucial in body weight regulation (6), we also measured interscapular BAT (iBAT) mass. AdLacZ-treated \( p22^{phox/fl} \) mice showed no change in iBAT mass after 10 weeks of HFD, whereas iBAT mass was significantly increased in DIO mice with targeted ablation of \( p22^{phox} \) in the PVN (Figure 3E). Neither iBAT nor WAT weights were altered by AdCre in NC-fed mice (Figure 3, A–C, E).

After the onset of obesity, \( p22^{phox} \) ablation in the PVN blunts further increases in adiposity and body weight gain. To determine if HFD-induced weight gain and adiposity could be arrested by removal of \( p22^{phox} \) from the PVN after the onset of obesity, a separate cohort of \( p22^{fl/fl} \) mice was fed HFD for 8 weeks to induce obesity prior to targeting the PVN with AdCre or AdLacZ. The mice in the 2 groups gained a similar amount of body weight during the first 8 weeks of HFD (Figure 4A). However, as body weight continued to increase in AdLacZ mice, AdCre-treated mice showed no further weight gain compared with AdLacZ mice over the next 10 weeks of HFD feeding (Figure 4A). This was independent of changes in food intake (Figure 4B). Corresponding to body weight, in mice with PVN injections of AdCre or AdLacZ after 8 weeks of HFD, WAT mass was lower and iBAT mass higher in the AdCre mice after 18 weeks of HFD (Figure 4, C and D).

\( p22^{phox} \) in the PVN is involved in the regulation of energy expenditure during the development of DIO. Next we investigated the underlying mechanism(s) of the beneficial effects of PVN \( p22^{phox} \) deletion on body weight and adiposity during HFD by measuring oxygen consumption (\( VO_2 \)) and heat production using indirect calorimetry at the end of the 10-week protocol. As expected for nocturnal animals, \( VO_2 \) and heat production were higher during the dark cycle (Figure 5, A and B). AdCre-targeted PVN injections did not alter \( VO_2 \) or heat production in mice fed NC (Figure 5, A and B). In addition, HFD had no effect on these parameters in AdLacZ-treated mice. However, targeted deletion of \( p22^{phox} \) in the PVN caused a significant...
increase in VO₂ and heat production throughout a 24-hour period (both light and dark cycles) during DIO (Figure 5, A and B). This indicates that prevention of HFD-induced increases in NADPH oxidase–dependent ROS production in the PVN elevates energy expenditure and thermogenesis.

Deletion of p22phox in the PVN during DIO elevates thermogenesis and promotes increased expression of Ucp-1 and Pgc-1α in iBAT and browning of scWAT. A) Average oxygen consumption (VO₂) and (B) heat production in AdLacZ- and AdCre-treated p22phox/fl mice after 10 weeks normal chow (NC) or diet-induced obesity (DIO). *P < 0.05 vs. NC and AdLacZ DIO; **P < 0.05 Dark vs. Light Cycle; n = 4–8. Gray area indicates dark cycle between 6 pm and 6 am. (C) Ucp-1 mRNA expression in interscapular brown adipose tissue (iBAT). *P < 0.05 vs. NC, n = 10–16. (D) Expression of thermogenic marker Pgc-1α in iBAT (n = 10–16). *P < 0.05 vs. NC and AdLacZ DIO; **P < 0.05 vs. NC. (E) Expression of Ucp-1, Pgc-1α, Prdm16, and Hsl mRNA in subcutaneous white adipose tissue (scWAT) in AdLacZ and AdCre mice after 10 weeks NC or high-fat diet (n = 11). *P < 0.05 vs. NC and AdLacZ DIO. All data are the mean ± SEM. All P values determined by 1-way ANOVA followed by Tukey’s post-hoc test.

Deletion of p22phox in the PVN during DIO induced browning in scWAT. A recent study showed that activation of the hypothalamic-adipocyte axis promotes a phenotypic switch of scWAT to a brown-like adipose tissue (30). Since deletion of p22phox in the PVN was associated with a significant reduction in WAT in the face of HFD (Figure 3, A–C), we speculated that browning and lipolysis of WAT may be involved (9). Markers of WAT browning including Ucp-1, Pgc-1α, and PR-domain-containing 16 (Prdm16) (30–32) were evaluated in scWAT. There was no significant increase in scWAT Ucp-1, Pgc-1α, or Prdm16 mRNA expression in DIO mice with PVN injection of AdLacZ (Figure 5E). In contrast, deletion of p22phox in the PVN during DIO caused a striking increase in scWAT Ucp-1 (~20-fold), Pgc-1α (~4-fold), and Prdm16 (~3-fold) mRNA expression (Figure 5E). None of these markers was altered in NC-fed mice. We also measured mRNA expression of hormone-sensitive lipase (Hsl), an enzyme necessary to promote lipolysis in WAT (33). HFD did not increase expression of Hsl in AdLacZ-treated mice, whereas AdCre-treated mice showed an ~6-fold increase in VO₂ and heat production throughout a 24-hour period (both light and dark cycles) during DIO (Figure 5, A and B). This indicates that prevention of HFD-induced increases in NADPH oxidase–dependent ROS production in the PVN elevates energy expenditure and thermogenesis.

p22phox deletion in the PVN increased Ucp-1 mRNA and peroxisome proliferator-activated receptor gamma coactivator 1-alpha mRNA (Pgc-1α) in iBAT during DIO. Given the increased iBAT mass along with elevated VO₂ and heat production in HFD-fed mice following selective removal of p22phox from the PVN, we investigated expression of adaptive thermogenesis markers in iBAT including Ucp-1 and Pgc-1α. Consistent with previous findings (28, 29), iBAT Ucp-1 mRNA was increased ~3-fold after 10 weeks of DIO in AdLacZ-injected p22phox/fl mice (Figure 5C). Deletion of p22phox in the PVN did not result in a further increase in iBAT Ucp-1 mRNA compared with AdLacZ DIO mice (Figure 5C). In contrast, Pgc-1α mRNA expression was increased ~4-fold in iBAT after deletion of p22phox in the PVN during DIO (Figure 5D). Pgc-1α expression was not significantly increased in AdLacZ DIO mice (Figure 5D), nor was it increased in NC mice.
As above, mice fed NC did not show changes in Hsl transcript levels. Ablation of p22phox in the PVN attenuates increased adiposity during HFD by activation of β3-adrenoceptors in iBAT and scW AT. Previous studies implicate sympathetic signals from the PVN in the regulation of thermogenesis by stimulating BAT and WAT activity (5, 13, 15, 34). Thus, we investigated if deletion of PVN p22phox alters norepinephrine (NE) levels in iBAT and scWAT. NE levels in iBAT and scWAT were lower in mice fed HFD than in mice fed NC (Figure 6, A and B). Deletion of p22phox in the PVN of NC-fed mice did not significantly alter NE in iBAT or scWAT (Figure 6, A and B). In contrast, deletion of p22phox in the PVN increased NE levels in both iBAT and scWAT after 10 weeks of DIO, suggesting that PVN ROS inhibits NE levels in adipose depots during HFD (Figure 6, A and B) (35).

Given the prominent role of β3-adrenoceptors (Adrb3) in thermogenic and lipolytic processes in both BAT and WAT (5, 6, 13, 34), we next examined expression of Adrb3 in iBAT and scWAT. AdCre injections did not change Adrb3 expression in p22phox−/− mice fed NC compared with AdLacZ control mice (Figure 6C). Further, DIO did not significantly increase Adrb3 mRNA in iBAT or scWAT in AdLacZ DIO mice (Figure 6C). In contrast, deletion of p22phox in the PVN of AdCre-treated mice led to striking increases in Adrb3 mRNA in both iBAT (~10-fold vs. AdLacZ NC) and scWAT (~4-fold vs. AdLacZ NC) during HFD (Figure 6C).
To investigate the functional role of an upregulation of β3-adrenoceptors in DIO mice lacking p22 phox we injected (i.p.) animals daily with SR59230A, a selective β3-adrenoceptor antagonist (36). Relative to vehicle-injected AdCre DIO mice, SR59230A significantly reduced Adrb3 mRNA in iBAT and scW AT of AdCre-treated DIO mice (Figure 6D). Blockade of β3-adrenoceptors also decreased VO2 and heat production in AdCre DIO mice compared with AdCre vehicle controls (Figure 6, E and F). The reductions in VO2 and heat production were independent of alterations in food intake or locomotor activity (Figure 6, G and H). Furthermore, SR59230A caused a significant reduction in iBAT mass (>50%) and Pgc-1α mRNA in DIO mice lacking p22 phox. β3-Adrenoceptor blockade did not alter iBAT mass or Pgc-1α mRNA in DIO without deletion of PVN p22phox (Figure 7, A and B), suggesting that β3-adrenoceptor-mediated mechanisms contribute to the increased iBAT mass and augmented Pgc-1α levels in DIO mice with deletion of PVN p22phox. Blockade of the β3-adrenoceptors also ameliorated scW AT Prdm16 and Hsl expression in DIO mice with deletion of PVN p22phox, indicating reduced browning and lipolysis of scWAT (Figure 7C). Blockade of β3-adrenoceptors did not affect these variables in AdLacZ DIO mice (Figure 7C).

Discussion
This study reveals a potentially novel role for NADPH oxidase–derived ROS in the PVN in the regulation of metabolic homeostasis and HFD-induced obesity. Selective deletion of p22phox in this brain region increases thermogenesis and BAT mass and induces browning and decreases of scWAT during HFD. Notably, these effects are independent of changes in caloric intake or locomotor activity.

Links between brain NADPH oxidase–derived ROS and cardiovascular disease are well established (23) and are emerging in DIO (22, 27). The subunit p22phox is not catalytically active, but is critical for proper functioning of all rodent NADPH oxidase isoforms (37). Increased expression of p22phox is an indicator of increased NADPH oxidase activity (23, 38), and elevated p22phox in the vasculature was recently linked to accelerated obesity during HFD (26). Here we observed HFD-induced elevations in p22phox expression and corresponding increases in ROS as indicated by DHE fluorescence in the PVN, both of which were abolished by viral gene transfer of Cre recombinase selectively to this brain region. Tissue samples for these studies were obtained 11 weeks after gene transfer, indicating that AdCre induced a highly stable reduction in p22phox throughout the course of the experiments. This is consistent with previous studies using a similar approach in different brain sites (23). Importantly, PVN-targeted AdCre did not alter p22phox expression in another important metabolic center of the brain, the arcuate nucleus or in the CTX, indicating the site selectivity of PVN deletion of p22phox.

The protective effects of p22phox deletion on weight gain and adiposity during DIO were independent of energy intake and locomotor activity. We also did not observe differences in other feeding parameters.
including the frequency of feeding, average meal size, or length of time spent feeding. Interestingly, caloric intake was only elevated in the first week of HFD. This finding is in agreement with previous studies showing that HFD-fed mice and rats have, after an initial elevation in caloric intake, similar caloric intake compared with NC-fed animals (26, 39). Similar observations have been made in primates (40). These findings suggest that rodents and primates adapt quickly to the increased nutrient availability and increased caloric intake is not sustained over a long period of time. The exact mechanisms of this caloric adaptation remain elusive. However, NADPH oxidase–dependent oxidative stress in the PVN during DIO does not seem to play a role in the caloric adaptation in our study since there was no difference in food intake between HFD DIO mice with or without deletion of PVN p22\textsuperscript{phox}.

Next we tested if removal of p22\textsuperscript{phox} can halt progression of DIO. For this we fed the animals HFD before transfection with adenoviral vectors. Our results show that further weight gain in obese animals was arrested upon deletion of p22\textsuperscript{phox} in the PVN despite continued HFD feeding. This effect on weight gain was again independent of changes in caloric intake. These studies indicate that deletion of p22\textsuperscript{phox} in the PVN not only prevents lean animals from becoming obese during HFD, but also slows further weight gain in obese animals.

Previous studies have implicated the PVN in control of sympathetic outflow to BAT (4, 5), and thereby the regulation of thermogenesis and energy expenditure (6). Here we determined that HFD-fed animals lacking PVN p22\textsuperscript{phox} exhibited increased iBAT mass and elevated energy expenditure and thermogenesis. We pursued this by investigating the expression of the adaptive thermogenesis markers Ucp-1 and Ppargc1a in iBAT. UCP-1 uncouples the respiratory chain in mitochondria and creates heat, and Ppargc1a is a coactivator gene required for brown fat thermogenesis (32). We confirmed previous findings that Ucp-1 mRNA is elevated in response to HFD in control mice (28, 29), but Ucp-1 mRNA did not increase further with deletion of PVN p22\textsuperscript{phox}. We cannot explain this finding, but note that Ppargc1a mRNA in iBAT was elevated in AdLacZ DIO mice and increased further in AdCre DIO mice lacking PVN p22\textsuperscript{phox}. Thus, despite the unexplained failure of iBAT Ucp-1 mRNA to increase with deletion of PVN p22\textsuperscript{phox}, we suggest that the data on Ppargc1a mRNA in iBAT support our conclusion that deletion of PVN p22\textsuperscript{phox} augments thermogenesis in iBAT.

Recent findings indicate that a phenotypic switch of WAT to a brown-like adipose tissue contributes to thermogenesis (12, 30). Given that the PVN is involved in the sympathetic regulation of WAT (13, 14), we investigated if deletion of p22\textsuperscript{phox} in this brain region induces browning of WAT. Increased expression of Prdm16, Ucp-1, and Ppargc1a in WAT correlates with the metabolic activity of these tissues, which is referred to as browning of white fat (11, 31, 32). We focused on scWAT, because it is especially prone to browning (11). Deletion of p22\textsuperscript{phox} in the PVN of DIO mice was associated with increased expression of each of these browning markers in scWAT. Browning of WAT was also associated with elevated lipolysis as indicated by augmented Hsl expression (41). Indeed, DIO mice with targeted deletions of p22\textsuperscript{phox} in the PVN showed increased Hsl mRNA in scWAT. These data suggest that a reduction in NADPH oxidase–dependent oxidative stress in the PVN elevates metabolic activity in scWAT during DIO. This contributes to the observed attenuation of WAT mass and increased thermogenesis.

Deletion of p22\textsuperscript{phox} in the PVN augmented \(\beta_3\)-adrenoceptor expression in iBAT and scWAT during DIO, and this was prevented by selective \(\beta_3\)-adrenoceptor blockade with SR59230A. This suggests that blunting of oxidative stress in the PVN during DIO leads to increased \(\beta_3\)-adrenoceptor activity that contributes to increased energy expenditure in both iBAT and scWAT (32). A recent study showed that NE stimulates blood flow to iBAT (42).

PVN-derived sympathetic signals are involved in the regulation of thermogenesis by stimulating BAT and WAT metabolism (5, 15). Adipose NE levels were higher in mice fed NC than in DIO mice and were not altered by deletion of p22\textsuperscript{phox} in the PVN, suggesting that PVN ROS was not appreciably limiting adipose NE in mice fed NC. In contrast, deletion of PVN p22\textsuperscript{phox} in DIO mice increased adipose NE levels, particularly in scWAT. These data suggest that PVN ROS modulate scWAT NE levels in DIO mice but not in mice fed NC. This may indicate that the increase in energy expenditure with deletion of PVN p22\textsuperscript{phox} may be due primarily to elevated metabolic activity of the scWAT. Although an indirect indicator of sympathetic activity, the adipose NE levels taken together with the increased Adrb3 expression in iBAT and scWAT of AdCre DIO mice suggest that PVN ROS modulate adipose NE levels in DIO mice but not in mice fed NC. Increased oxidative stress in certain brain regions, including the PVN, has been shown to increase sympathetic nerve activity (43–45). In contrast, our data suggest that in DIO mice, blunting of oxidative stress in the PVN contributes to elevated sympathetic activity in adipose tissue regions. Recent evidence shows that some neurons in the

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PVN inhibit sympathetic outflow (8). We speculate that deletion of \( p22^{phox} \) and reduction of ROS in the PVN may increase sympathetic outflow to adipose tissues by reducing inhibitory signals. In support of this concept are recent findings that scavenging ROS increased the excitability of spinal GABA neurons in mice (46).

A recent study in human subjects showed that \( \beta_3 \)-adrenoceptor–mediated mechanisms elevate resting metabolic rate and activate BAT and WAT metabolism (47). Our findings that SR59230A attenuated \( Adrb3 \) mRNA in BAT and scWAT, blunted the increased VO\textsubscript{2} and heat production, and reduced scWAT browning in AdCre-treated DIO mice are consistent with the conclusion that \( \beta_3 \)-adrenergic mechanisms are at play in the increased thermogenesis and energy expenditure observed in DIO mice lacking \( p22^{phox} \) in our study. It should be noted that SR59230A crosses the blood brain barrier and acts on CNS \( \beta_3 \)-adrenoceptors (48, 49). Thus, the possibility of the involvement of CNS \( \beta_3 \)-adrenoceptor–dependent mechanisms following SR59230A injection cannot be excluded. However, given that \( \beta_3 \)-adrenoceptors are expressed in the rodent hypothalamus at very low levels (50), it is likely that peripheral adipose tissue \( \beta_3 \)-adrenoceptor–dependent mechanisms are primarily responsible for the observations in our study.

Although we and others have demonstrated the importance of sympathetic signaling in adipose tissue (5, 13, 34), we recognize the potential importance of oxidative stress in the PVN in the regulation of the hypothalamic-pituitary-adrenal and hypothalamic-pituitary-thyroid axes during HFD. The PVN plays a major role in the activity of both of these hormonal systems (51–53). Obesity alters the activity of both hormonal systems (54, 55). High-fat feeding per se increases the activity of the hypothalamic-pituitary-adrenal axis (56). Chronic elevation of glucocorticoids contributes to obesity (57). DIO furthermore increases circulating thyroid hormones (58). The role of oxidative stress during obesity in the activation of these hormonal systems remains undefined.

Our data demonstrate that selective reduction in \( p22^{phox} \)-derived ROS in the PVN during HFD feeding increases iBAT thermogenesis and browning of scWAT via activation of a \( \beta_3 \)-adrenoceptor–dependent pathway, thus blunting gain in body weight and adiposity during HFD (Figure 8). While we cannot exclude a role of ROS in metabolic processes in other regions of the brain, our findings reveal a potentially novel role for hypothalamic oxidative stress in modulating \( \beta_3 \)-adrenoceptor signaling in the development of obesity, with the hypothalamic PVN playing a pivotal role.

**Methods**

*Animals.* All studies were performed on male mice harboring a conditional allele of \( p22^{phox} \) (\( p22^{phox/^f} \)) (23) obtained from David G. Harrison (Vanderbilt University, Nashville, Tennessee, USA) and used to establish our own colony. Mice were fed an NC diet until the start of experimental procedures and were singly housed with a 12-hour light/dark cycle. All studies were conducted according to procedures approved by the Cornell University IACUC.

*Adenoviral targeting of \( p22^{phox} \) to the PVN.* Six-week-old \( p22^{phox/^f} \) mice underwent microinjection of an adenovirus encoding Cre-recombinase (AdCre, \( 4 \times 10^{10} \) plaque-forming units/ml) or titer-matched LacZ (AdLacZ) bilaterally into the PVN as described (43). We used adenovirus as the gene transfer vector since NADPH oxidase is expressed in both neurons and glia, and this virus targets both cell types (59, 60). Briefly, mice were anesthetized (ketamine 150 mg/kg + xylazine 15 mg/kg, i.p.) and placed in a stereotaxic device (Stoelting). After drilling a hole in the appropriate skull location, AdCre or AdLacZ was injected bilaterally into the PVN using a glass micropipette (coordinates: –0.7 mm bregma, ± 0.2 mm midline, 4.6 mm dorsal). Mice were given 7–10 days for recovery to allow for viral expression before the start of HFD. We and oth-
Measurement of ROS in the brain. DHE fluoroscopy was used to measure ROS in the PVN as described in previous studies (43, 45). After decapitation, brains were immediately frozen on dry ice. Micropunches of the PVN, arcuate nucleus, and CTX were isolated using the coordinates of Paxinos and Franklin (64) and methods utilized by our laboratory and others (43, 62). Briefly, brains were embedded in optimal cutting temperature (OCT) freezing medium and coronally cryosectioned in the appropriate location based on anatomical landmarks. A single micropunch (Stoelting) was obtained for the arcuate nucleus (0.25 mm), whereas bilateral micropunches were collected for the PVN (0.5 mm) and somatosensory CTX (0.75 mm). Brain tissue from 2 mice was pooled per biological sample. RNA was extracted using TRIzol reagent according to the manufacturer’s protocol (Invitrogen). RNA with an A260/A280 ratio between 1.6 and 2.0 was used for reverse transcription with the qScript cDNA kit (Quanta BioSciences). Quantitative rtPCR was performed in triplicate with an ABI 7500 Fast Thermocycler (Applied Bioscience) using SYBR Green (Quanta BioSciences). All rtPCR primers were designed using PrimerQuest from Integrated DNA Technologies (Table 1). Data were analyzed using the ΔΔCt method and expressed as fold change (65). All results were normalized to 18S.

Table 1. Target genes and primer sequences for quantitative rtPCR

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>18S</td>
<td>F: 5'-GTAAGCGCTGGAAGCCACATT-3' R: 5'-GCCAATTCAGAGGCTGACTT-3'</td>
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<tr>
<td>P22phox</td>
<td>F: 5'-TTCTTTTACCTAACAGTTTGCCTT-3' R: 5'-GACGTTTCTAGGAGGCTGACTT-3'</td>
</tr>
<tr>
<td>Ucp1</td>
<td>F: 5'-GAGCAAGTCCACTTCCACAGTTT-3' R: 5'-TCGATGAGGCTGACTT-3'</td>
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<td>Adrb3</td>
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</tr>
<tr>
<td>Hsf1</td>
<td>F: 5'-GAGCAAGTCCACTTCCACAGTTT-3' R: 5'-TCGATGAGGCTGACTT-3'</td>
</tr>
<tr>
<td>Prdm16</td>
<td>F: 5'-GAGCAAGTCCACTTCCACAGTTT-3' R: 5'-TCGATGAGGCTGACTT-3'</td>
</tr>
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Quantitative real-time polymerase chain reaction (rtPCR). All brain tissues were removed immediately after decapitation and frozen on dry ice. Micropunches of the PVN, arcuate nucleus, and CTX were isolated using the coordinates of Paxinos and Franklin (64) and methods utilized by our laboratory and others (43, 62). Briefly, brains were embedded in optimal cutting temperature (OCT) freezing medium and coronally cryosectioned in the appropriate location based on anatomical landmarks. A single micropunch (Stoelting) was obtained for the arcuate nucleus (0.25 mm), whereas bilateral micropunches were collected for the PVN (0.5 mm) and somatosensory CTX (0.75 mm). Brain tissue from 2 mice was pooled per biological sample. RNA was extracted using TRIzol reagent according to the manufacturer’s protocol (Invitrogen). RNA with an A260/A280 ratio between 1.6 and 2.0 was used for reverse transcription with the qScript cDNA kit (Quanta BioSciences). Quantitative rtPCR was performed in triplicate with an ABI 7500 Fast Thermocycler (Applied Bioscience) using SYBR Green (Quanta BioSciences). All rtPCR primers were designed using PrimerQuest from Integrated DNA Technologies (Table 1). Data were analyzed using the ΔΔCt method and expressed as fold change (65). All results were normalized to 18S.

Measurement of NE in the brain. NE quantification in adipose tissue. NE was quantified using an ELISA in duplicate according to the manufacturer’s protocol (IBL America). Tissues were homogenized by sonication in homogenization buffer as described previously (35), and the clear homogenates were stored at –80°C until further use. The measurements were normalized to the protein concentration of each sample that was determined by a bicinchoninic acid assay.
**Data analysis.** Data are expressed as the mean ± SEM. To determine statistical significance, we performed a 2-way ANOVA followed by a Sidak multiple comparison post-hoc test for time-course experiments. In all other experiments, a 1-way ANOVA followed by Tukey's post-hoc test was used. P values less than 0.05 were considered significant.

**Author contributions**

HEL, ALM, and RLD designed the studies. HEL, JS, CH, CNY, and AC performed and analyzed the experiments. HEL, ALM, and RLD wrote the paper. JS, CH, CNY, ALM, and RLD participated in important discussions of the data. All authors approved the manuscript.

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