

Anti-CRF Antibody-Mediated HPA Axis Modulation Induces Selective Fat Mass Loss with Lean Mass Preservation

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Keywords: HPA Axis, obesity, glucocorticoids, corticosterone, metabolism, corticotropin-releasing factor, obesity

Conflict of interest statement. TEG, ZK, YL, ERL, EGK, ADK, KAS, HSF, and YR are inventors on patents or patent applications relating to anti-CRF antibodies and their uses. TEG and ERL are co-founders and equity holders of Andante biologics that has licensed technology related to this work. No funds from Andante biologics were used to support these studies.

Abstract

The hypothalamic-pituitary-adrenal (HPA) axis integrates central neural processing and responses to stress with endocrine and metabolic responses. We utilized a monoclonal antibody binding corticotrophin-releasing factor (anti-CRF) to achieve durable HPA axis modulation. In mice, anti-CRF treatment decreased plasma corticosterone, food-intake, body weight and fat mass, but preserved lean mass and improved metabolic parameters including insulin sensitization. These body composition effects were observed in middle aged mice on normal chow or high fat diet, aged mice, and *Mc4r*^{-/-} and *ob/ob* mice. Single-dose studies demonstrated impacts on weight gain and body composition lasting beyond the period of pharmacological target engagement. Multi-organ transcriptomic and proteomic analyses revealed organ-specific effects of anti-CRF and are consistent with altered glucocorticoid receptor (GR) signaling as a mediator of the observed phenotypes. These studies support anti-CRF-mediated HPA-axis modulation as a metabolic and anti-obesity therapeutic approach distinct from incretins.

Introduction

Acute activation of the hypothalamic-pituitary-adrenal (HPA) axis is adaptive and promotes survival during stress (1, 2). In contrast, chronic HPA axis activation and glucocorticoid (GC) dysregulation are associated with numerous metabolic, immune, and neuropsychiatric disorders (1, 2). HPA axis activity is tightly regulated by GC-mediated negative feedback at the hypothalamus and pituitary (3-5), with cellular and tissue-level GC resistance increasingly recognized as a common feature of chronic stress, metabolic disease and a driver of accelerated aging (6-9). Prolonged excess GC exposure, as in Cushing's syndrome, causes visceral adiposity, muscle wasting, insulin resistance, immune dysfunction, and brain atrophy (10, 11). More subtle elevations in GC levels and HPA axis activity are associated with obesity, increased BMI, and aging, which all associated with GC resistance (12-18).

Given the central role of the HPA axis in integrating stress, metabolic, immune, and neuroendocrine responses, considerable effort has been devoted to developing therapies that modulate its activity (19, 20). However, pharmacologic approaches targeting CRHR1, the glucocorticoid receptor (GR, NR3C1), or 11 β HSD1 have generally failed to produce sustained, robust, suppression of HPA axis activity (21-23). Consequently, despite substantial evidence implicating HPA axis dysfunction in metabolic disease (24-26), whether durable suppression of this pathway can safely improve metabolic health remains unresolved.

We previously identified a highly selective, high-affinity anti-CRF antibody (CTRND05, anti-CRF) that dose-dependently suppresses HPA axis activity under both basal and stress conditions (27). In young male mice maintained on a low-fat diet (LFD), anti-CRF increased lean mass and body weight gain, with a trend toward reduced fat mass, without altering food intake or energy expenditure. Here, we examined the long-term effects of anti-CRF-mediated HPA axis suppression across multiple mouse models, including LFD, high-fat diet (HFD), HFD plus chronic stress, aged LFD, *Mc4r*^{-/-}, and *ob/ob* cohorts. We assessed body weight, body composition, food

intake, organ weights, metabolic biomarkers, and responses to glucose and insulin challenge. We additionally performed multi-organ transcriptomic and proteomic profiling to define systems-level pharmacodynamic responses to anti-CRF treatment and identify pathways associated with its metabolic effects.

Our findings demonstrate that long-term anti-CRF treatment consistently lowers GC levels, reduces adiposity, preserves or increases lean mass, and improves insulin sensitivity across diverse metabolic contexts. Systems-level analyses support rebalanced GR signaling and normalization of GC-responsive pathways across brain, muscle, liver, and adipose tissue as central mechanisms underlying these effects.

Results

Long-Term Impacts of Anti-CRF-Mediated HPA Axis Modulation

Multiple cohorts of male and female B6-C3H F1 mice were treated with anti-CRF or an IgG1 control (Figure 1). LFD and aged-LFD cohorts received weekly anti-CRF (Figure 1A, B). HFD mice were placed on diet at 32 weeks of age and treated with either weekly anti-CRF or a single loading dose beginning at 50 weeks (Figure 1C). Across all cohorts, anti-CRF produced consistent reductions in body weight and fat mass while preserving or increasing lean mass (Figure 1A–C). Sex-dependent differences were observed. In LFD and aged-LFD cohorts, males showed smaller reductions in body weight and fat mass but greater relative increases in lean mass than females. In contrast, HFD males exhibited larger treatment responses than females. Following a single anti-CRF dose, females showed reduced weight gain for approximately six weeks before returning to control-like trajectories, but fat mass remained reduced. In males, the single dose reduced body weight, weight gain trajectory, and body composition measures throughout the study. Given the ~7-day half-life of anti-CRF and its ability to suppress GCs for approximately six weeks (Figure 3A) (27). These findings indicate that metabolic benefits persist well beyond pharmacologic suppression of the HPA axis.

Anti-CRF also produced consistent effects on organ mass (Figure 2A). Weekly treatment reduced liver and adipose tissue weights while preserving splenic mass. Skeletal muscle mass was maintained or increased, and brain mass increased by ~4–5% across cohorts. Mesenteric adipose tissue was generally the most responsive fat depot. Similar but smaller effects were observed following single-dose treatment. Because liver mass was consistently reduced by anti-CRF, we examined correlates of hepatic weight. Across HFD and LFD cohorts, mesenteric fat mass was the strongest predictor of liver weight, consistent with known links between visceral adiposity and ectopic lipid accumulation. In aged mice, body weight showed the strongest correlation (Figure S1A–C).

Food intake was reduced by approximately 10–20% across all cohorts (Figure 2B). While these effects were maintained during weekly dosing, sex differences emerged following single-dose treatment. Female HFD mice exhibited reduced food intake for up to 15 weeks after dosing, whereas food intake normalized more rapidly in males. Plasma corticosterone (CORT) levels were consistently reduced in weekly-dosed LFD, aged-LFD, and HFD cohorts (Figure 3A). In single-dose HFD mice, CORT remained suppressed at 58 weeks but returned toward control levels by 68 weeks. Control cohorts also displayed expected age-, diet-, and sex-dependent differences in CORT levels (Figure S1D).

Metabolic profiling in weekly treated HFD mice improved metabolic health (Figure 3B). Anti-CRF slightly lowered fasting glucose and produced marked reductions in insulin and HOMA-IR. Glucagon, leptin, and Fgf21 levels were also reduced, consistent with decreased visceral adiposity and reduced hepatic stress (28, 29). PYY and IL-6 were unchanged, whereas TNF- α showed a small but significant increase.

Anti-CRF Improves Glucose Homeostasis and Insulin Sensitivity

To directly assess glucose regulation, we performed oral glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) in additional long-term HFD cohorts (Figure 4). A cohort exposed to

chronic psychological stress was included to determine anti-CRF effects under combined metabolic and stress-related challenges. Body weight trajectories and organ weight changes were highly consistent with previous studies (Figure 4A and Figure S2A). Again, a single anti-CRF dose reduced weight gain throughout the post-treatment period. Heart weights were reduced in weekly treated HFD mice, whereas skeletal muscle mass was again increased. Anti-CRF also lowered circulating CORT levels in weekly treated animals (Figure S2B).

Weekly anti-CRF improved both glucose tolerance and insulin sensitivity (Figure 4B-E). Single-dose treatment also improved GTT and ITT responses, although the ITT AUC did not reach statistical significance. In chronically stressed HFD males, glucose handling and insulin sensitivity were impaired relative to unstressed controls and improved by anti-CRF treatment. To confirm tissue-level changes in insulin signaling, we measured insulin-stimulated Akt phosphorylation in liver and skeletal muscle from HFD males (Figure 4F and Figure S2C) (30, 31). Anti-CRF markedly increased the pAkt-S473 to total-Akt ratio in both tissues, whereas HFD controls showed little response. These findings are consistent with enhanced insulin sensitivity in liver and skeletal muscle following HPA axis suppression and mirror improvements observed in GTT and ITT studies. The absence of Akt activation in untreated HFD mice is consistent with profound insulin resistance.

Systems-Level Assessments of Anti-CRF

To define systems-level effects of anti-CRF mediated HPA-axis modulation, we performed bulk RNA sequencing in brain, hypothalamus, skeletal muscle, liver, and mesenteric adipose tissue and proteomic analyses in brain and skeletal muscle. Marked sex-dependent responses were observed across tissues, necessitating sex-stratified analyses. Complete datasets are provided in Supplemental Tables S2–S7. Below, we summarize the transcriptomic and proteomic findings with emphasis on: (i) the overall effects of HFD and anti-CRF in each tissue, (ii) links to GC receptor (GR)-responsive pathways, (iii) sex-dependent responses, and (iv) integration of

transcriptomic and proteomic data in brain and muscle. Gene set enrichment analyses for all tissues are provided in Supplemental Tables S8–S14. Notably, at approximately 8 total months on diet, this represents one of the longest time-on-diet studies of diet-induced obese mice compared to mice maintained on a LFD over the same time course. Initial analyses demonstrated major sex-associated differences across the tissues, necessitating sex-based comparisons. This sex-based analysis reduced overall power and thus we present the data as non-FDR rate corrected p-values (FDR corrected p_{adj} values are provided in the supplemental data tables).

Brain Transcriptomics. HFD produced substantial transcriptomic remodeling in the brain relative to LFD controls (Figure 5A-F, Tables S2, S7, and S8). Females exhibited a greater predominance of downregulated genes than males. Anti-CRF treatment also altered brain gene expression under both dietary conditions, although its effects were more pronounced in LFD mice than in HFD mice. Correlation analyses demonstrated that anti-CRF largely preserved the overall HFD transcriptional signature while reducing the magnitude of many HFD-associated changes (Figure 5B). Anti-CRF treatment in LFD mice also induced a subset of transcriptional responses that overlapped with those observed following HFD exposure, suggesting partial convergence of downstream adaptive pathways.

To identify core anti-CRF-responsive genes, we examined DEGs shared across dietary conditions and sexes. 26 common DEGs were identified (Figure 5C,D), many of which have previously been reported as GC-responsive genes. ChEA analyses revealed strong enrichment for Nr3c1-associated regulatory networks (Figure 5E), supporting altered GR signaling as a major pharmacodynamic consequence of anti-CRF treatment (32). Many of these genes, including *Sult1a1*, *Gjb6*, *Phyh*, *Fam107a*, *Plin4*, *Hif3a*, *Prodh*, *Etnppl*, and *Mfsd2a*, have previously been identified as GC-responsive transcripts (33, 34). Figure 5F highlights treatment- and sex-dependent regulation of genes involved in GR signaling, energy balance, and brain metabolism.

Hypothalamic Transcriptomics. Both HFD and anti-CRF altered hypothalamic gene expression relative to LFD controls (Figure 5G-L, Tables S3, S7, and S9). Compared with whole brain, transcriptional responses were less sex-dependent and showed a more balanced distribution of up- and downregulated genes. Anti-CRF produced relatively modest effects under LFD conditions but generated broader transcriptional responses in HFD mice.

Global correlation analyses suggested that anti-CRF had limited impact on the overall structure of HFD-associated transcriptional programs despite producing substantial gene-level changes (Figure 5H). Comparison of anti-CRF-responsive genes across dietary conditions identified shared DEGs (Figure 5I,J), which were strongly enriched for Nr3c1 target genes (Figure 5K). Because anti-CRF reduced food intake, we also examined genes involved in hypothalamic regulation of feeding behavior (Figure 5L). Treatment- and diet-dependent changes were observed in *Crh*, *Npy*, *Pomc*, *Pcsk1*, *Agrp*, *Fkbp5*, and *Nr3c1*, highlighting coordinated effects of anti-CRF on hypothalamic pathways controlling energy balance and neuroendocrine function.

Muscle Transcriptomics. Skeletal muscle exhibited some of the largest transcriptomic responses to both HFD and anti-CRF treatment (Figure 6; Tables S3, S7, and S10). Anti-CRF treatment in HFD mice generated transcriptional profiles that remained highly similar to HFD controls but generally showed reduced fold-change magnitudes (Figure 6A). In contrast to brain and hypothalamus, anti-CRF-induced changes in muscle were strongly correlated with HFD-associated transcriptional programs under both dietary conditions (Figure 6B).

Comparison of anti-CRF-responsive genes identified 185 overlapping DEGs across dietary conditions (Figure 6C,D), substantially more than observed in CNS tissues. These shared genes were highly enriched for Nr3c1-regulated pathways (Figure 6E). Shared anti-CRF-responsive genes included regulators of GR signaling, metabolism, and muscle remodeling, including *Nr3c1*,

Fkbp5, *Pdk4*, and *Plin4*. Representative genes with links to the observed physiological and proteomic phenotypes are highlighted in Figure 6F.

Liver Transcriptomics). HFD and anti-CRF substantially altered the liver transcriptome, with marked sex-dependent responses (Figure S3A–E; Tables S4, S7, and S11). Compared with CNS tissues and muscle, anti-CRF produced more heterogeneous transcriptional effects in liver with few genes were shared across treatment groups. Nevertheless, anti-CRF generally attenuated HFD-associated transcriptional responses, with stronger effects observed under HFD conditions than in LFD mice (Figure S3B-D).

ChEA analyses identified enrichment of Nr3c1-associated pathways, particularly in females, along with broader nuclear hormone receptor signaling networks (Figure S3E). Representative gene clusters demonstrated anti-CRF-, diet-, and sex-dependent regulation of pathways linked to adipocyte biology, fibrosis, interferon signaling, cytokine responses, and immune-cell populations including T cells, neutrophils, and monocytes (Figure S3F). These findings support broad hepatic remodeling by anti-CRF that includes both GC-responsive and immune-related pathways.

Adipose Tissue Transcriptomics. Adipose tissue exhibited pronounced diet- and sex-dependent transcriptional responses to both HFD and anti-CRF treatment (Figure S4; Tables S6, S7, and S12). Anti-CRF produced relatively modest effects in LFD animals but generated broader transcriptional changes in HFD mice that largely paralleled existing HFD-associated programs (Figure S4B-D). As observed in other tissues, responses under LFD conditions were more strongly sex-dependent than those observed under HFD conditions.

Shared anti-CRF-responsive genes were enriched for Nr3c1-associated pathways and related nuclear hormone receptor networks (Figure S4E), supporting altered GR signaling as a common feature of the anti-CRF response. Representative gene sets implicated regulation of insulin signaling, lipid storage and metabolism, cellular differentiation, mitochondrial function, adrenergic signaling, and epigenetic regulatory pathways (Figure S4F). These data indicate that anti-CRF

broadly influences adipose tissue metabolic and signaling programs while interacting with the transcriptional adaptations induced by HFD.

Brain Proteomics. DIA-TIMS-TOF proteomics quantified 6,878 proteins in brain (Figure 7; Tables S2, S7, and S13). Across all comparisons, substantially fewer differentially abundant proteins (DAPs) were identified than differentially expressed transcripts, indicating more modest proteomic than transcriptomic responses (Figure 7A). Nevertheless, anti-CRF treatment produced consistent alterations across dietary conditions and sexes with a subset of shared DAPs showing consistent regulation across dietary conditions and sexes (Figure 7B,C).

ChEA analyses demonstrated enrichment for Nr3c1-associated pathways among anti-CRF-responsive proteins (Figure 7D), supporting altered GR signaling as a common feature of the treatment response. Correlation analyses further indicated that anti-CRF modestly attenuated HFD-associated proteomic changes while preserving the overall structure of the HFD proteomic signature (Figure 7E). Together, these findings suggest that anti-CRF produces reproducible but relatively subtle remodeling of the brain proteome consistent with its broader effects on GC-responsive pathways.

Muscle Proteomics. DIA-TIMS-TOF proteomics quantified 2,877 proteins in skeletal muscle (Figure 8; Tables S5, S7, and S14). As observed in brain, proteomic responses were smaller than transcriptomic responses and displayed substantial sex dependence (Figure 8A). Female HFD mice exhibited the largest number of altered proteins, whereas overlap among anti-CRF-responsive DAPs across dietary conditions was more limited (Figure 8B,C).

Unlike the transcriptomic datasets, overlapping DAPs showed less consistent enrichment for Nr3c1-regulated pathways (Figure 8D), suggesting additional post-transcriptional influences on the muscle response. Correlation analyses indicated that anti-CRF partially attenuated HFD-associated proteomic remodeling and produced broadly similar proteomic signatures across dietary conditions, particularly in females (Figure 8E). Overall, these findings support broad but

context-dependent effects of anti-CRF on muscle protein networks that only partially mirror the corresponding transcriptomic changes.

RNA–Protein Relationships. We examined global RNA-protein correlations (Figure 9). In muscle, HFD-induced RNA and protein changes were uncorrelated (Figure 9A). Anti-CRF treatment produced weak but detectable RNA-protein correlations. In female muscle, the negative y-intercept in HFD mice (-0.24) suggested overall suppression of protein abundance relative to transcript levels, an effect partially normalized by anti-CRF treatment. Similar analyses in brain revealed almost no RNA-protein correlation in HFD mice and weak correlations with anti-CRF treatment (Figure 9B). Unlike muscle, brain y-axis intercepts remained near zero across all conditions. These findings demonstrate extensive transcriptomic remodeling by HFD and anti-CRF, whereas proteomic changes were more modest and poorly correlated with transcript abundance.

Functional Clustering of Muscle Proteomic Results. We performed an in-depth analysis of diet- and anti-CRF-induced alterations in the muscle proteome (Figure 10). Using an AI-assisted functional clustering approach, DAPs were grouped by biological function and directionality of change for each treatment condition and sex. Average log₂ FC were calculated for each cluster relative to sex-matched LFD IgG1 controls. Heatmaps summarizing these functional clusters are shown in Figure 10A (female) and Figure 10B (male). Individual cluster heatmaps for each DAP within the cluster are provided in Figure S5.

Functional clustering revealed extensive sex-dependent remodeling of the muscle proteome in response to both HFD and anti-CRF treatment (Figure 10A,B). Female HFD mice exhibited broader proteomic disruption than males, with prominent decreases in pathways related to muscle structure and function, mitochondrial biology, proteostasis, RNA processing, and extracellular matrix regulation. In contrast, male HFD muscle displayed fewer alterations and greater evidence of adaptive metabolic remodeling, particularly in lipid metabolism and mitochondrial pathways.

Anti-CRF broadly attenuated many HFD-associated proteomic changes while producing overlapping signatures under both dietary conditions. In females, treatment frequently reversed pathways disrupted by HFD, whereas in males anti-CRF more often reduced the magnitude of HFD-associated alterations. Common themes across sexes included regulation of mitochondrial function, muscle structure, and protein homeostasis. Despite substantial sex differences in individual pathways, the overall direction of anti-CRF-induced remodeling was remarkably similar in male and female HFD muscle, supporting a shared physiological response to HPA-axis modulation. Heatmaps for individual DAPs in each cluster are provided in Figure S5.

To provide greater mechanistic insight, representative heatmaps from these functional clusters are shown in Figure S7A. Several key effects of anti-CRF emerged:

1. Evidence for restored GC sensitivity. Anti-CRF consistently increased *Nr3c1* and decreased *Fkbp5* (FKBP51), changes consistent with enhanced GC responsiveness (12, 35). Many of the most altered proteins are known GR-regulated transcriptional targets (33, 34).
2. Metabolic reprogramming. Alterations in *Pdk4*, *Plin5*, and other metabolic regulators support a shift from lipid oxidation and mitochondrial oxidative phosphorylation toward glycolytic metabolism (36).
3. Mitochondrial remodeling. Respiratory chain proteins were decreased in female HFD but increased in male HFD muscle. Anti-CRF attenuated these sex-dependent responses. RNA and protein changes were completely uncoupled for the 113 downregulated mitochondrial proteins in female HFD muscle ($R^2 = 0.00$), consistent with complex post-transcriptional regulation. Impacts of anti-CRF are consistent with known roles of GC on mitochondria (37).
4. Muscle structure and contractile proteins. Proteins involved in muscle architecture and function were broadly reduced in female HFD mice but largely unchanged in males. Anti-

CRF regulated many of these proteins similarly across diets and sexes despite divergent HFD responses.

5. Coagulation and ECM proteins. Reduced levels of coagulation, platelet, and some ECM-associated proteins in female HFD muscle may reflect dysfunction in non-muscle tissues, as many of these proteins are not synthesized locally in skeletal muscle.
6. Neuropathy-related changes. Alterations in axonal and myelin-associated proteins are consistent with a diabetic neuropathy phenotype. These effects were more pronounced in female HFD mice and were attenuated by anti-CRF.
7. Proteostasis and RNA processing. Broad disruptions in translation, RNA processing, and proteostasis pathways were evident in female HFD muscle and were largely normalized by anti-CRF. Similar changes were generally absent in males.
8. Broad actions beyond classic atrogenes. Collectively, these findings demonstrate that anti-CRF exerts complex, systems-level effects on skeletal muscle that extend well beyond regulation of canonical GC-responsive atrogenes (38).

Given the weak global correspondence between RNA and protein abundance, we next examined RNA-protein relationships among the most altered DAPs (Figure 10C,D). In females, anti-CRF modestly increased RNA-protein correlations and shifted the regression intercept from -0.62 to 0.02. In males, anti-CRF also strengthened RNA-protein correlations but shifted the positive intercept below zero. These findings suggest that anti-CRF partially restores coordination between dysregulated transcripts and protein abundance in HFD muscle.

Functional Clustering Analyses of Brain Proteomic Data. Functional clustering analyses identified multiple brain protein networks altered by both HFD and anti-CRF treatment (Figure 11A,B,S6). These responses were strongly sex dependent. HFD affected pathways related to extracellular matrix biology, coagulation and immune signaling, lipid and redox metabolism, and cellular homeostasis, with distinct patterns in females and males. Anti-CRF generally preserved the

direction of HFD-associated changes while attenuating their magnitude. Under LFD conditions, anti-CRF also induced sex-dependent proteomic responses that partially overlapped with those observed in HFD mice, particularly within metabolic, extracellular matrix, and immune-related pathways. These findings indicate that anti-CRF influences multiple aspects of brain biology through mechanisms that are both sex-dependent and only partially explained by dietary status.

To provide greater mechanistic insight, representative functional cluster heatmaps are shown in Figure S7B. Several notable effects of anti-CRF emerged:

1. Evidence for altered GR signaling. Anti-CRF decreased Fkbp5 (FKBP51) and increased Nr3c1, consistent with reduced GC exposure and enhanced GR responsiveness. Hsd11b1 was increased in male brain, while Crhbp was reduced in male anti-CRF LFD mice.
2. Plin4 is a sensitive anti-CRF target. Plin4 (Perilipin-4), a regulator of lipid droplet stability and known GR target, was among the most strongly decreased proteins in both brain and muscle following anti-CRF treatment (39).
3. Metabolic pathways are broadly affected. Changes in apolipoproteins (Apoa4, Apoh, and Apoa1), likely reflecting circulating protein levels, were prominent and sex dependent. Several glycogen metabolism proteins were decreased by anti-CRF. Mitochondrial responses were heterogeneous, although proteins such as Prodh and Gpt2 were consistently reduced.
4. Immune-related pathways show strong sex differences. Complement, coagulation, and immune proteins exhibited pronounced sex-dependent responses to anti-CRF.
5. Subtle effects on neural cell types and signaling. Anti-CRF influenced markers of interneurons, neuromodulation, neuronal signaling, plasticity, and oligodendrocytes. Reduced parvalbumin (Pvalb) in HFD mice is consistent with previous reports of HFD-induced dysfunction in inhibitory interneurons (40). While most changes were not

consistently observed across sexes and conditions, Glp1r protein levels appeared elevated in all groups.

6. Brain mass changes remain unexplained. No clear protein alterations accounted for the small but consistent increase in brain mass following anti-CRF treatment.

Finally, we examined whether anti-CRF altered the relationship between transcript and protein abundance (Figure 11C,D). In female mice, RNA-protein HFD versus LFD but became strongly positive following anti-CRF treatment in HFD. For the top DAPs altered by anti-CRF there are poor or anti-correlations in the HFD versus LFD comparison, but strong correlations of these same RNAs and proteins in upon anti-CRF treatment in HFD. These data suggest that anti-CRF may restore the perturbed balance between select dysregulated transcripts and protein levels in the HFD brain.

Anti-CRF in *Mc4r*^{-/-} and *ob/ob* mice.

We evaluated anti-CRF impacts in monogenic models of obesity with disrupted hypothalamic satiety signaling: *Mc4r*^{-/-} and *ob/ob* (leptin-deficient) mice (41, 42). Anti-CRF treatment markedly slowed weight gain in both models, reduced fat mass, and preserved lean mass. In female *Mc4r*^{-/-} mice, lean mass increased in absolute terms (Figure 12). Food intake was reduced in female and male *Mc4r*^{-/-} mice and in female *ob/ob* mice (Figure 13A). In male *ob/ob* mice, however, the initial reduction in food intake was not sustained throughout treatment. Across all cohorts, anti-CRF attenuated the rise in circulating corticosterone observed in controls, and in female *Mc4r*^{-/-} mice reduced corticosterone below baseline levels (Figure 13B). Glucose tolerance improved in female *Mc4r*^{-/-} mice but not in the other cohorts (Figure 13C). Despite this, insulin and glucagon concentrations were markedly reduced in all anti-CRF-treated groups, indicating metabolic benefits. To determine whether preservation of lean mass translated into functional improvements, we assessed grip strength. Anti-CRF improved grip strength over time in all cohorts except male *ob/ob* mice, in which grip strength relative to body weight was like controls

(Figure 13D). These findings were highly reproducible in an independent validation cohort that included *Mc4r*^{-/-}, *Mc4r*^{+/-}, *ob/ob*, *ob/wt*, and wild-type mice (Figure S8). Consistent with our previous observations in young wild-type animals, anti-CRF had little effect on body weight in wild-type or *ob/wt* mice. In contrast, anti-CRF reduced weight gain in *Mc4r*^{+/-} mice, a model that closely resembles the partial MC4R loss-of-function mutations that represent the most common form of monogenic obesity in humans (43).

Discussion

Across multiple obesity models, sustained HPA axis modulation through peripheral anti-CRF antibody administration improved metabolic health. Anti-CRF consistently reduced circulating corticosterone (CORT), decreased adiposity, preserved or increased lean mass, improved insulin sensitivity, and lowered insulin and glucagon levels. Single-dose studies demonstrated reproducible, durable effects that extended well beyond the pharmacokinetic window of target engagement, suggesting persistent physiological reprogramming. Together, these findings support chronic CRF neutralization as a viable strategy for improving metabolic fitness.

Systems-level omic analyses are consistent with restoration of GR signaling as the main mechanism underlying these effects. Across tissues, anti-CRF treatment reduced Fkbp5 (Fkbp51) and increased Nr3c1 expression, changes consistent with enhanced GR sensitivity (12, 35). These signatures were accompanied by normalization of numerous GC-responsive pathways. Given the well-established role of Fkbp51 as a negative regulator of GR signaling, our data support a model in which chronic HPA axis activation promotes a state of GC resistance that contributes to metabolic dysfunction, whereas anti-CRF restores adaptive GC responsiveness through sustained reductions in GC exposure.

There was no evidence of anti-CRF-mediated toxicity. Previous studies demonstrated preservation of stress-induced CORT responses despite reductions in basal GC levels, indicating that anti-CRF modulates rather than ablates HPA axis function (27). Residual CORT production

likely reflects incomplete CRF neutralization within the hypophyseal portal circulation and ACTH release driven by alternative regulatory inputs. Although responses varied quantitatively according to sex, age, and dietary context, the overall metabolic effects of anti-CRF were remarkably consistent across cohorts. Future studies more closely looking at circadian HPA axis dynamics following anti-CRF will be needed.

Our current results are distinct from results in young male LFD B6 mice where anti-CRF increased lean mass and body weight gain. These differences are likely attributable to starting body composition, diet, and age, as the lack of impact on body weight in younger mice is reproduced in the wild-type B6 controls used in the cohorts in Figure S8. Future, studies will be needed to further establish how parameters such as strain, diet, and age alter the impacts of anti-CRF.

Several findings suggest that anti-CRF acts through additional mechanisms extending beyond reduced food intake (44). Though food consumption was decreased in most cohorts, anti-CRF remained effective in both *Mc4r*^{-/-} and *ob/ob* mice, models with disrupted hypothalamic satiety signaling (41, 42). The magnitude and persistence of improvements in body composition, insulin sensitivity, and metabolic biomarkers exceeded those expected from modest reductions in food intake alone (45), though these studies will need to be extended with more rigorous metabolic monitoring and pair-feeding studies. These findings argue that chronic GC excess and resistance represent a convergent downstream mechanism across diverse obesity etiologies. The enhanced response observed in aged mice is also consistent with age-related increases in allostatic load, adiposity, and FKBP51 expression, suggesting that anti-CRF may be particularly effective in physiological states characterized by prolonged HPA axis activation (12, 20, 46, 47).

Our findings are also not readily predicted by canonical views of CRF signaling in the hypothalamus. Acute central CRF administration is anorexigenic; therefore, inhibition of CRF might be expected to increase feeding and weight gain (48, 49). Instead, anti-CRF reduced adiposity and improved metabolic health. These observations suggest that in chronic obesity,

sustained GC exposure and resistance become drivers of pathology, outweighing the acute effects of CRF on feeding circuits. In this context, prolonged CRF suppression may restore homeostatic regulation rather than simply basal satiety signals. Indeed, similar phenotypes have been reported in *Crf* and *Fkbp5* loss-of-function models, and our transcriptomic evidence support a potent effect on hypothalamic satiety-related transcripts (50-54).

Binding of CRF within the hypophyseal portal circulation likely underlies the primary pharmacologic effect of anti-CRF. Whether any observed effects are mediated by direct targeting of CRF within the brain is an open question. Approximately 0.1% of circulating antibody is predicted to penetrate the CNS, and the low nanomolar concentrations of anti-CRF predicted to be in the brain could engage central CRF (27, 55).

A striking observation was the disconnect between transcriptomic and proteomic responses. HFD-induced changes in RNA abundance showed little relationship to corresponding protein changes in muscle or brain, highlighting extensive post-transcriptional regulation of translation, protein turnover, and proteostasis. Anti-CRF partially restored RNA-protein concordance. More broadly, these findings reinforce growing evidence that transcript abundance alone is an incomplete or even misleading surrogate for protein expression and biological function (56-59). The integrated transcriptomic and proteomic datasets generated here therefore provide a valuable resource for identifying tissue-specific pathways and candidate mediators of anti-CRF action.

Compared with incretin-based therapies, anti-CRF appears mechanistically distinct. Incretins primarily improve metabolic health through reductions in food intake and consequent weight loss (60). Anti-CRF also improved body composition and metabolic parameters but produced direct evidence of insulin sensitization in both liver and skeletal muscle, accompanied by marked reductions in fasting insulin, glucagon, and Fgf21 levels. Anti-CRF additionally preserved or increased lean mass and preferentially reduced visceral adiposity. Single dose studies in HFD

show impacts of anti-CRF beyond the period of target engagement suggesting metabolic reprogramming. Future studies will need to investigate underlying mechanisms; given known epigenetic modifications of downstream GR target genes such as *Fkbp5*, interrogation of anti-CRFs impact on the epigenome will be informative.

The preservation of lean mass alongside reductions in adiposity may have broader implications for age-related metabolic disease and sarcopenic obesity. Decades ago, Sapolsky, Krey, and McEwen proposed the “GC cascade hypothesis” mechanistically linking chronic stress, HPA axis activation, GCs and aging (61), with subsequent work demonstrating that GCs influence multiple hallmarks of aging (8, 9). Our findings in aged mice provide support for this framework and suggest that anti-CRF may offer a useful experimental approach for testing the contribution of chronic HPA axis activation to aging-related decline.

This study represents one of the longest diet-induced obesity paradigms reported in middle-aged mice and is complemented by extensive multi-tissue transcriptomic and proteomic profiling. Collectively, these data demonstrate that antibody-mediated CRF neutralization can safely and durably improve body composition and metabolic fitness across multiple contexts of metabolic dysfunction. Unlike downstream receptor antagonists, anti-CRF achieves broad modulation of HPA axis activity while preserving physiological responsiveness to stress. These findings support CRF neutralization as a distinct therapeutic strategy for obesity and other conditions characterized by chronic HPA axis activation and GC resistance.

Methods

Sex as a biological variable. The studies use male and female mice with statistical analyses within sex.

Mice, diets and handling. The following mouse strains were utilized in this study (corresponding Jackson Labs stock numbers are listed in parentheses): Wild type C57BL/6J (000664), WT C57BL/6J (000664) x C3H/HEJ (000659), B6.Cg-Lepob/J (*ob/ob*, 000632), B6.129S4-

Mc4rtm1Low/J (*Mc4r*^{-/-}, 032518). C57BL/6J x C3H/HeJ F1 hybrid strain mice were prepared by breeding a male breeder mouse from this line from our in-house colony with a C3H/HeJ female purchased from Jackson Laboratories. For *ob/ob* and *Mc4r*^{-/-} mice, homozygous animals were purchased from Jackson laboratories and used directly whereas; heterozygous animals were bred in house to produce homozygous, heterozygous, and wild type offspring for studies in Figure S8. Same sex littermates were housed in groups of 3-4 and cluster randomized to treatment. Animals were maintained on chow diets unless otherwise specified (Teklad 2918x; protein 24% kcal/g, Fat 18% kcal/g, Carbohydrate 58% kcal/g). High-fat diets (Research Diets; catalog #D12492i protein 20% kcal/g, Fat 60% kcal/g, Carbohydrate 20% kcal/g) were provided to animals assigned to diet-induced obesity groups. Animals had ad libitum access to food and water except for described fasting windows prior to blood collection.

Animals were maintained at 23°C +/- 1 at 40-60% humidity. Health status was normal for all animals that reached experimental endpoints. For the LFD and HFD cohorts, animals were weighed, and food consumption was quantified twice per week. For the monogenetic model cohorts animals were weighed, and food consumption was quantified once per week. For restraint stress, mice were placed into rodent restrainers 2-times per week (Broome-Style, Plas-Labs or Braintree Scientific) for 30 min, with the restrainer being kept in the mouse's home cage during this time. Stress was performed between ZT 18-22. For grip strength assessments, we used the fore-limb. The mouse was placed on the metal grid of the grip tester (BioSeb, FL, USA) until it gripped the grid. The mouse was then gently pulled by the tail base until it could no longer hold the grid. Each measurement was conducted 3X with 1-2 minutes intervals for three consecutive days. The maximum force generated was used for statistical analysis.

All blood collections were performed between Zeitgeber Time 18-23. At study endpoint, mice underwent decapitation and organs dissected rinsed in PBS and weighed on a scale (#AL54; Mettler Toledo). The left lobe of the liver was harvested from each mouse.

Anti-CRF and IgG1 Control Antibodies. The anti-CRF antibody (CTRND05) was previously described (27). Over the course of studies we utilized fiber cell production from the original hybridoma, and recombinant antibody generated by Genscript following large scale transient transfection. For hollow fiber cell production, we used the bioreactor Duet Pump (P3202, FiberCell Systems, New Market, MD), with the cartridge (C-2008, FiberCell Systems). According to the manufacturer's protocol, 1×10^8 hybridoma cells were seeded with chemically defined media into the cartridge. Antibody accumulating in the ECS was harvested yielding an average of 250 mg/month/hollow fiber cartridge. Anti-CRF and anti-ricin IgG1 control were prepared by this method.

Antibodies produced in the bioreactor were affinity purified over a HiTrap protein G column (17040501, Cytiva, Marlborough, MA) using a GE AKTA Prime Plus FPLC and eluted with 200 mM glycine pH 2.5 and neutralized with 1M Tris pH 8.8. IgG-enriched eluates were collected based on the A280 from the AKTA and neutralized to pH 7.4 and buffer exchanged over a Hiprep 26/10 desalting column (17508701, Cytiva) into PBS pH 7.4 to complete the final product. Final purified concentration of antibody product was 2.0-5.0 mg/mL.

Body composition analysis. Body composition (fat mass, lean mass, and body water) was quantified every 4 weeks using EchoMRI Quantitative Magnetic Resonance Body Composition Analyzer (Echo Medical Systems, Houston, Texas). Calibrations were performed on corn oil within a clear glass container, per manufacturer's instructions.

Glucose- and insulin-tolerance tests (GTT/ITT). Glucose tolerance was assessed in 5 h fasted mice after oral gavage of 2g/kg glucose (DIO mice) or i.p. injection of 2g/kg glucose (Monogenetic models). Insulin tolerance was assessed in 5 h fasted mice after i.p. injection of 1.0 U Insulin per kilogram body weight (Humalin R, Eli Lilly). Tail vein blood glucose was measured using a handheld glucometer (TheraSense Freestyle) at baseline and after 15, 30, 60 and 120 min.

Corticosterone (CORT) Measurement. MP Bio corticosterone double antibody RIA Kit (07120102) or Invitrogen Cortisone Competitive ELISA Kit (EIA-COR) were used according to instructions for studies in the non-genetic models. Mouse plasma was diluted 1:100 in assay buffer for nonstress basal conditions, 1:200 for recovery time points, and 1:400 for maximum-stress time points.

Fasting Hormone Measurements. Plasma from fasted mice or terminal mice was analyzed using the MSD U plex U-PLEX Metabolic Combo 1 (mouse) on the MESO QuickPlex SQ 120.

Westerns Blots. Frozen skeletal muscle were homogenized in RIPA buffer containing protease inhibitor cocktail and Halt phosphatase inhibitor cocktail (Thermo Fisher Scientific) and ultracentrifuged at 100,000 $\times g$ for 1 h. 15 μg of RIPA lysate was separated on 4–20% Tris-Glycine gel (Novex, Invitrogen) and transferred to the Immobilon-FL PVDF membrane (MilliporeSigma). Membranes were blocked in 0.5% casein, incubated with primary antibodies overnight at 4°C pAkt and Akt antibodies: 1:1000 (#4060T and #4691T, respectively, Cell Signaling); Beta-tubulin antibody: 1:2000 (ab6046, Abcam) and protein bands were visualized using the Li-Cor Odyssey M Infrared Imaging system (Li-Cor Biosciences, Lincoln, NE, USA). Band intensity was quantified using ImageJ software (National Institutes of Health).

RNA-seq and analysis. Harvested organs were snap-frozen in liquid nitrogen. Tissues were pulverized under liquid nitrogen using a mortar and pestle, and total RNA extracted with Trizol reagent (Thermo Fisher Scientific). RNA was passed over a Qiagen RNeasy column with on-column DNase treatment. A subsequent DNase treatment using Turbo DNA-free kit (Thermo Fisher Scientific) was performed. RNA was quantified using the Qubit 4 fluorometer and the RNA HS assay (Thermo Fisher Scientific), and quality was determined using the Fragment Analyzer Automated CE System and the Standard Sensitivity RNA Analysis kit (Agilent Technologies). 1 μg of total RNA was polyA enriched and subjected to library preparation using the TruSeq RNA Sample Prep Kit v2 (Illumina). Library size was determined with the High-Sensitivity NGS Fragment Analysis Kit (Agilent Technologies). Libraries were quantified (KAPA iosystems) and

pooled to minimize batch effects from library preparation and sequencing and to achieve 30–50 million reads per sample.

The resulting FASTQ files were aligned against the mouse genome using STAR v2.7.9a (62) and GRCm39. Subsequent analyses were performed in R v4.5.2. Differential gene expression analysis was performed with DESeq2 v1.48.2 (63). Differentially expressed genes between treated versus control groups within each tissue were defined as $p\text{-value} \leq 0.05$ and an absolute $\log_2\text{FC} \geq 0.2$. Transcriptomic group sizes were $n=5$ for LFD/IgG1/M and LFD/anti-CRF/M; $n=4$ for LFD/IgG1/F and LFD/anti-CRF/F and $n=6$ for all other groups. Gene set enrichment analysis was performed with Enrichr v3.4 (32). Pathways and terms reaching a $p\text{-value} \leq 0.05$ were considered. FDR corrected p values (p_{adj}) are provided in all supplemental data files. Venn diagrams were generated with ggVennDiagram v1.5.5. All other transcriptomic plots were generated with ggplot2 v4.0.0 and cowplot v1.2.0. No original code is reported for these analyses.

DIA-MS Proteomics. Tissues were homogenized in 8M urea buffer (1:10) with 1x Halt protease inhibitors and 1x Halt phosphatase inhibitors plus one scoop of stainless-steel beads with homogenization blender (2 x 5 min; speed 12). Homogenized samples were centrifuged at 13,300 rpm for 1 min, supernatants transferred to fresh 1.5mL tubes, centrifuged at 13,300 rpm for 1 min, sonicated 5 sec on and 5 sec off for 3 cycles, centrifuged at 13,300 rpm for 10 min, and transferred 110 μL into a 2 mL deep well plate. 10 μL of homogenates plus 90 μL 8M urea buffer were incubated while shaking (800 rpm) for 2 min then centrifuged for 1 min at 2,400 rpm. BCA (bicinchoninic acid) assay was used to determine total protein concentration, and all lysates were normalized to 4 $\mu\text{g}/\mu\text{L}$.

50 μL of normalized lysates (200 μg) were transferred to 2 mL deep well plate for digestion and incubated while shaking (800 rpm) 25°C for 30 min as follows 1) with 5 μL of 50 mM DTT (Sigma, D0632), with 5 μL of 100 mM IAA (Sigma I1149) in the dark. Subsequently, 42.5 μL 100mM Tris-HCl (pH8.0) and 6.7 μL LysC (5 mAU/ μL) were added and the sample incubated on the shaker

overnight, followed by 1) addition of 290 μ L 100 mM Tris (pH8.0), add 13.33 μ L Trypsin (1 μ g/ μ L), and overnight shaking and 2) dilution with 410 μ L of 4% phosphoric acid and incubation on shaker (1500 rpm) for 20 min. Centrifuge at 2500 rpm for 1 min and check for pH $\leq$ 2. SPE clean up using MCX column plate (Waters Oasis MCX 96-well Plate, 10 mg Sorbent per Well, SKU: 186000259) was performed with the following steps: 0.5mL 100mM Ammonium formate with 2% FA (equilibrate), load total volume of samples and discard flow through, 0.75mL 100mM Ammonium formate with 2% FA (wash 1), 0.75mL 100% Methanol (wash 2), 0.55mL 5% Ammonium hydroxide in Methanol (elute). Eluants were dried to completion using a SpeedVac.

Samples (500 ng) of digested mouse brain or muscle were loaded and eluted by an Evosep One LC system coupled with a Bruker timsTOF HT. Data were acquired at 30 SPD with Performance Column (8 cm $\times$ 150 μ m $\times$ 1.5 μ m, part number EV1109) in DIA mode with narrow mobility window 0.85–1.35 V s cm⁻² (volts x seconds/square cm). Database searches and protein quantification were performed on 104.d files (48 brain, 4 brain pooled standards, 48 muscle, 4 muscle pooled standards) using Data-Independent Acquisition by Neural Networks (DIANN; version 1.9.1; C20) (64). Full log of search parameters can be found on Synapse (<https://www.synapse.org/Synapse:syn73668067>); briefly, library-free, fully tryptic parameters with 0.01 FDR, fixed modification for cysteine carbamidomethylation, variable modification for methionine oxidation and N-terminal acetylation, and mouse database (82,718 proteins; 25,220 genes).

Differential Protein Analysis. Gene-level protein quantification files were used to calculate differential protein abundances. Rows were dropped if values were not present across at least half of the samples in each experimental group. Data were log₂ transformed and analyzed for abundance using imputation-free, empirical Bayes moderated t-statistics test provided in the Limma package (65). Differentially abundant proteins were defined with a p-value $\leq$ 0.05 and an

absolute $\log_2\text{FC} \geq 0.2$. All downstream analyses followed workflows as in DEG analysis. Proteomic group sizes were $n=5$ for LFD/IgG1/F and LFD/anti-CRF/F and $n=6$ for all other groups.

Functional Clustering of DAPs. DAPs ($p < 0.05$) for each condition and directionality (e.g., muscle female HFD IgG1 v LFD IgG1 with $\log_2\text{FC} > 0$) were uploaded to Chat GPT5.2 with the instruction to “group the DAPs into functional clusters”. When numerous small functional clusters or very large functional clusters were generated, these were iteratively refined by manually grouping smaller related clusters or uploading the larger cluster to Chat GPT 5.2 with a query to be more granular with respect to the broad functional categorization. Small clusters that did not add biological relevance were excluded; nevertheless, more than 90% of DAPs were assigned a cluster. Following this initial functional clustering, all clusters were refined by inspection of membership by several of the authors and by reuploading to Chat GPT5.2 in a new session with the explicit instruction to not use previous data. Effectiveness of the AI-assisted clustering was assessed by running the DAP clusters through Enrichr (32) to ensure that the assignment and cluster naming was consistent with top terms identified by GO ontologies, Reactome pathways 2024, and KEGG 2026.

Statistical Analysis. Except for the systems data, all the statistical analyses and graph creations were done in GraphPad Prism 11. Methods for systems analyses are described above. Tests used are indicated in legends. In Table S1, details of all non-omic statistical tests are provided. All comparisons are within sex with almost all the studies reported using comparable groups of male and female mice.

Study Approval. All animal procedures were approved by the University of Florida and Emory Institutional Animal Care and Use Committees in accordance with NIH guidelines.

Data availability. A supporting data values Table is provided that contains individual values used to generate the graphs. All unique/stable reagents generated in this study are available from the Lead Contact. RNA sequencing data are available in the NCBI Gene Expression Omnibus (GEO):

GSE317085 (brain), GSE317086 (fat), GSE317092 (hypothalamus), GSE317093 (liver), GSE317099 (muscle). Proteomics data are deposited in MassIVE under dataset identifier MSV000102636.

Author Contributions.

ZAK designed studies, conducted most of the most experiments, acquired the data and helped with writing and editing the manuscript. TI conducted the experiments on the Mc4r and *ob/ob* mice and contributed to data analyses. CMW, NTS, and LW conducted and helped with the analyses of the proteomic data. YR, YL, JL HSF, DR, TBL, ERL, XL, KFA, AG, KDD, KAS provided reagents and assisted with acquiring data and tissues. KNM and TEG designed and analyzed the transcriptomic studies. TEG, AK, EGK, JL, and JLB helped designed the studies and analyzed data. TEG, ZK and KNM prepared figures and wrote the initial drafts of the manuscript. All authors reviewed the draft manuscript and contributed to edits.

Funding Support

This work is the result of NIH funding, in whole or in part, and is subject to the NIH Public Access Policy. Through acceptance of this federal funding, the NIH has been given a right to make the work publicly available in PubMed Central.

NIH Award Numbers RF1AG064942 to (TEG, JLB), NIH R01AG072714 and NIH R01AG067429 (to JLB), and NIH R35HL150750 (to EGK). This work was also supported by the Georgia Research Alliance (TEG and EGK), The McKnight Brain Research Foundation (JLB), and Emory Gouizueta Brain Health Institute (TEG).

Acknowledgements

The authors thank Benoit Giasson and Brenda Moore for their contributions to the development of the anti-CRF antibody and Karyn Esser for help with body composition assessments. We thank the UF and Emory animal care services staff for their support of these projects and the Emory Multiplexed Immunoassay Core (EMIC) for assisting with MSD assays.

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Figures and Figure Legends

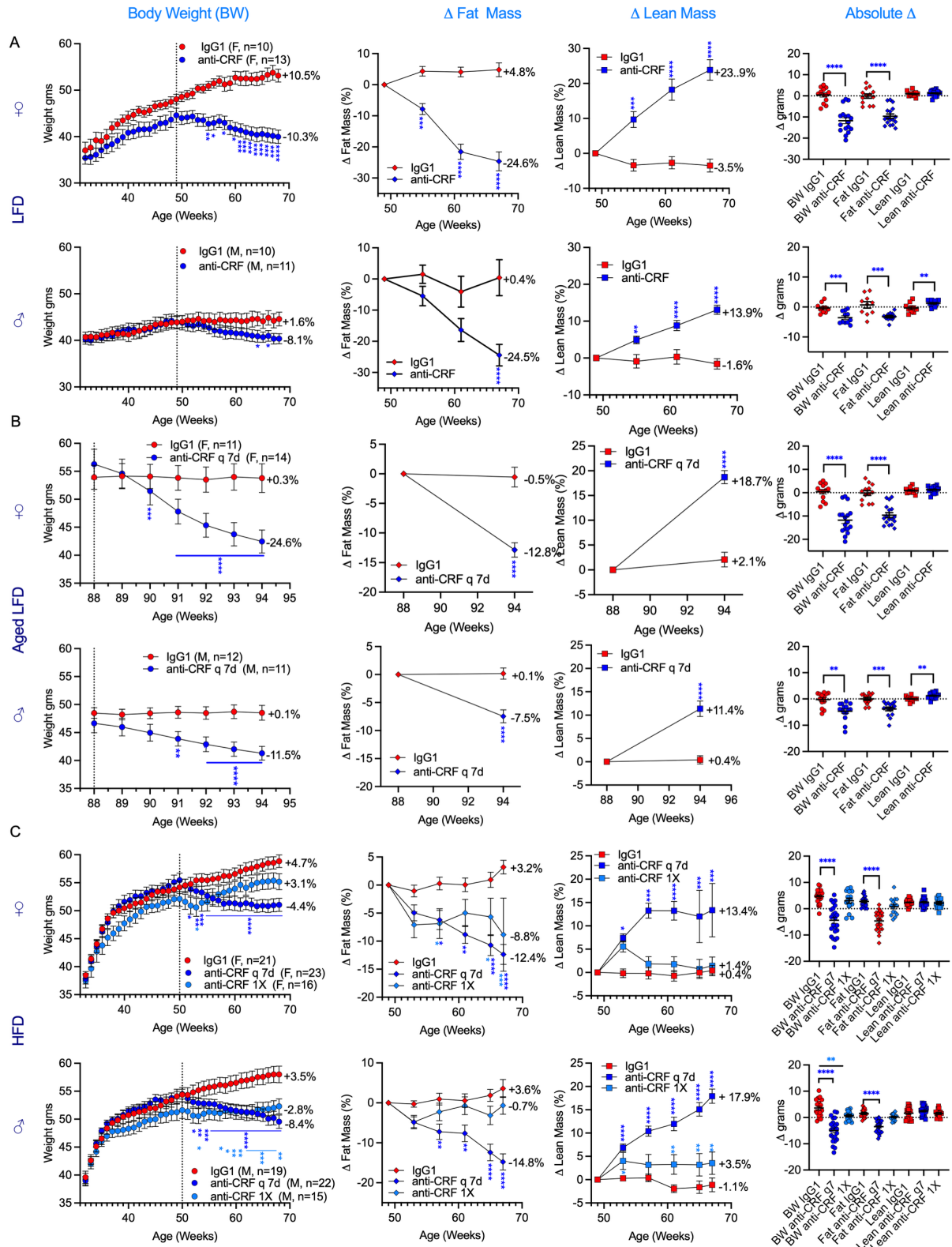


Figure 1. Anti-CRF treatment alters weight trajectories in mice, with reductions in fat mass and lean mass preservation. (A-C) Weight, change in fat mass percentage and lean mass percentage, and absolute changes at endpoint are depicted. Vertical dashed lines indicate start of treatment.

Numbers indicate percent change from start of treatment. Data are $\pm$ SEM, except for absolute weights where individual values are shown. (A) Low fat diet (LFD) cohort. Statistical comparisons are shown by two-way ANOVA with Šídák's multiple comparisons test and reveal impacts of anti-CRF on weight ($p=0.0172$, female; $p=0.0064$, male) and lean mass ($p<0.0001$ male and female). For absolute changes, multiple unpaired t tests were conducted and corrected for FDR. (B) Aged LFD cohort. Statistical comparisons are shown by two-way ANOVA with Šídák's multiple comparisons test and show impacts of anti-CRF on weight, fat and lean mass ($p<0.0001$, female and, male). Analyses of absolute changes used multiple unpaired t tests were conducted and corrected for FDR. (C) High fat diet (HFD cohort). This cohort includes a single dose group in which anti-CRF is only administered at 50 weeks of age (anti-CRF 1X). Statistical comparisons are shown for each time point after treatment by two-way ANOVA with Dunnett's multiple comparisons test. Two-way ANOVA for female mice shows impacts of anti-CRF on body weight ($p<0.0001$), fat mass ($p=0.0090$) and lean mass ($p=0.0530$). Two-way ANOVA for male mice shows impacts of anti-CRF on weight ($p<0.0001$), fat ($p<0.0001$) and lean mass ($p<0.0001$). For analyses of absolute change in body weight, fat and lean mass, a two-way ANOVA was used with Dunnett's multiple comparisons test compared to control. Full statistical reports are provided in Table S1. Significance is noted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

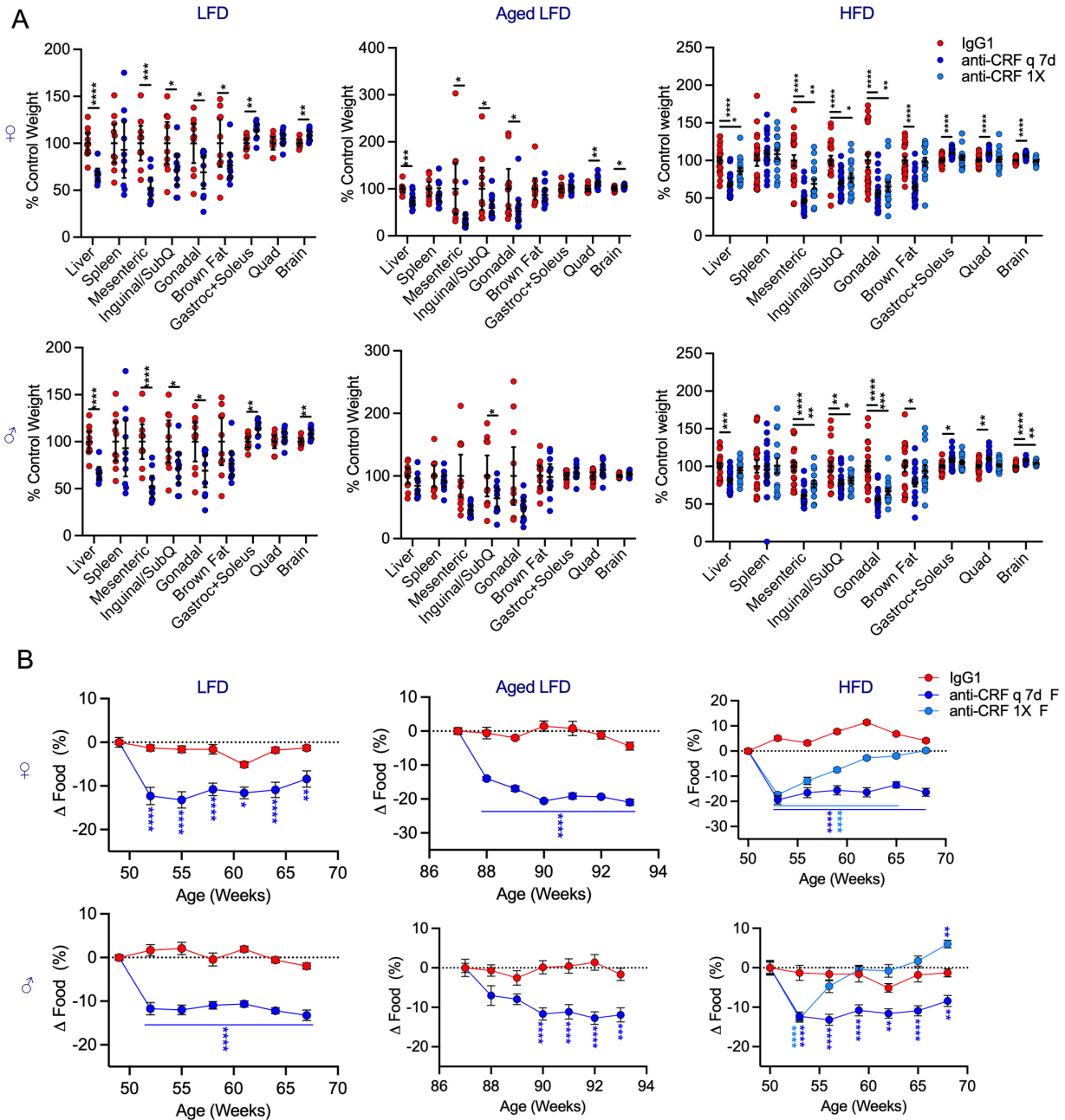


Figure 2. Anti-CRF impacts on organ weights and food intake, (A) Organs weights at endpoint from the mice in the cohorts in Figure 1 depicted as percent control IgG1 +/- SEM. LFD and aged LFD cohort data were analyzed using unpaired t-tests and FDR correction; HFD cohort data was analyzed by two-way ANOVA with Dunnett's multiple comparisons test. (B) Food consumption over time. Two-way ANOVA showed impacts of anti-CRF on food intake over time in the LFD females and males ($p < 0.0001$), in the aged LFD cohort, anti-CRF shows impacts on food intake in females and males ($p = 0.0015$). Significance at each 3-week interval was assessed using Šidák's multiple comparisons test. In the HFD cohorts, two-way ANOVA showed anti-CRF impacts on food intake for females and males ($p < 0.0001$). Significance at each 3-week interval was assessed using Dunnett's multiple comparisons. Significance is noted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

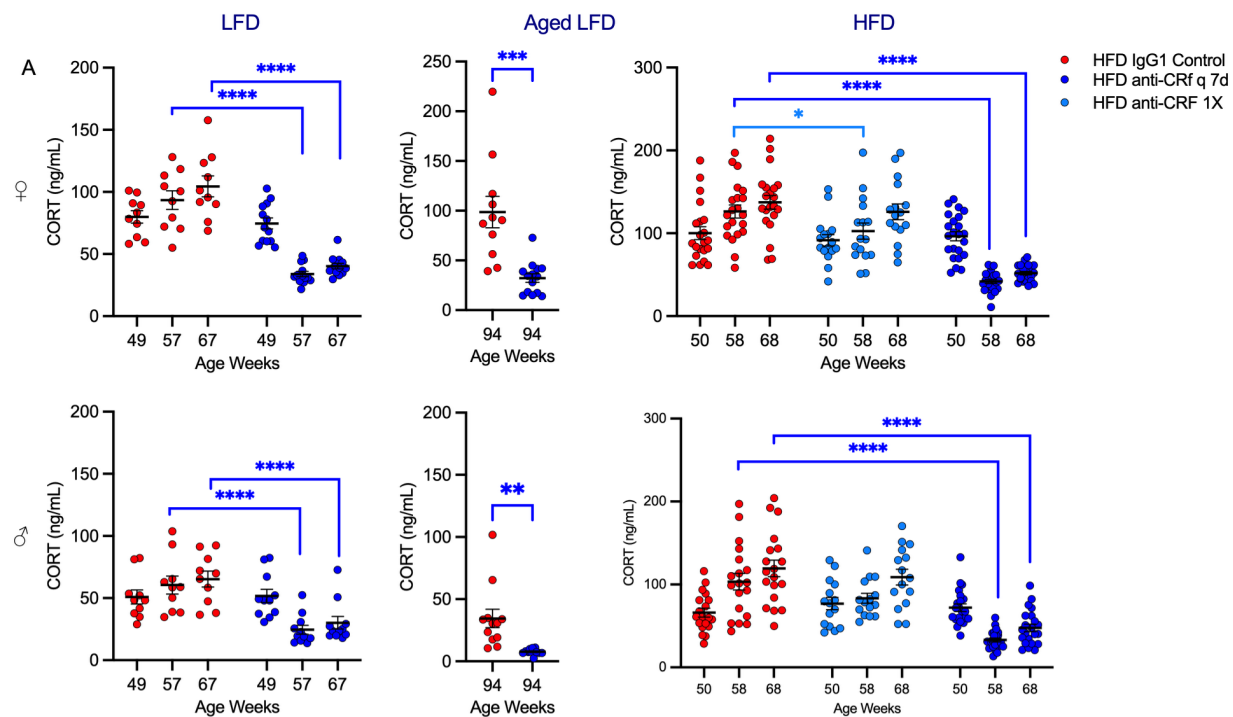
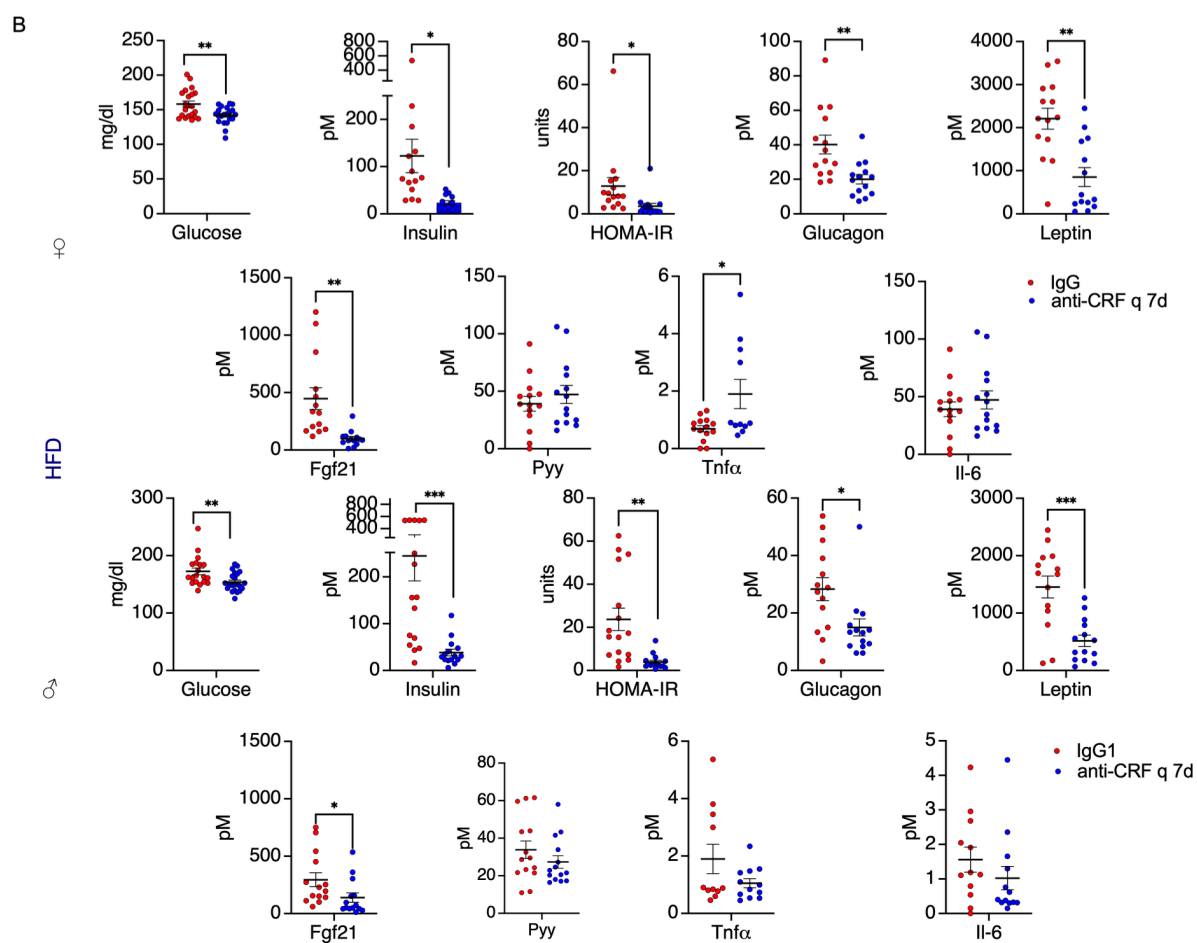


Figure 3. Anti-CRF effects on corticosterone and plasma metabolic markers and measures. (A) Plasma corticosterone levels. In the LFD cohort, two-way ANOVA showed significant lowering of CORT by anti-CRF over time in females ($p < 0.0001$) and males ($p = 0.0018$). Significance at each timepoint was assessed using Šídák's multiple comparisons test. In the aged LFD cohort, anti-CRF also showed significant impacts of anti-CRF on CORT after 6 weeks of treatment (week 94), parried t-test. In the HFD cohorts, two-way ANOVA showed weekly anti-CRF reduced CORT over time in females and males ($p < 0.0001$). Single dose anti-CRF also reduced CORT at the 58-week time point in female HFD cohort. Significance at each interval was assessed using Dunnett's multiple comparisons. (B) Plasma levels of glucose, insulin, HOMA-IR, glucagon, leptin, Fgf21, Pyy, Il-6, and TNF- α in HFD mice at 17.5 months are shown for each sex. Unpaired two tailed t-tests, FDR two-stage step-up (Benjamini, Krieger, and Yekutieli). Significance is noted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Full statistical reports are provided in Table S1.

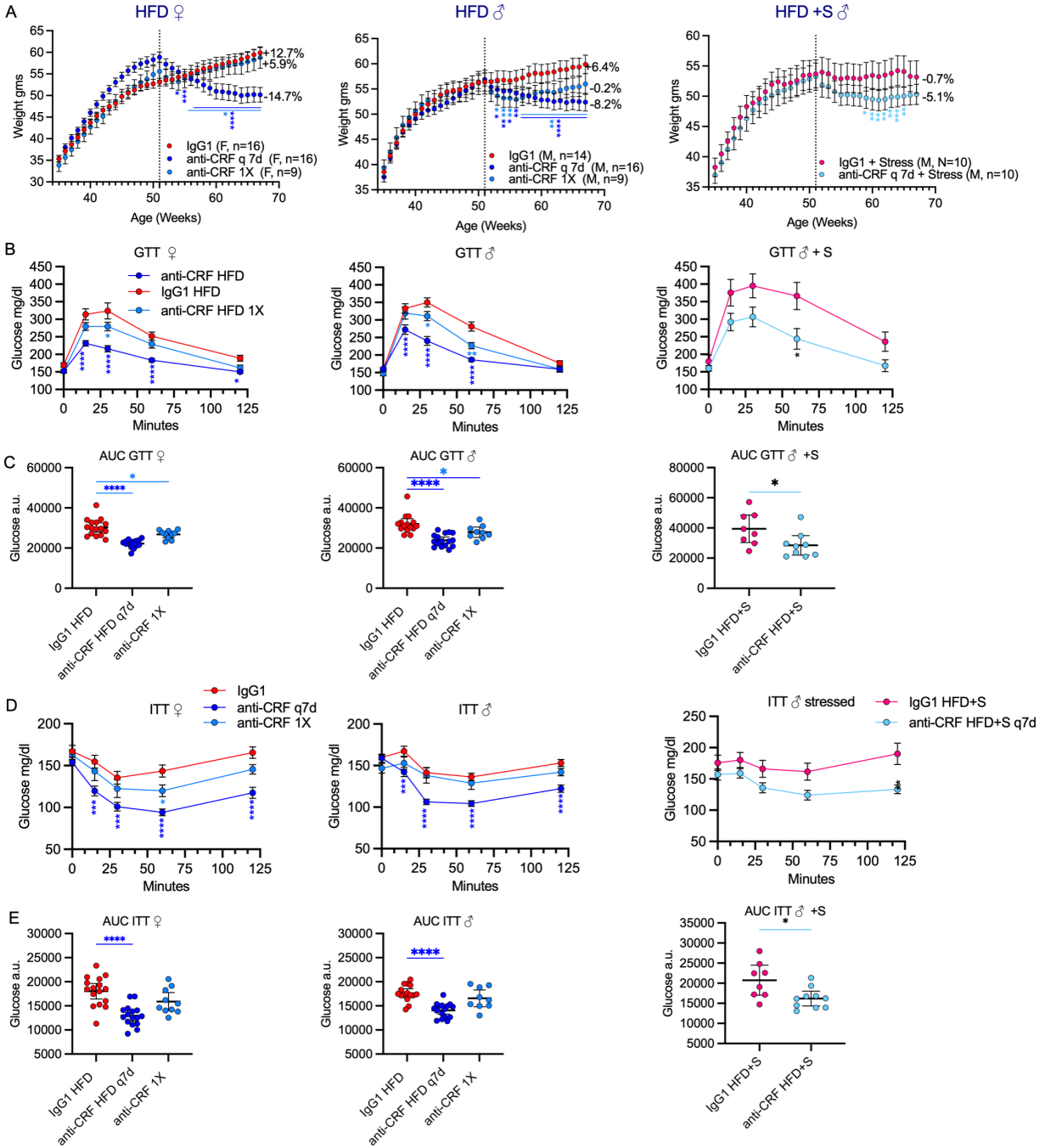
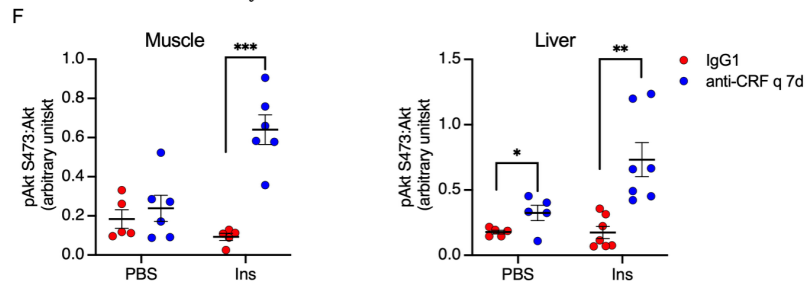


Figure 4. Anti-CRF improves glucose tolerance and insulin sensitivity. (A) Body weight in new HFD cohorts and a HFD plus chronic stress male cohort (HFD+S). Vertical dashed lines indicate start of treatment. Numbers indicate percent change from start of treatment. Data are $\pm$ SEM, except for absolute weight. Two-way ANOVA shows a significant impact of anti-CRF treatment on weight trajectory for female and male mice HFD+S male mice ($p < 0.0001$) and weight in the HFD+S male mice ($p < 0.0001$). Significance at each time point assessed by two-way ANOVA with Dunnet's (HFD) or Šídák's (HFD +S) multiple comparison test. (B) Oral Glucose Tolerance Tests (GTT). Two-way ANOVA demonstrates impacts of anti-CRF treatment on glucose levels in the HFD female ($p = 0.0017$), HFD male cohorts ($p < 0.0001$) and the HFD+S male cohorts ($p < 0.0001$) with Dunnet's (HFD) or Šídák's (HFD +S) multiple comparison identifying changes at individual time points. (C) GTT AUC. ANOVA with Dunnet's multiple comparison test for HFD cohorts and unpaired t-test with Welch's correction for the HFD+S cohort. (D) Insulin Tolerance Tests (ITT). Two-way ANOVA demonstrates impacts of anti-CRF treatment on insulin stimulated glucose levels with time and treatment ($p < 0.0001$) in female and glucose levels over time in males ($p = 0.0025$) and HFD+S males ($p < 0.0001$) with Dunnet's (HFD) or Šídák's (HFD +S) multiple comparison revealing significant changes at individual time points. (E) ITT AUC. ANOVA with Dunnet's multiple comparison test for HFD cohorts and unpaired t-test with Welch's correction for the HFD+S cohort. (E) Quantitative analyses of Western blots for muscle and liver pAkt-S473 to total-Akt ratio from male mice following insulin challenge. Unpaired T-tests with False Discovery Rate (FDR), two-stage step-up (Benjamini, Krieger, and Yekutieli) test. Significance is noted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Full statistical reports are provided in Table S1.

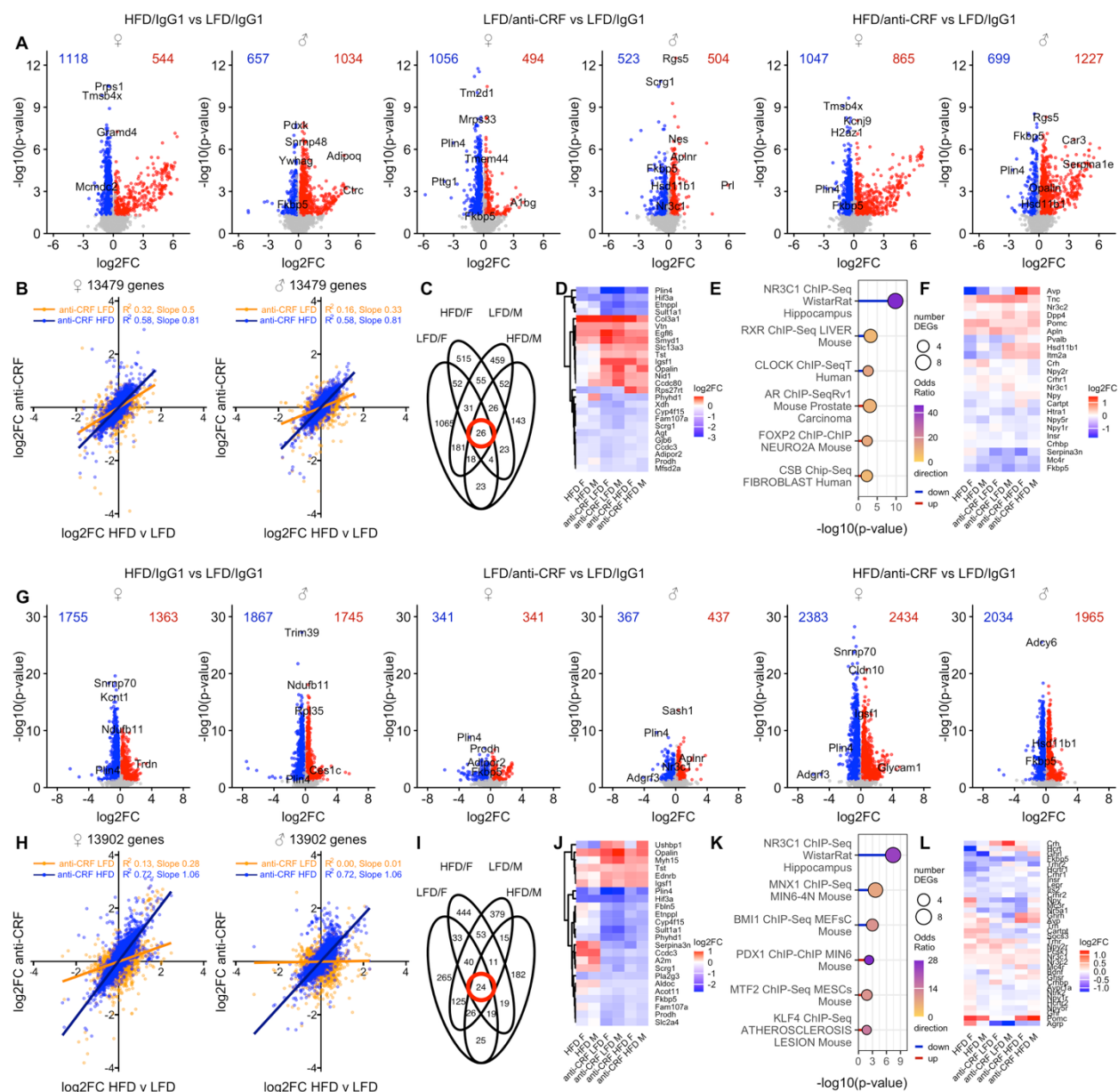


Figure 5. Transcriptomic analysis of the effect of HFD and anti-CRF treatment in the brain and hypothalamus. (A, G) Volcano plots ($-\log_{10}$ p value, y axis; $\log_2$ fold change (FC), x-axis) of differentially expressed genes ($p < 0.05$) in the brain (A) and hypothalamus (G). Pair-wise comparisons are shown in HFD treatment (HFD/IgG1 versus LFD/IgG1) F and M; anti-CRF in LFD (LFD/anti-CRF versus LFD/IgG1) F and M; anti-CRF in HFD (HFD/anti-CRF versus LFD/IgG1) F and M. Down- and up-regulated genes (DEGs) are noted in blue and red, respectively, with the total number of down- and up-regulated DEGs at the top of each plot in the same colors. (B, H) Plots and linear correlation analyses of the RNA $\log_2$ FC of HFD IgG1 (x-axis), anti-CRF LFD (y-axis, orange), and anti-CRF HFD (y-axis, blue) versus LFD IgG1 control in brain (B) and hypothalamus (H) are shown. (slope differing from 0, $p < 1e^{-9}$ for all comparisons). $\log_2$ FC values were restricted between -2.0 and 2.0 for the HFD v LFD female comparisons in the brain and -2.5 and 2.5 for the hypothalamus. (C, I) Venn diagram of overlapping genes of anti-CRF versus IgG1 comparisons in LFD/F, HFD/F, LFD/M and HFD/M treated animals, respectively

in brain (C) and the hypothalamus (I). (D,J) The genes within the intersection of all four groups (circled in red) were used to generate heatmaps of expression data (log2 FC). (E,K) Enrichment analyses of the genes identified in C and H using ChIP-X Enrichment Analysis version 3 (ChEA). (F, L) Heatmap of select genes of interest showing sex and treatment effects on expression levels (log2 FC).

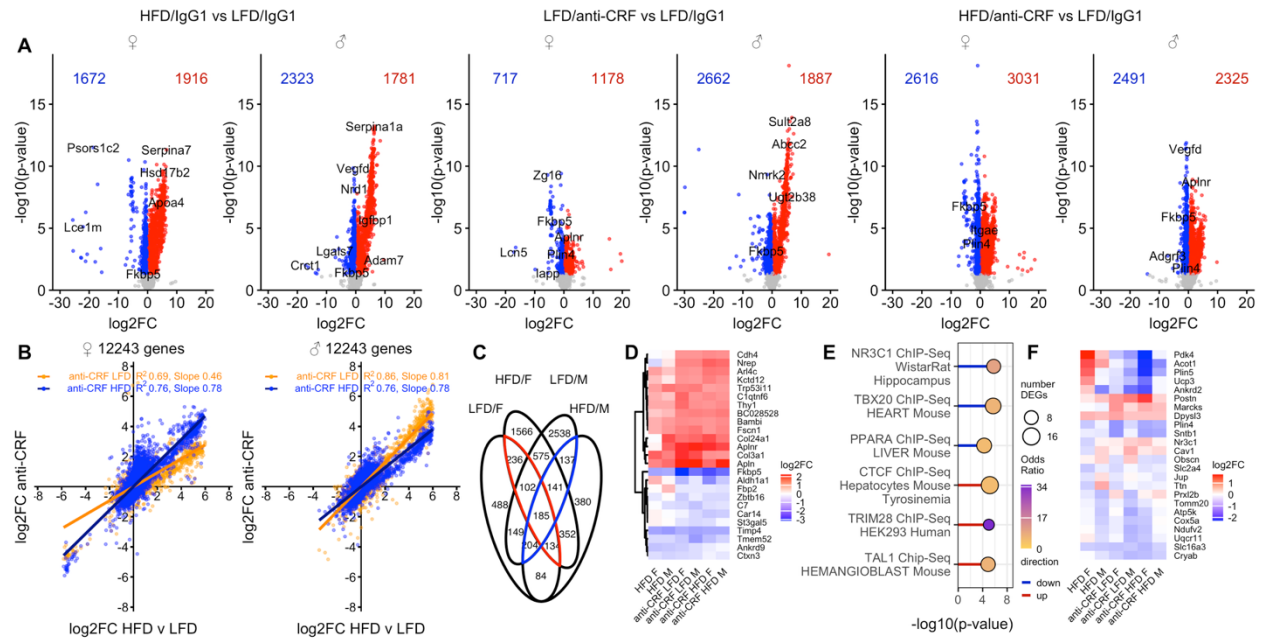


Figure 6. Transcriptomic analysis of the effect of HFD and anti-CRF treatment in skeletal muscle (A) Volcano plots ($-\log_{10} p$ value, y-axis; $\log_2\text{FC}$, x-axis) of differentially expressed genes ($p < 0.05$). Pair-wise comparisons are shown in HFD treatment (HFD/IgG1 versus LFD/IgG1) F and M; anti-CRF in LFD (LFD/anti-CRF versus LFD/IgG1) F and M; anti-CRF in HFD (HFD/anti-CRF versus LFD/IgG1) F and M. Down- and up-regulated genes (DEGs) are noted in blue and red, respectively, with the total number of down- and up-regulated DEGs at the top of each plot in the same colors. (B) Plots and linear correlation analyses of the RNA $\log_2\text{FC}$ of HFD IgG1 (x-axis), anti-CRF LFD (y-axis, orange), and anti-CRF HFD (y-axis, blue) versus LFD IgG1 control are shown. (slope differing from 0, $p < 1e^{-9}$ for all comparisons). $\log_2\text{FC}$ values were restricted between -6.0 and 6.0 for the HFD versus LFD female comparisons. (C) Venn diagram of overlapping genes of anti-CRF versus IgG1 comparisons in LFD/F, HFD/F, LFD/M and HFD/M treated animals. (D) A subset of the genes within the intersection of all four groups (circled in red) were used to generate heatmaps of expression data ($\log_2\text{FC}$). (E) Enrichment analyses of the genes identified in C and H using ChIP-X Enrichment Analysis version 3 (ChEA). (F) Heatmap of select genes of interest showing sex and treatment effects on expression levels.

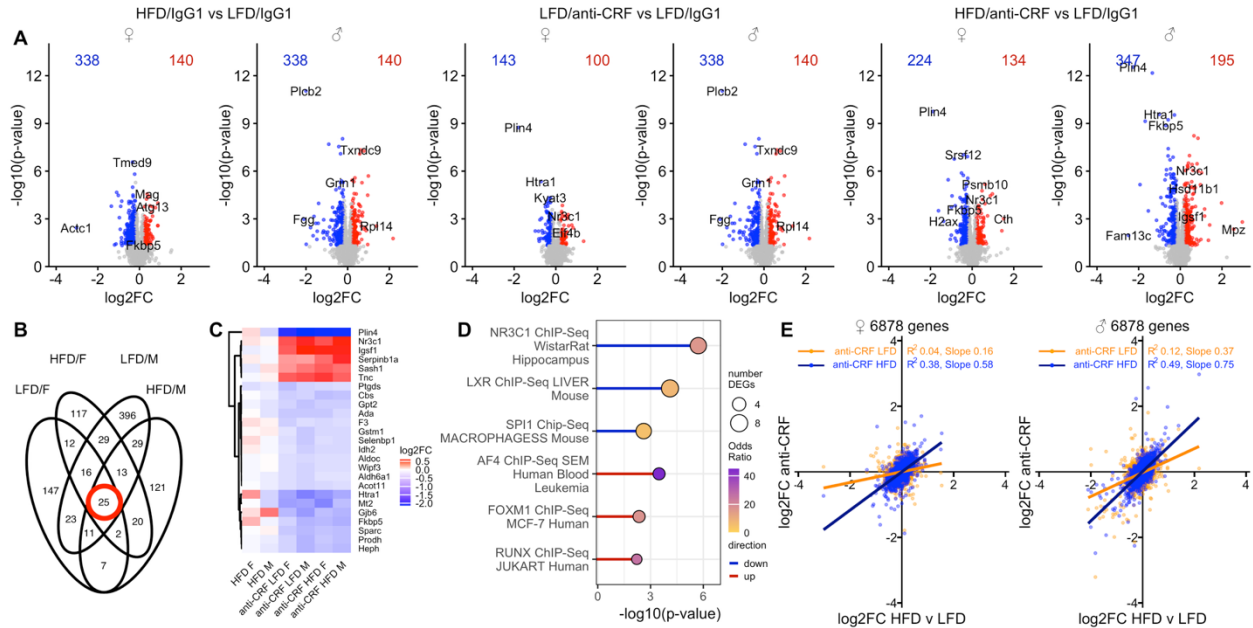


Figure 7. Proteomic analysis of the effect of HFD and anti-CRF treatment in brain. (A) Volcano plots of differentially abundant proteins (DAPs) in brain. Pair-wise comparisons are shown in HFD treatment (HFD/IgG1 versus LFD/IgG1) F and M; anti-CRF in LFD (LFD/anti-CRF versus LFD/IgG1) F and M; anti-CRF in HFD (HFD/anti-CRF versus LFD/IgG1) F and M. Down- and up-regulated DAPS are noted in blue and red, respectively, with the total number of proteins with decreased and increased levels compared to LFD/IgG1 at the top of each plot in the same colors. (B) Venn diagram of overlapping DAPS of anti-CRF versus IgG1 comparisons in LFD/F, HFD/F, LFD/M and HFD/M treated animals, respectively. (C) The 25 proteins within the intersection of all four groups (circled in red) were used to generate heatmaps of expression data (log2 FC). (D) Enrichment analyses of DAPS identified in (B) using ChIP-X Enrichment Analysis version 3 (ChEA). (E) Plots and linear correlation analyses of the RNA log2FC of HFD IgG1 (x-axis), anti-CRF LFD (y-axis, orange), and anti-CRF HFD (y-axis, blue) versus LFD IgG1 control in brain (slope differing from 0, $p < 1e^{-9}$ for all comparisons).

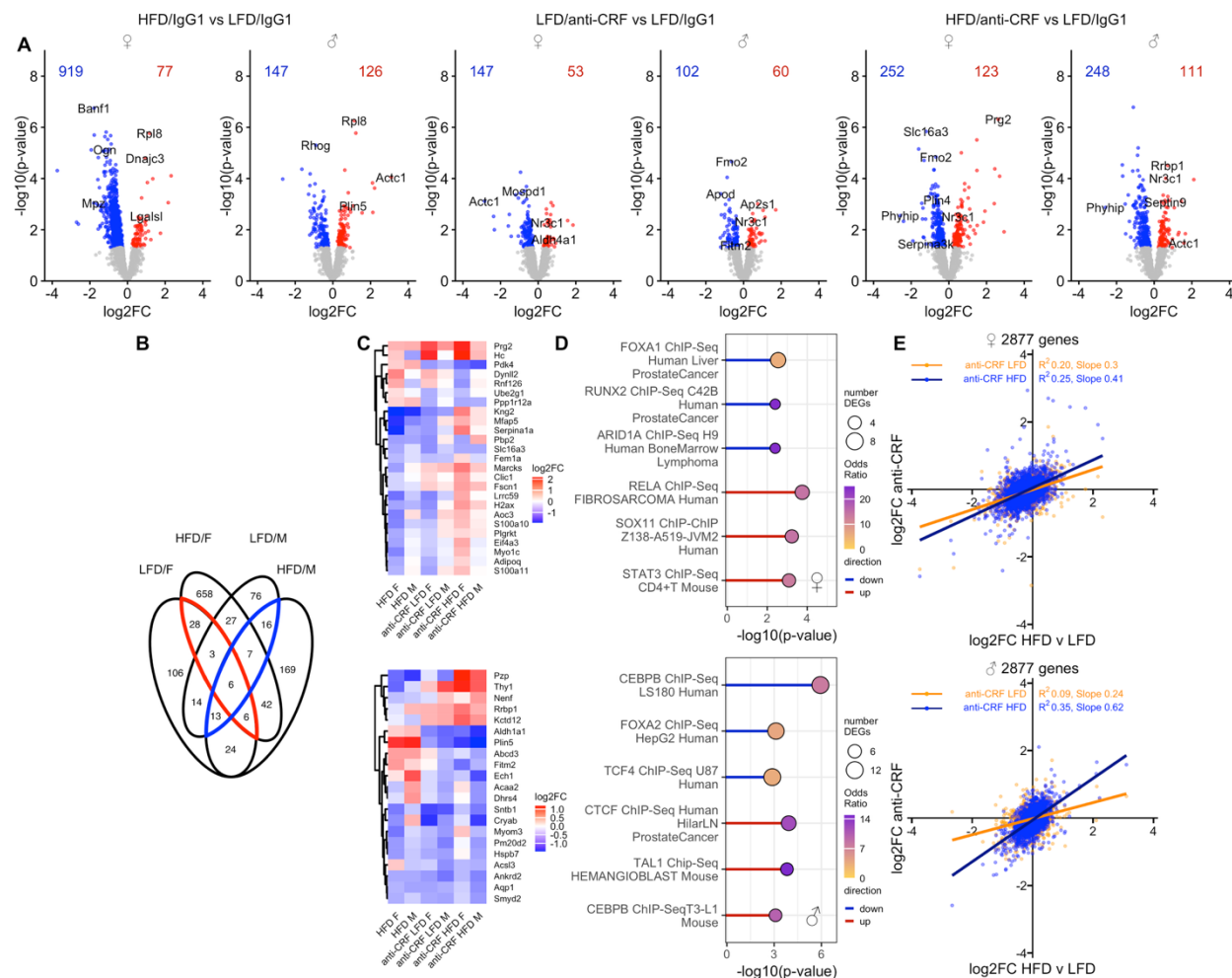


Figure 8. Proteomic analysis of the effect of HFD and anti-CRF treatment in skeletal muscle. (A) Volcano plots of differentially abundant proteins (DAPs) in muscle. Pair-wise comparisons are shown in HFD treatment (HFD/IgG1 versus LFD/IgG1) F and M; anti-CRF in LFD (LFD/anti-CRF versus LFD/IgG1) F and M; anti-CRF in HFD (HFD/anti-CRF versus LFD/IgG1) F and M. Down- and up-regulated DAPs are noted in blue and red, respectively, with the total number of proteins with decreased and increased levels compared to LFD/IgG1 at the top of each plot in the same colors. (B) Venn diagram of overlapping proteins of anti-CRF versus IgG1 comparisons in LFD/F, HFD/F, LFD/M and HFD/M treated animals, respectively. Given the small number of overlapping proteins ($n = 6$) at the intersection of all groups, female (red oval, 53 DAPs) and male (blue oval, 42 DAPs) intersects were examined separately. (C) Subsets of the overlapping proteins in female and male muscle were used to generate heatmaps of expression data (log2 FC). (D) Enrichment analyses of DAPs identified in (B) using ChIP-X Enrichment Analysis version 3 (ChEA). (E) Plots and linear correlation analyses of the RNA log2FC of HFD IgG1 (x-axis), anti-CRF LFD (y-axis, orange), and anti-CRF HFD (y-axis, blue) versus LFD IgG1 control in brain (slope differing from 0, $p < 1e^{-9}$ for all comparisons).

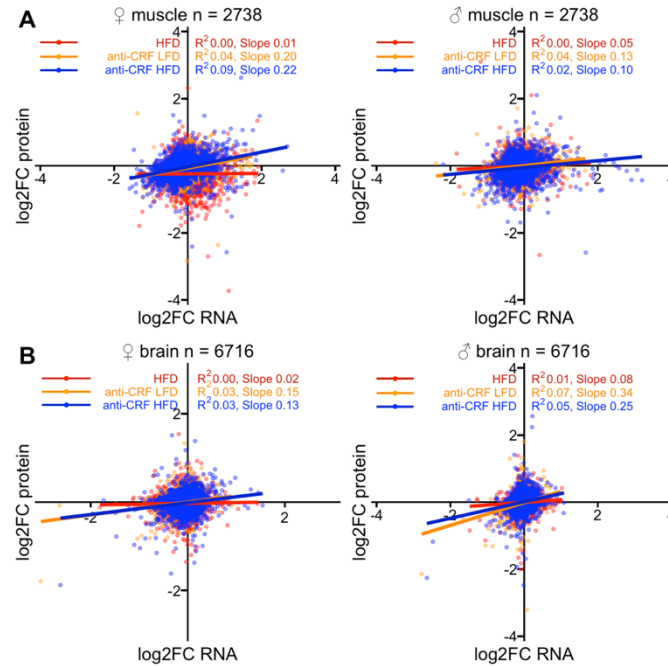


Figure 9. Poor correlations between RNA and protein changes in muscle and brain. Plots of log2 FC RNA (x-axis) versus log2 FC protein (y-axis) and linear regression analysis for HFD treatment (red), anti-CRF in LFD (orange), and anti-CRF in HFD (blue). (A) Female and male muscle. (B) Female and male brain.

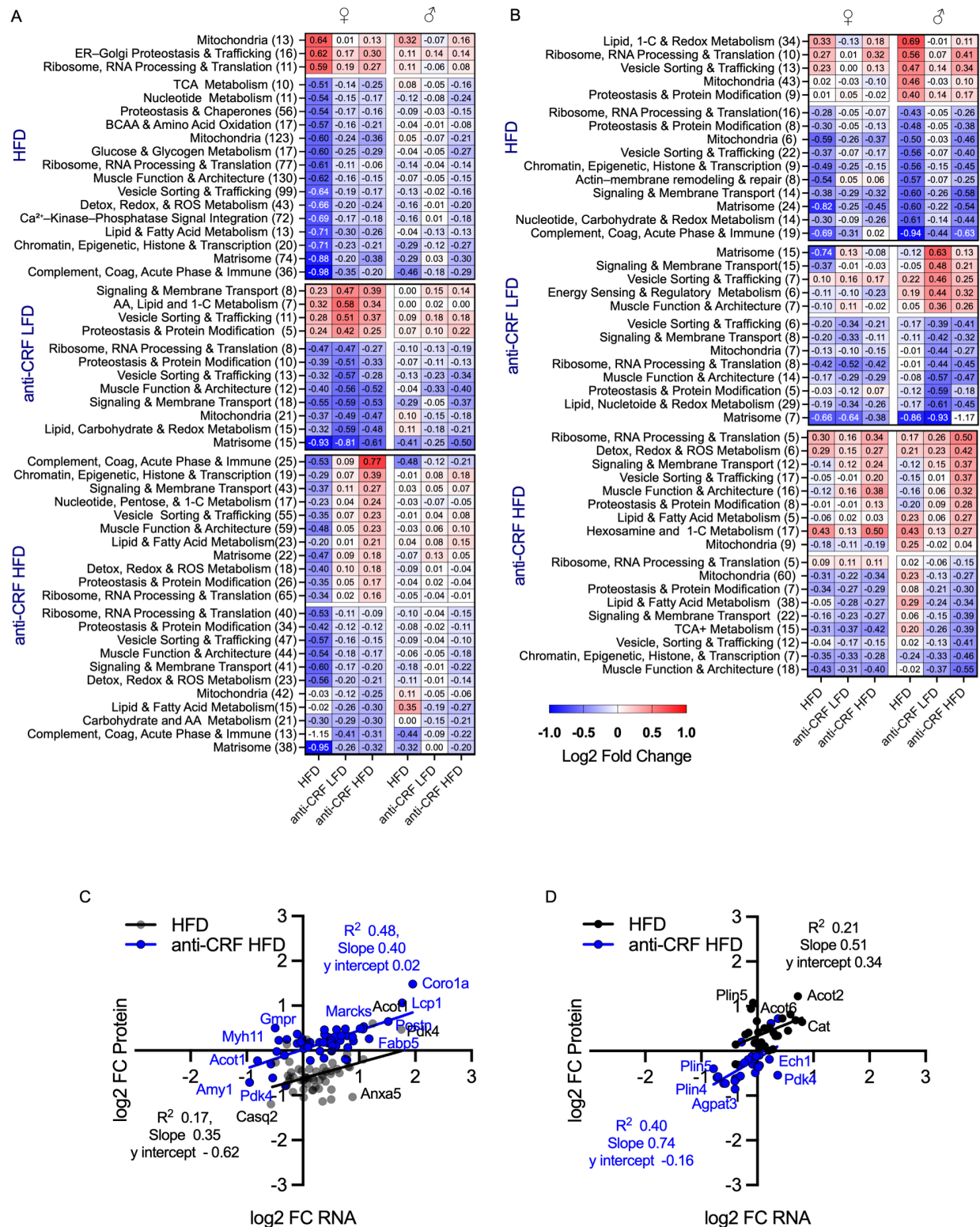


Figure 10. Functional clustering of DAP changes of HFD and anti-CRF treatment in muscle. (A, B) Functional clustering of up and down regulated DAPs identified in the muscle of females (A)

and male (B) in these condition (HFD IgG1 vs LFD IgG1, anti-CRF LFD vs LFD, anti-CRF HFD vs HFD IgG1). Heatmaps show the average log2 FC change of the DAPs using values from comparisons against the LFD IgG1 condition. Individual DAPs in each module are shown in Supplemental Figure S5A-F. Additional DAPS are also shown in heat maps in Supplemental Figure (S6A). (C,D) Plots and linear regression analysis of log2 FC RNA (x-axis) versus log2 FC protein (y-axis) are shown of HFD treated (black) and anti-CRF in HFD (blue). DAPS in the anti-CRF in HFD group are restricted to $p_{adj} < 0.01$ in females and to $p_{adj} < 0.05$ in males.

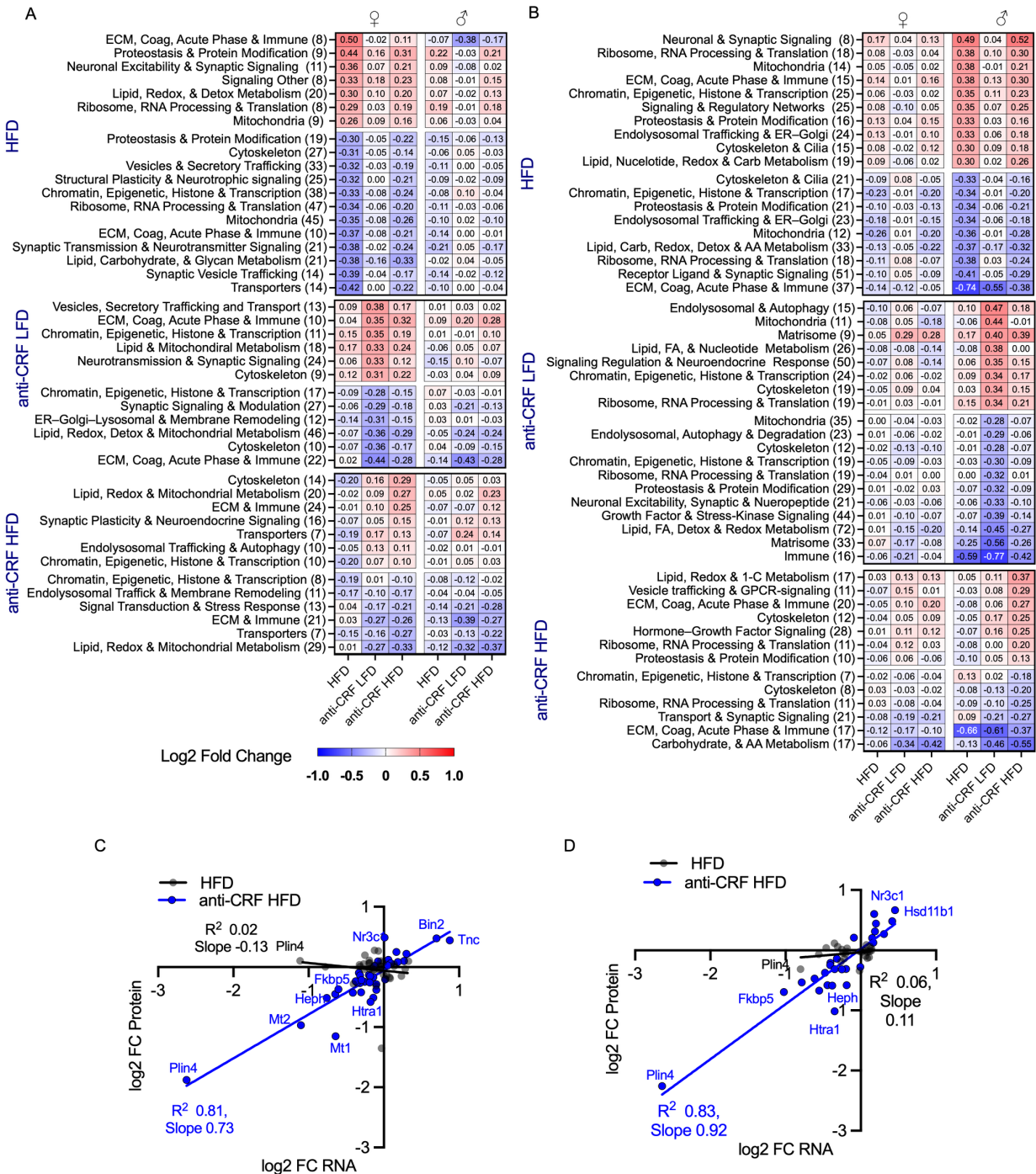


Figure 11. Functional clustering of DAP changes of HFD and anti-CRF treatment in brain. (A, B) Functional clustering of up and down regulated DAPs identified in the brain of females (A) and male (B) in these condition (HFD IgG1 vs LFD IgG1, anti-CRF LFD vs LFD, anti-CRF HFD vs HFD IgG1). Heatmaps show the average log2 FC change of the DAPs using values from comparisons against the LFD IgG1 condition. Individual DAPs in each module are shown in Supplemental Figure S6A-F. Additional DAPS are also shown in heat maps in Supplemental Figure (S7B). (C,D) Plots and linear regression analysis of log2 FC RNA (x-axis) versus log2 FC protein (y-axis) are shown of HFD treated (black) and anti-CRF in HFD (blue). For brain, DAPS

in the anti-CRF in HFD group are restricted to $p_{\text{adj}} < 0.01$ in females and to $p_{\text{adj}} < 0.05$ in males. Significance is noted as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Full statistical reports are provided in Table S1.

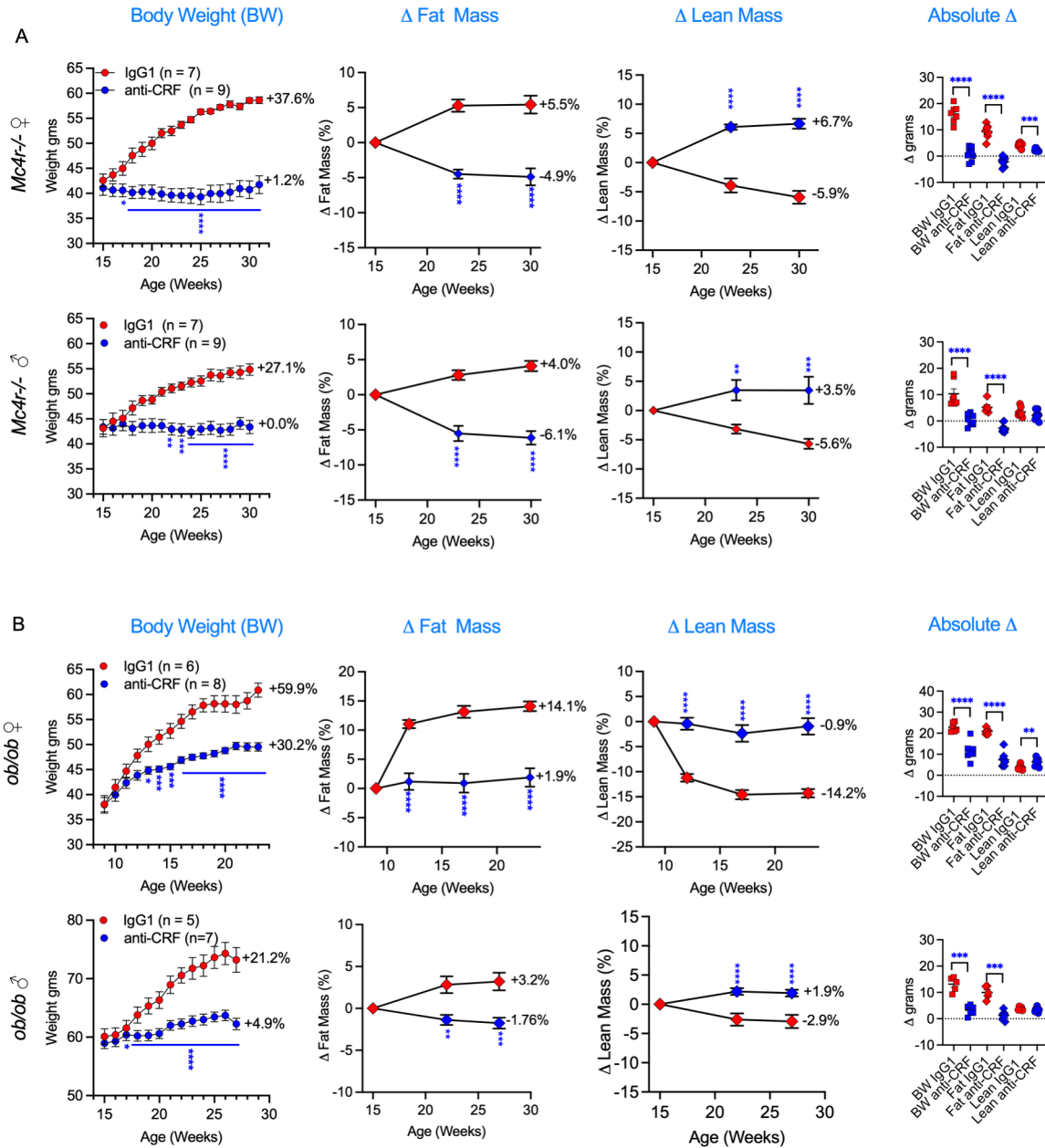


Figure 12. Anti-CRF treatment impacts in *Mc4r^{-/-}* and *ob/ob* mice. (A,B) Weight over time, change in fat mass percentage, change in lean mass percentage and absolute changes at endpoint are depicted for *Mc4r^{-/-}* (A) and *ob/ob* mice (B) for each sex. Number of mice in each group is shown in the legend for the plot of body weight over time. The first time point indicates the start of treatment with anti-CRF or IgG1 control with measures taken before administration on that day, and the numbers at the end of the plots indicate percent change from start of treatment. Absolute change in weights show individual points at end of study. Data are shown as $\pm$ SEM, except for absolute weight. (A) Two-way ANOVA with Šídák's multiple comparisons test for body weight, fat mass, and lean mass change versus time show significant impacts of anti-CRF on weight ($p < 0.0001$), fat ($p < 0.0001$) and lean mass ($p < 0.0001$ (F); $p = 0.0075$ (M)) in *Mc4r^{-/-}* mice. Absolute change in body weight, fat and lean mass were assessed using multiple unpaired t-tests and corrected for FDR. (B) Two-way ANOVA for body weight, fat mass, and lean mass change versus

time show significant impacts of anti-CRF on weight ($p < 0.0001$), fat ($p < 0.0001$ (F); $p = 0.0013$ (M)) and lean mass ($p < 0.0001$ (F); $p = 0.0009$ (M)) in *ob/ob* mice. Two-way ANOVA for male mice for body weight, fat mass, and lean mass change versus time show significant impacts of anti-CRF on body weight ($p < 0.0001$), fat mass ($p = 0.0013$) and lean mass ($p = 0.0009$) in *ob/ob* mice. Absolute changes in body weight, fat and lean mass were assessed using multiple unpaired t-tests and corrected for FDR. Significance is noted as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Full statistical reports are provided in Table S1.

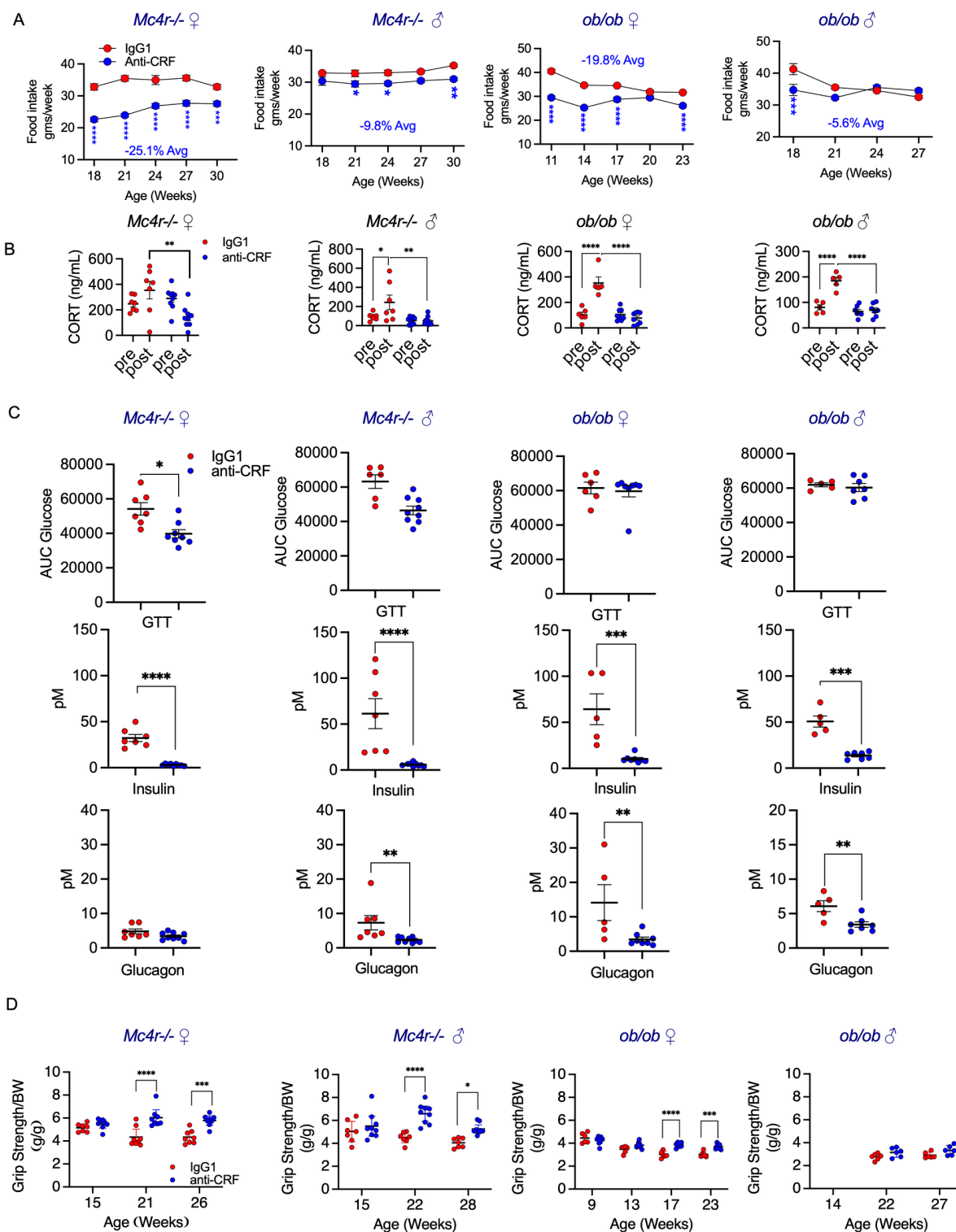


Figure 13. Anti-CRF treatment reduces food intake, improves select metabolic markers, and increases or maintains grip strength in *Mc4r*^{-/-} and *ob/ob* mice. (A) Food consumption over time. In *Mc4r*^{-/-}, two-way ANOVA showed significant impacts of anti-CRF on food intake over time in females ($p=0.0092$) and in males on food consumption ($p<0.0001$). In *ob/ob* mice, anti-CRF food

altered intake over time in females ($p < 0.0001$) and males ($p = 0.0015$). Significance at each interval was assessed using Šídák's multiple comparisons test. (B) Corticosterone levels before (pre) and after treatment (post) in this cohort with statistical significance assessed by one-way ANOVA. (C) GTT was conducted, and plasma levels of insulin and glucagon were measured following a 6 hour fast. AUC graphs are shown for the GTT. Statistical analyses by paired two-tailed t-tests. For insulin and glucagon plasma levels, unpaired two tailed t-tests with an FDR two-stage step-up were used. (D) Grip strength normalized to body weight was assessed in each cohort. Two-way ANOVA shows significant impacts of anti-CRF on food intake over time in *Mc4r^{-/-}* females ($p = 0.0222$); and *ob/ob* females ($p < 0.0001$). In *ob/ob* males, impacts of anti-CRF over time were not significant, but impacts of anti-CRF were ($p = 0.0100$). Significance is noted as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Full statistical reports are provided in Table S1.

Supplemental Figures are included.

Supplemental Data Tables

Supplemental Table S1. Details of statistical analyses for non-omic data.

Supplemental Table S2. brain_FandM_DEGs_DAPs.xlsx. Related to Figure 5 and 7.

Supplemental Table S3. hypothalamus_DEGs.xlsx. Related to Figure 5.

Supplemental Table S4. muscle_DEGs_DAPs.xlsx. Related to Figure 6 and 8

Supplemental Table S5. liver_FandM_DEGs.xlsx. Related to Supplemental Figure S3

Supplemental Table S6. fat_FandM_DEGs.xlsx. Related to Supplemental Figure S4

Supplemental Table S7. Venn_overlaps_enrichr_transcriptome_proteome.xlsx. Related to Figures 5-8 and S3-4.

Supplemental Data Tables S8-S14. Gene set enrichment analysis from each tissue.

Supplemental Table S8. brain_transcriptomics_enrichr.xlsx. Related to Figure 5

Supplemental Table S9. hypothalamus_transcriptomics_enrichr.xlsx. Related to Figure 5.

Supplemental Table S10. muscle_transcriptomics_enrichr.xlsx. Related to Figure 6.

Supplemental Table S11. liver_transcriptomics_enrichr.xlsx. Related to Supplemental Figure S3.

Supplemental Table S12. fat_transcriptomics_enrichr.xlsx. Related to Supplemental Figure S4.

Supplemental Table S13. brain_proteomics_anti-CRFvslgG1_enrichr.xlsx. Related to Figure 7.

Supplemental Table S14. muscle_proteomics_anti-CRFvslgG1_enrichr.xlsx. Related to Figure 8.