Chapter 4

EXPLORING NOVEL THERAPEUTICS WITH GENETICALLY ENGINEERED REPORTERS

Summary

A variety of psychiatric and metabolic disorders are associated with the dysfunction of neuropeptide signaling pathways, whose malfunction can result in a variety of mental illnesses and metabolic disorders. Neuropeptide receptors are druggable targets (typically GPCRs) that have received heavy investment from the pharmaceutical industry. Many of these drugs have failed in the clinic, however, resulting in abandonment of this approach. One potential reason for failure is that each neuropeptide often has multiple, functionally redundant receptors. Therefore, inhibiting just one receptor may not suffice to have any effect. An alternative strategy is to target the *processing/release* of the neuropeptide from neurons, rather than blocking its receptor. We have developed new technology that allows the detection of localization, expression and release of specific neuropeptides. Here we propose to use this technology to develop an *in vitro* platform for high-throughput screening of drug libraries for CRISPR sgRNAs, specific recombinant antibodies and rational design of novel neuropeptide derivatives. In the long term, we expect to develop a large-scale drug screening platform that automates the research and development of neuropeptide-targeted lead compounds.

Introduction

Neuropeptides are a class of neural signaling molecules that play a pivotal role in brain function and human health. Malfunctioning of neuropeptide pathways can potentially result in a variety of mental illnesses triggered by stress, and metabolic disorders, including obesity (Griebel & Holsboer, 2012;

Hökfelt et al., 2003). For example, it is widely believed that disrupted cholecystokinin (CCK), neurokinin (NK) and corticotropin-release factor (CRF) pathways cause depression and anxiety (Bowers et al., 2012; Schank et al., 2012); abnormal neuropeptide Y (NPY) and Agouti-Related Peptide (AGRP) signaling results in feeding disorders which can potentially lead to obesity (Arora & Anubhuti, 2006; Dhillo & Bloom, 2001), Calcitonin gene-related peptide (CGRP) and substance P are thought to be related to the transmission of pain (Hökfelt et al., 2001; Russell et al., 2014). Peripheral dysregulation of neuropeptides, such as PYY, affects satiety sensation (Acuna-Goycolea & Van Den Pol, 2005; Batterham et al., 2003; Pedragosa-Badia et al., 2013). The list goes on. A huge battery of drugs has been developed in the hopes that targeting neuropeptide pathways will lead to novel therapies for neuropsychiatric, neurodegenerative or neurometabolic disorders.

Neuropeptide receptors are druggable targets (typically GPCRs) that have received heavy investment from the pharmaceutical industry (Jacobson, 2015; Tyndall & Sandilya, 2005). Many of these drugs have failed in the clinic, however, resulting in abandonment of this approach by many pharma companies (Eiger et al., 2022). One potential reason for failure is that each neuropeptide often has multiple, functionally redundant receptors (van den Pol, 2012). Therefore, inhibiting just one receptor may not suffice to have any effect. An alternative strategy is to target the *processing/release* of the neuropeptide from neurons, rather than blocking its receptor. We have developed new technology that allows the detection of release of specific neuropeptides (Ding et al., 2019). Here we propose to use this technology to develop an *in vitro* platform for high-throughput screening of drug libraries for inhibitors of specific neuropeptide synthesis/processing and/or release. This proposal is mechanistically testable owing to (1) the characterizations of neuropeptide biogenesis, sorting, trafficking, maturation and release, also (2) the establishment of NPLER/NPRR platforms as a hypothesis generator and testbed (details in Chapter 3). In the long term, we expect to develop a

large-scale drug screening platform that automates the research and development of neuropeptide-targeted lead compounds.

We explored the potential of some latest biotechnologies as new, transformative therapeutics for treating neuropeptide relevant disorders. These techniques regulate the neuropeptide pathway at multiple levels (Figure 1). In this chapter, I will focus on the progress on CRISPR-Cas9 screening, as well as two new protein engineering techniques that alter the binding, trafficking and release properties of neuropeptides. RNAi-based neuropeptide regulation was discussed in Chapter 3.

Results

Regulation of NPY with CRISPR-Cas9 in PC12 cells

CRISPR-Cas9 technology is widely used for genome editing and engineering (Doudna & Charpentier, 2014; Hsu et al., 2014; Jiang & Doudna, 2017). We attempted to integrate NPLER imaging platform with CRISPR-Cas9 to facilitate functional screening of neuropeptide-targeting sgRNAs. We aimed to regulate the expression of Neuropeptide Y (NPY), because (1) we have established a full-fledge NPY screening platform (Chapter 3), and (2) NPY sgRNA candidates are available from databases (details in Materials and Methods). However, the puromycin selection of N46 cells was unsuccessful for the CRISPR construct. We turned to PC12 cells for pilot experiments. The rat origin of PC12 cells removed some sgRNA candidates that only recognize mouse NPY. Fortunately, 1 out of 3 sgRNAs dramatically reduced the fluorescent intensity of NPY antibody staining in PC12 cells (A-B). The median fluorescence of control sgRNA (35.26 unit, n=20) was twice as high as NPY sgRNA (17.54 unit, n=19) (Figure 2B).

CRISPR-Cas9 was designed to interrupt NPY biogenesis at the DNA level (Figure 1). We wondered why the complete removal of NPY staining was not seen. Hypotheses include (1) PC12 cells are diploid. Cas9 may not function as efficiently to interact with two alleles; (2) Residual mRNA or synthesized peptides linger after NPY DNA was disrupted. Further experiments to support or deny these hypotheses will involve the establishment of stable cell line, pinpointing cut site of sgRNAs, and forcing out residual neuropeptide via intense neuronal activations. NPLER-assisted sgRNA screening will be developed based on the mentioned pilot experiments. The antibody-free approach will potentially generalize its implementation to any neuropeptide of choice.

Interference of neuropeptide pathways with novel intravesicular antibodies

Recombinant antibody technology sprung from interfacing molecular biology, protein engineering and immunology to facilitate manipulation of antibody fragments (Boss et al., 1984; Elgundi et al., 2017; Kontermann & Müller, 1999). The idea of assembling heavy and light chains of Fv fragment as a means of antigen binding (Figure 3A), later named scFv (single-chain variable fragment), inspired us to explore the potential of interfering neuropeptide prior to their release from the DCVs. If a scFv binds an neuropeptide with high affinity and specificity, it may be possible to sort the scFv molecules into the DCVs via specific sorting domains uncovered in Chapter 3. Neuropeptides, once scFV-bound, are potentially hindered from binding downstream receptors. In theory, the "competitive binding" model works better for neuropeptides due to their small size and limited epitopes. I named these hypothetical molecules iD-Ab (intra-DCV Antibodies) (Figure 3B).

Commercialized neuropeptide antibodies are mostly polyclonal (specifically, rabbit). Only few antibody sequences were made available. In our desk research, sequences of 6 NPY antibodies were uncovered from various public sources. We identified the motif of these antibodies through parallel

comparisons with other sequences to identify the positions of variable fragments. The heavy and light chain from these fragments were concatenated with designed linkers in between to form NPY iD-Ab candidates #1-6. NPLER^{NPY28mTq2} served as control, as it shared with all iD-Ab candidates the configuration of sorting domains (Figure 3C$_1$). N46 cells were transfected with the control or one of six iD-Abs, stained with anti-NPY antibody, and compared for fluorescent intensity. In the "competitive binding" model, an iD-Ab that efficiently binds NPY within the DCVs will block or reduce the binding with the staining antibody. In another word, low staining fluorescence bode well for good iD-Ab candidates.

Cell death was seen predominantly in #4, also moderately in #2. #1 and #6 candidates showed no significant differences with the control (Figure 3C$_2$). Cells transfected with #2 or #3 have peculiarly strong immunofluorescence. #5 exhibited reduced staining and healthy cell morphology (Figure 3D). Detailed analysis of two candidates, #3 and #5 confirmed that the fluorescence increased and decreased relative to the control group, respectively (Figure 3E). Parallel experiments were performed in PC12 cells (Figure 3F), where no statistically significant difference was seen between #3 and control, #5 again showed reduced immunofluorescence (Figure 3G), implying that iD-Ab #5 may serve as a novel molecule to target NPY at the intravesicular level.

Engineered neuropeptides as next-generation drug candidates

Peptide YY (PYY), together with Neuropeptide Y and pancreatic polypeptide (PP), constitutes the neuropeptide Y (NPY) family of biologically active peptides (Holzer et al., 2012). Though structurally similar with NPY, PYY is expressed exclusively in endocrine cells of the digestive system and preferentially binds a different receptor, NPY2R (Gantz et al., 2007; Rangwala et al., 2019). Physiologically, PYY exerts an anorexigenic effect through (1) indirect inhibition of feeding-

promoting AgRP/NPY neurons in the arcuate nucleus (Acosta et al., 2011; Batterham et al., 2002, 2003; Beutler et al., 2017; Sloth et al., 2007), (2) cause taste aversion (Halatchev & Cone, 2005) Administration of PYY reduces food intake in mouse, rat and human (Batterham et al., 2002; Chelikani et al., 2005; Pittner et al., 2004). Ablation of PYY in mice causes obesity (Boey et al., 2008). The plasma level of PYY is inversely correlated with Body Mass Index in human, as lean group shows higher plasma PYY than the obese group in both pre- and post-prandial conditions (Batterham et al., 2003; Guo et al., 2006). However, research and development of PYY-derived therapeutics fall behind, mostly due to (1) the chemical instability caused by rapid proteolysis and (2) severe sides effects from ascended doses, an indirect result of (1) (Adrian et al., 1986; Toräng et al., 2015). Therefore, we wondered if our established platform would assist the exploration of novel therapeutics.

We first tested if cells in our platform express endogenous PYY. Antibody staining results confirmed that PYY does not express in N46 cells (Figure 4A) and PC12 cells (Figure 4D), consistent with the reported exclusivity of gut expression (Batterham et al., 2003). We then generated an expression plasmid encoding a full-length mouse PYY driven by CAG promoter (CAG-Native PYY). Surprisingly, the PYY staining signals in the transfected N46 cells show strong accumulation in the nucleus and peri-nucleus regions. We reasoned that the embryonic feature of this cell line may result in an underdeveloped, or different neuropeptide sorting machineries for an exogenous neuropeptide, so we performed a parallel experiment in PC12 cells and observed that the majority of fluorescence locate within the nucleus, inferred from the DAPI staining (Figure 4E). The absence of dispersed puncta along the processes, a pattern commonly shared by neuropeptides, led to our belief that PYY does not sort and/or traffic properly in the tested cells. We investigated the structure and domains of PYYs and other neuropeptides, and transformed native PYY with domain swapping and residual

modifications. Expression of the engineered PYY with the same plasmid configuration (CAG-Engineered PYY) removed the abnormal accumulation in N46 cells (Figure 4C) and PC12 cells (Figure 4F). The expression pattern of the engineered PYY, as distributed puncta in soma and processes, highly suggests that the engineering practices enhance the sorting/trafficking of an exogenous neuropeptide.

Intravenous and intraperitoneal administration of PYY reduces body weight (Chelikani et al., 2005, 2007). We therefore adapted the PYY plasmids for AAV expression. In addition, we included a scramble control consists of identical amino acid composition of the neuropeptide but with shuffled sequence. The control is presumably deprived of binding any neuropeptide receptors. All three transgenes were packaged in AAV-PHP.S for peripheral tissue expression (Chan et al., 2017a). CAG promoter was used to enable strong gene expression. 6-month-old aged male mice underwent unilateral retro-orbital viral injection, and single-housed for seven weeks to monitor their body weight change (Figure 5A). All three groups (native PYY, engineered PYY and control) experienced loss of weight in the first three weeks after injection, likely due to some metabolic change by the surgery or viral expression. However, all three groups showed rebound of body weight between week 4 and 7. The control group gained the most weight as expected, while native PYY and engineered PYY did not differ in their weight-loss effect. It is yet immature to conclude that engineering PYY exhibits no superiority, as the immunohistochemical results from the postmortem fat tissue of experimental animals demonstrate that engineered PYY show better distribution pattern than the native counterpart (Figure 5B), a piece of phenomenon seen in cell lines (Figure 4). The inguinal white adipose tissue (IngWAT) does not express PYY endogenously but they can release neuropeptides (Figure 5B) (Wang et al., 2008), exogenous expression of native PYY result in a tangled-fiber pattern while in the case of engineered, PYY staining much resemble the distribution

of secretory granules in adipocytes. We further employed ELISA to measure the released PYY in the blood serum. The engineered PYY group shows a strikingly high level of serum PYY (~8.04 ng/ml) compared to the other two group (~0.48 ng/ml and ~0.41 ng/ml) (Figure 5C). Data were inferred from a parallel standard curve generated with standardized PYY provided (Figure 5C). The difference in serum concentration is likely due to the enhanced expression, or optimized release properties of PYY molecules in non-intestinal tissues after engineering. However, it remains unclear why the elevated PYY level did not lead to weight loss in animals. One possibility is the saturation of PYY created a "ceiling effect" in weight change; another possibility is the usage of AAV-PHP.S may have introduced unknown influence on the metabolic states of animals.

Discussion

The scientific advance and clinical significance of neuropeptides prompted us to explore early-stage therapeutics by integrating multiple cutting-edge biotechnologies to our imaging platform. The progress of our attempts, including CRISPR-Cas9, novel recombinant antibody, neuropeptide engineering, as well as the RNAi approach described previously (Chapter 3), are summarized (Table 1). We yielded at least one good candidate from each category of technology in the preliminary screening and engineering. The NPLER/NPRR imaging platform could further (1) empower large-scale screening of siRNAs, sgRNAs and scFvs that specifically target neuropeptides, and (2) assist rational design of novel neuropeptide derivatives with altered sorting/trafficking efficiencies, release properties and receptor affinities.

In summary, we propose to establish a novel and scalable drug-screening platform to help advance the discovery of new drugs for regulating neuropeptide action in a therapeutic context. It is yet

difficult to estimate the potential market size directly, as the platform is not itself a product for customers, but rather a vehicle to facilitate pharmaceutical R&D processes. Nevertheless, the market size for indications that could be treated with neuropeptide-related drugs is very large, as it includes obesity, stress/anxiety, depression, and chronic pain. The products from our platform will be several drug leads that, respectively, target a specific type of neuropeptide. These leads will undergo further *in vitro* validation, and hopefully in animal studies and for patients.

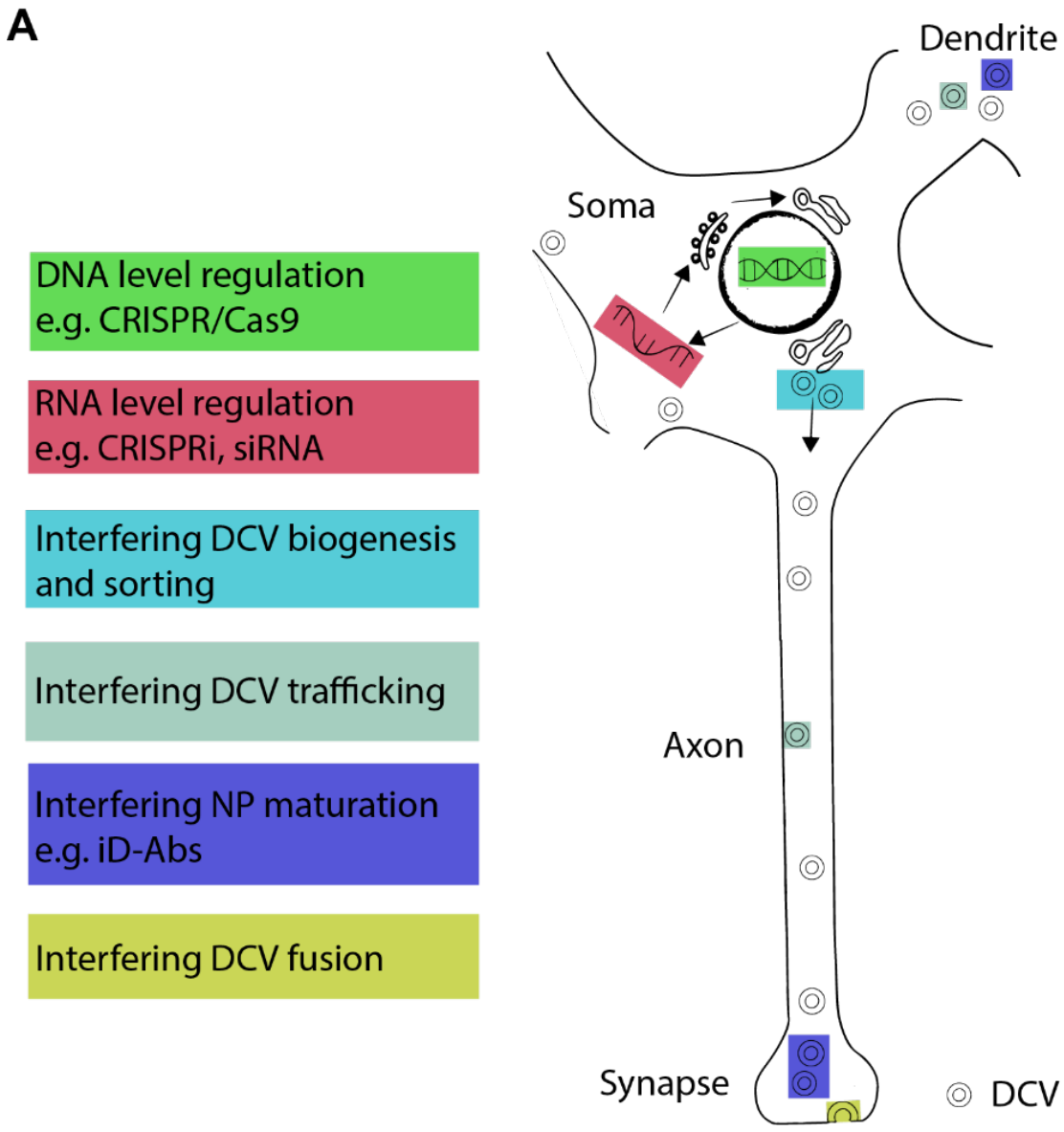

Figure 1: Means to target neuropeptide pathways in a peptidergic neuron.

(A) The complex nature of neuropeptide genesis, sorting, trafficking and release enable multiple ways of targeting neuropeptide pathways. These means are color coded and placed into a peptidergic neuron illustration.

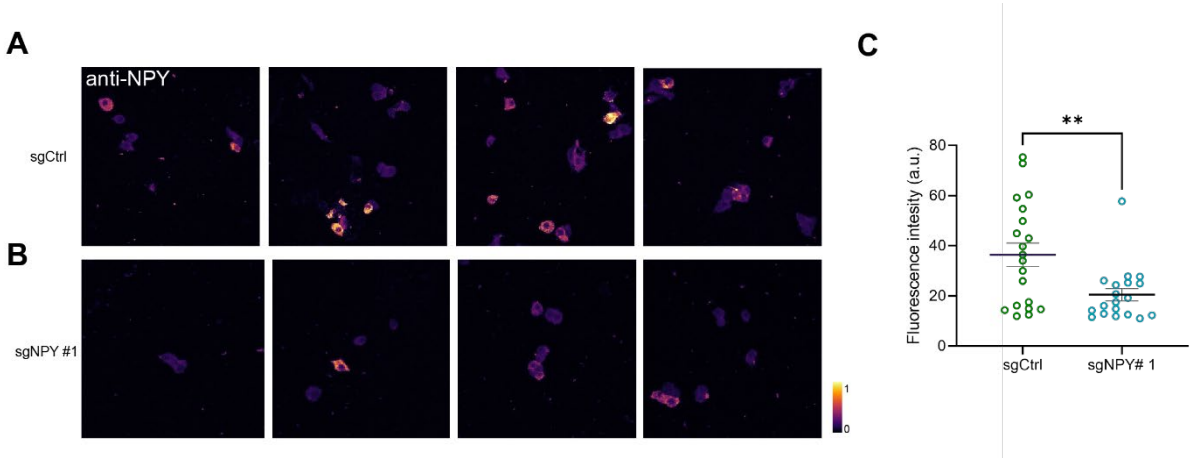

Figure 2: Regulating NPY expression in PC12 cells with CRISPR-Cas9.

(A-B) Example images of PC12 cells transfected with Cas9 and control sgRNA **(A)** or Cas9 and sgRNA targeting Neuropeptide Y **(B)**, details are shown in **Materials and Methods**. Images were pseudo-colored to highlight contrast. **(C)** sgNPY #1 downregulates NPY expression in PC12 cells. Quantification and comparison of fluorescence in intact cells (N=19-20) of **(A,B)**. (**P<0.01, Mann-Whitney *U* test).

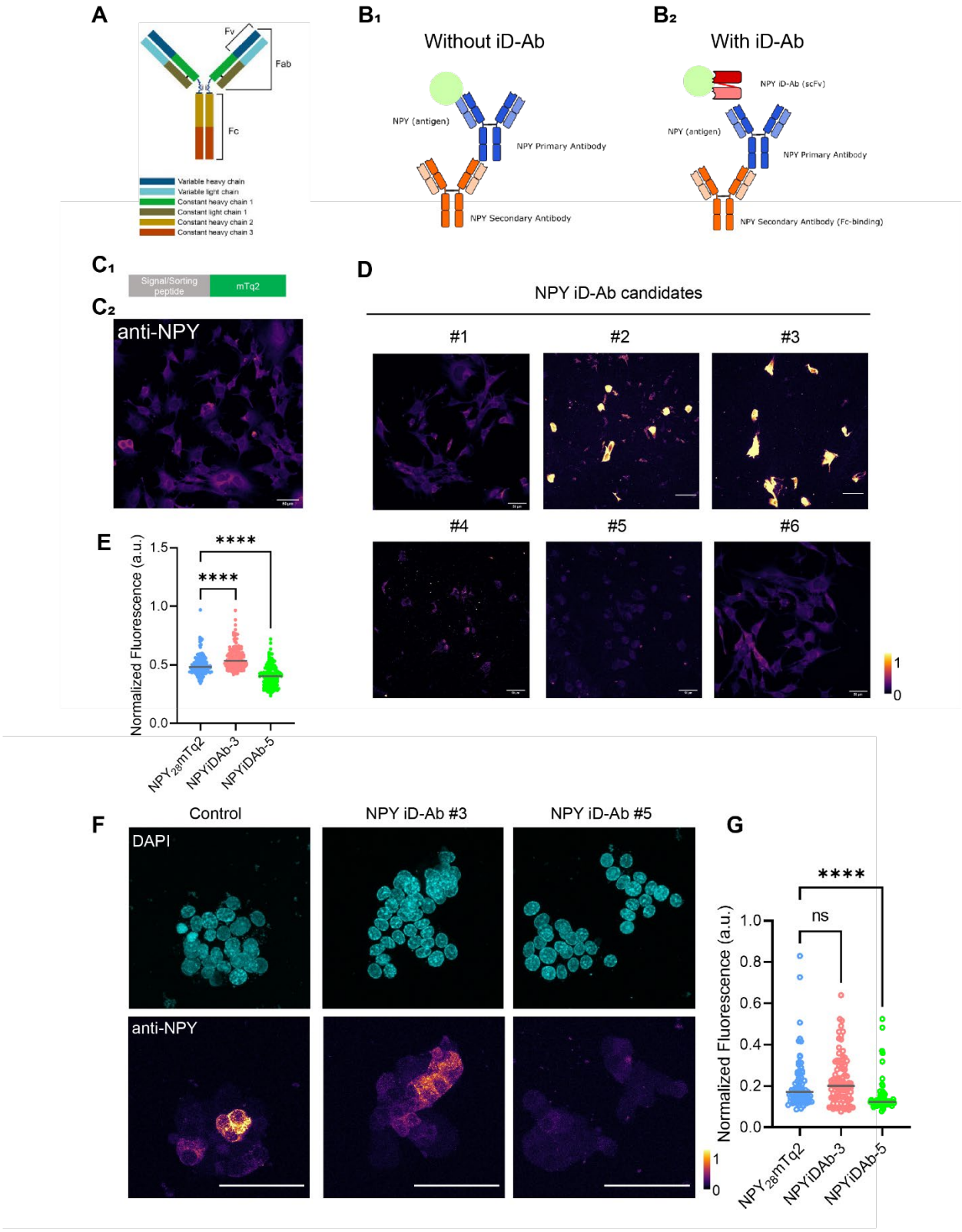

Figure 3: Targeting neuropeptide signaling with intra-DCV Antibodies (iD-Abs).

(A) Illustration of an antibody molecule, which consists of Fab and Fc regions. Fv (variable) regions on the Fab arm recognizes and binds antigen. **(B)** Principle of intra-DCV antibodies (iD-Abs). The canonical primary-secondary antibody binding provides means to visualize and amplify the presence of antigens **(B1)**. If two Fv regions of an antibody are concatenated in lieu of a linker to form a single-chain antibody, and such an antibody is engineered to sort and transport into the DCVs like neuropeptides, it may competitively bind the antigen (neuropeptide) **(B2)** to block or attenuate its interaction with downstream GPCR receptors. These engineered single-chain antibody candidates were named intra-DCV antibody (iD-Ab). **(C)** N46 cells were transfected with $NPLER^{NPY}$ (CMV-NPY_{28}mTq2) **(C1)** and stained for NPY as control **(C2)** for further iD-Ab candidate screening. **(D)** 6 iD-Abs that potentially target NPY were tested via immunocytochemistry, similar to **(C2)**. Among all candidates, #4 exhibited toxicity to N46 cells, #1 and #6 showed no difference, #2 and #3 had increased staining signals and cell morphology was abnormal in the former case. #5 showed decreased anti-NPY signals, making it a potential good candidate for NPY signaling interruption. **(E)** Quantification of two candidates of interest, iD-Ab #3 and #5, as well as their comparisons with control in **(C1)** confirmed the significance of the increase/decrease of fluorescence. (****P<0.0001, Mann-Whitney *U* test). **(F)** Validation of iD-Ab #3 and #5 in PC12 cells and corresponding quantifications of comparisons **(G)**, with #3 showing no change of fluorescence, but #5 decreased as in N46 cells in **(D,E)** (ns: not significant, ****P<0.0001, Mann-Whitney *U* test). Scale bar, 50 μm.

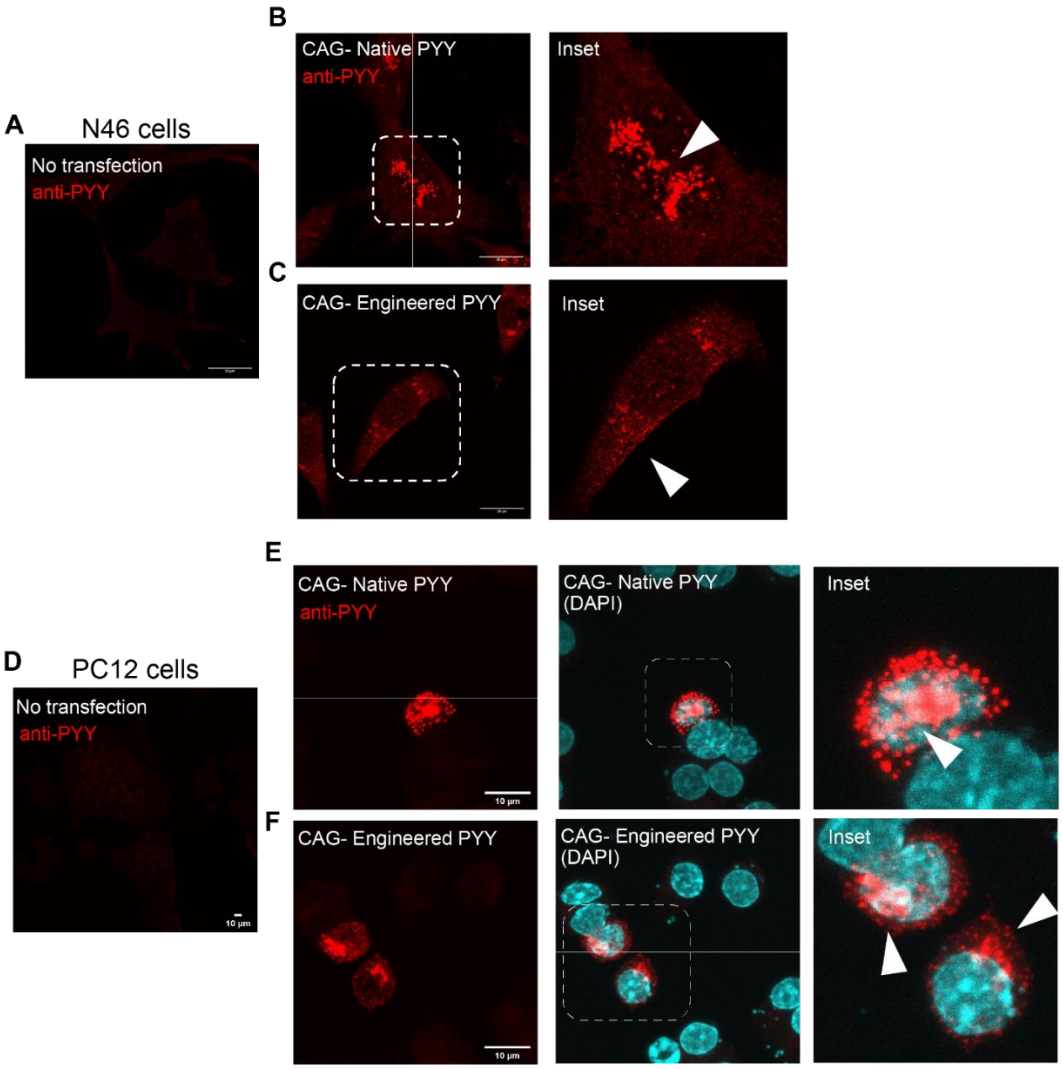

Figure 4: Neuropeptide engineering reduces organelle retention of Peptide YY (PYY) in cell lines.

(A) N46 cells do not express endogenous PYY. **(B)** Exogenous expression of PYY in its native form result in strong accumulation of stained signals at the center of cells, likely in ER, golgi apparatus or nucleus. **(C)** Engineered PYY attenuates accumulation of stained signals in **(B)**. Similar case was seen in PC12 cells, as endogenous PYY was also not seen **(D)**, exogenous expression exhibits a nucleus patch **(E)**, as referenced by the DAPI staining. **(F)** The size of the patch reduces dramatically with engineered PYY expression. Demonstrative regions of interest were indicated with arrow signs. Scale bar, 10 µm.

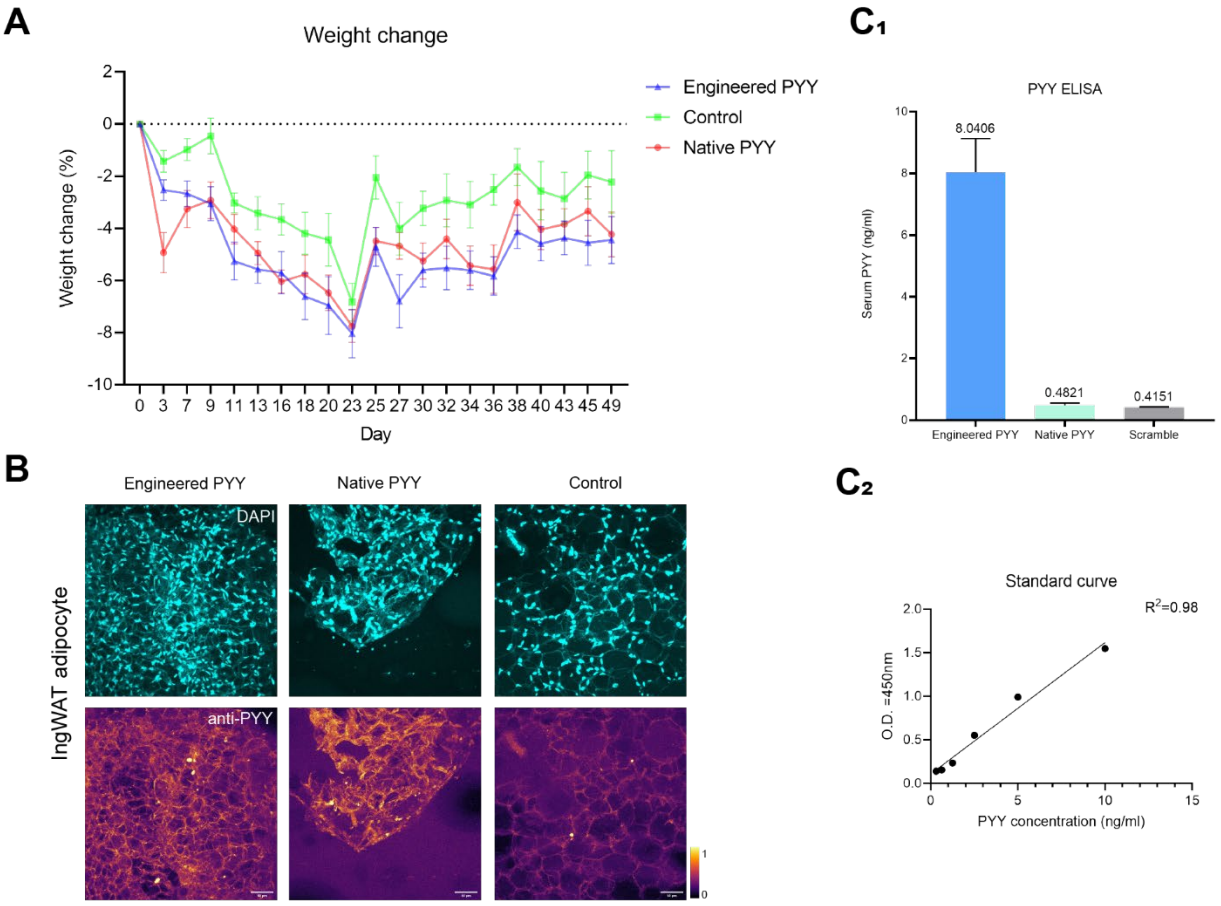

Figure 5: Engineered PYY shows improved tissue distribution and serum concentration.

(A) Weight change plot of experimental mice injected with viruses carry control gene, native PYY transgene and engineered PYY transgene, respectively (N=8 for each group). Day 0 was set as a day after the viral injection. All groups experienced severe weight loss between Day 0 and Day 23, yet the two PYY groups showed less rebound between Day 23 and Day 49. No statistically significant differences were found between native and engineered PYY in terms of their weight loss effects in this experiment. **(B)** PYY IHC of Inguinal white adipose tissue (IngWAT) of each group showed better tissue distribution of PYY staining signals of engineered PYY. Native PYY group exhibited unknown tangled patterns. Images were pseudo-colored to highlight contrast. Scale bar, 50 μm. **(C)** PYY ELISA of blood serum from experimental groups showed that engineered PYY outperforms native PYY as the serum PYY concentration is nearly 20-fold higher **(C1)**, standard curve of the ELISA experiment conveyed strong regressive power of the experiment **(C2)**.

	RNAi KD	CRISPR/Cas9	iD-Ab
NPY	-	+	+
NkB	+	?	-

Table 1: Summary of research progress in NkB and NPY regulation in this study.

Materials and Methods

Molecular cloning

pCAG-PYY and other engineered forms are constructed by replacing CMV promoters in pCMV with CAG promoter through Gibson assembly. Native and engineered PYY DNA molecules were synthesized (Integrated DNA Technologies). All other practices were described in Chapter 3.

CRIPSR experiments

All sgRNA candidates were designed using CHOPCHOP (https://chopchop.cbu.uib.no) (Labun et al., 2019) and top candidates were synthesized and cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene #62988). 24 hours after transfection, medium was replaced and puromycin was added to the new medium to reach final concentration of 1.5μg/ml (PC12 cells) or 3μg/ml (N46 cells). Fresh puromycin-free medium was used to terminate the selection process. All other cell culture practices were identical as in Chapter 3. The best candidate sgNPY #1 targets the sequence GAGGGGTACCCCTCCAAGCCGG. Please note that this sequence is present in both rat and mouse NPY genes, rendering it theoretically effective in both species.

Animal experiments

All experimental procedures involving the use of live mice or their tissues were carried out in accordance with NIH guidelines and approved by the Institute Animal Care and Use Committee (IACUC) and the Institute Biosafety Committee (IBC) at the California Institute of Technology (Caltech). All experimental animals were single-housed 6-month old C57BL/6N male mice (The Jackson Laboratory, #000664). Weight of each mouse was measured with a food scale 3 times a week, 9 weeks in total.

Virus

All AAVs used in this study were customized in this paper with AAV-PHP.S serotype that facilitates peripheral expression (Chan et al., 2017b). They were packaged at the HHMI Janelia Research Campus virus facility, and a high original titer were diluted with clean PBS on the day of use. Viruses were delivered via unilateral retro-orbital injection of 10^{11} genome copies per animal.

Immunocytochemistry (ICC)

For Immunocytochemistry, all reagents and protocols were described in Chapter 3 except the usage of Rabbit anti-PYY (1:500, Bioss Antibodies BS-2265R).

Immunohistochemistry (IHC) and ELISA

Inguinal white adipose *tissues* (*ingWAT) were mined from freshly sacrificed animals and prepared according to (de Jong et al., 2015). The tissues were embedded in OCT compound (Fisher Scientific,* #23-730-571) and *frozen at -80*℃ *overnight* prior to sectioning. To prepare mouse blood serum for ELISA experiments, whole blood was collected right after animal sacrificed, immediately mixed with heparin, and centrifuged for 15 minutes at 1500 rpm at 4℃, clear supernatant was aspirated and stored at -80℃. Assays using the ELISA kits for PYY (Invitrogen EH387RB) was performed according to the user manuals.

Statistical analysis

All data analysis was performed with Graphpad Prism 9, Microsoft Excel and custom Matlab codes. Data underwent normality tests before analysis (Kolmogorov-Smirnov test, Shapiro-Wilk test, D'Agostino & Pearson test), those passed all tests are presented as mean ± s.e.m and used for

parametric comparisons. Otherwise, data are shown as median ± 95% CI and Mann-Whitney U test was used for comparison. Weight data were normalized to day 0 and presented as percentage of change in Figure 5A.

References

Acosta, A., Hurtado, M. D., Gorbatyuk, O., Sala, L. La, & Duncan, M. (2011). Salivary PYY: A Putative Bypass to Satiety. *PLoS ONE*, *6*(10), 26137. https://doi.org/10.1371/journal.pone.0026137

Acuna-Goycolea, C., & Van Den Pol, A. N. (2005). Peptide YY3-36 inhibits both anorexigenic proopiomelanocortin and orexigenic neuropeptide Y neurons: Implications for hypothalamic regulation of energy homeostasis. *Journal of Neuroscience*, *25*(45), 10510–10519. https://doi.org/10.1523/JNEUROSCI.2552-05.2005

Adrian, T. E., Sagor, G. R., Savage, A. P., Bacarese-Hamilton, A. J., Hall, G. M., & Bloom, S. R. (1986). Peptide yy kinetics and effects on blood pressure and circulating pancreatic and gastrointestinal hormones and metabolites in man. *Journal of Clinical Endocrinology and Metabolism*, *63*(4), 803–807. https://doi.org/10.1210/jcem-63-4-803

Arora, S., & Anubhuti. (2006). Role of neuropeptides in appetite regulation and obesity - A review. *Neuropeptides*. https://doi.org/10.1016/j.npep.2006.07.001

Batterham, R. L., Cohen, M. A., Ellis, S. M., Le Roux, C. W., Withers, D. J., Frost, G. S., Ghatei, M. A., & Bloom, S. R. (2003). Inhibition of Food Intake in Obese Subjects by Peptide YY $_{3-36}$. *New England Journal of Medicine*, *349*(10), 941–948. https://doi.org/10.1056/NEJMoa030204

Batterham, R. L., Cowley, M. A., Small, C. J., Herzog, H., Cohen, M. A., Dakin, C. L., Wren, A. M., Brynes, A. E., Low, M. J., Ghatei, M. A., Cone, R. D., & Bloom, S. R. (2002). Gut hormone PYY3-36 physiologically inhibits food intake. *Nature*, *418*(6898), 650–654. https://doi.org/10.1038/nature00887

Beutler, L. R., Chen, Y., Ahn, J. S., Lin, Y. C., Essner, R. A., & Knight, Z. A. (2017). Dynamics of Gut-Brain Communication Underlying Hunger. *Neuron*, *96*(2), 461-475.e5. https://doi.org/10.1016/j.neuron.2017.09.043

Boey, D., Lin, S., Enriquez, R. F., Lee, N. J., Slack, K., Couzens, M., Baldock, P. A., Herzog, H., & Sainsbury, A. (2008). PYY transgenic mice are protected against diet-induced and genetic obesity. *Neuropeptides*, *42*(1), 19–30. https://doi.org/10.1016/j.npep.2007.11.003

Boss, M. A., Kenten, J. H., Wood, C. R., & Emtage, J. S. (1984). Assembly of functional antibodies from immunoglobulin heavy and light chains synthesised in E. coli. *Nucleic Acids Research*, *12*(9), 3791–3806. https://doi.org/10.1093/nar/12.9.3791

Bowers, M. E., Choi, D. C., & Ressler, K. J. (2012). Neuropeptide regulation of fear and anxiety: Implications of cholecystokinin, endogenous opioids, and neuropeptide Y. *Physiology and Behavior*. https://doi.org/10.1016/j.physbeh.2012.03.004

Chan, K. Y., Jang, M. J., Yoo, B. B., Greenbaum, A., Ravi, N., Wu, W. L., Sánchez-Guardado, L., Lois, C., Mazmanian, S. K., Deverman, B. E., & Gradinaru, V. (2017a). Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous systems. *Nature Neuroscience*, *20*(8), 1172–1179. https://doi.org/10.1038/NN.4593

Chan, K. Y., Jang, M. J., Yoo, B. B., Greenbaum, A., Ravi, N., Wu, W. L., Sánchez-Guardado, L., Lois, C., Mazmanian, S. K., Deverman, B. E., & Gradinaru, V. (2017b). Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous systems. *Nature Neuroscience*, *20*(8), 1172–1179. https://doi.org/10.1038/nn.4593

Chelikani, P. K., Haver, A. C., & Heidelberger, R. D. (2005). Intravenous infusion of peptide YY(3-36) potently inhibits food intake in rats. *Endocrinology*, *146*(2), 879–888. https://doi.org/10.1210/en.2004-1138

Chelikani, P. K., Haver, A. C., & Reidelberger, R. D. (2007). Intermittent intraperitoneal infusion of peptide YY(3-36) reduces daily food intake and adiposity in obese rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *293*(1), R39–R46. https://doi.org/10.1152/ajpregu.00164.2007

de Jong, J. M. A., Larsson, O., Cannon, B., & Nedergaard, J. (2015). A stringent validation of mouse adipose tissue identity markers. *American Journal of Physiology - Endocrinology and Metabolism*, *308*(12), E1085–E1105. https://doi.org/10.1152/AJPENDO.00023.2015

Dhillo, W. S., & Bloom, S. R. (2001). Hypothalamic peptides as drug targets for obesity. In *Current Opinion in Pharmacology*. https://doi.org/10.1016/S1471-4892(01)00110-2

Ding, K., Han, Y., Seid, T. W., Buser, C., Karigo, T., Zhang, S., Dickman, D. K., & Anderson, D. J. (2019). Imaging neuropeptide release at synapses with a genetically engineered reporter. *ELife*, *8*. https://doi.org/10.7554/elife.46421

Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science*. https://doi.org/10.1126/science.1258096

Eiger, D. S., Pham, U., Gardner, J., Hicks, C., & Rajagopal, S. (2022). GPCR systems pharmacology: a different perspective on the development of biased therapeutics. *American Journal of Physiology-Cell Physiology*, *322*(5), C887–C895. https://doi.org/10.1152/ajpcell.00449.2021

Elgundi, Z., Reslan, M., Cruz, E., Sifniotis, V., & Kayser, V. (2017). The state-of-play and future of antibody therapeutics. *Advanced Drug Delivery Reviews*, *122*, 2–19. https://doi.org/10.1016/J.ADDR.2016.11.004

Gantz, I., Erondu, N., Mallick, M., Musser, B., Krishna, R., Tanaka, W. K., Snyder, K., Stevens, C., Stroh, M. A., Zhu, H., Wagner, J. A., MacNeil, D. J., Heymsfield, S. B., & Amatruda, J. M. (2007). Efficacy and Safety of Intranasal Peptide YY3–36 for Weight Reduction in Obese Adults. *The Journal of Clinical Endocrinology & Metabolism*, *92*(5), 1754–1757. https://doi.org/10.1210/jc.2006-1806

Griebel, G., & Holsboer, F. (2012). Neuropeptide receptor ligands as drugs for psychiatric diseases: The end of the beginning? *Nature Reviews Drug Discovery*. https://doi.org/10.1038/nrd3702

Guo, Y., Ma, L., Enriori, P. J., Koska, J., Franks, P. W., Brookshire, T., Cowley, M. A., Salbe, A. D., DelParigi, A., & Tataranni, P. A. (2006). Physiological Evidence for the Involvement of Peptide YY in the Regulation of Energy Homeostasis in Humans*. *Obesity*, *14*(9), 1562–1570. https://doi.org/10.1038/oby.2006.180

Halatchev, I. G., & Cone, R. D. (2005). Peripheral administration of PYY3-36 produces conditioned taste aversion in mice. *Cell Metabolism*, *1*(3), 159–168. https://doi.org/10.1016/j.cmet.2005.02.003

Hökfelt, T., Bartfai, T., & Bloom, F. (2003). Neuropeptides: Opportunities for drug discovery. *Lancet Neurology*. https://doi.org/10.1016/S1474-4422(03)00482-4

Hökfelt, T., Pernow, B., & Wahren, J. (2001). Substance P: A pioneer amongst neuropeptides. *Journal of Internal Medicine*. https://doi.org/10.1046/j.0954-6820.2000.00773.x

Holzer, P., Reichmann, F., & Farzi, A. (2012). Neuropeptide Y, peptide YY and pancreatic polypeptide in the gut-brain axis. *Neuropeptides*. https://doi.org/10.1016/j.npep.2012.08.005

Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. *Cell*. https://doi.org/10.1016/j.cell.2014.05.010

Jacobson, K. A. (2015). New paradigms in GPCR drug discovery. *Biochemical Pharmacology*. https://doi.org/10.1016/j.bcp.2015.08.085

Jiang, F., & Doudna, J. A. (2017). CRISPR–Cas9 Structures and Mechanisms. *Annual Review of Biophysics*, *46*(1), 505–529. https://doi.org/10.1146/annurev-biophys-062215-010822

Kontermann, R. E., & Müller, R. (1999). Intracellular and cell surface displayed single-chain diabodies. *Journal of Immunological Methods*, *226*(1–2), 179–188. https://doi.org/10.1016/S0022-1759(99)00062-9

Labun, K., Montague, T. G., Krause, M., Torres Cleuren, Y. N., Tjeldnes, H., & Valen, E. (2019). CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Research*, *47*(W1), W171–W174. https://doi.org/10.1093/NAR/GKZ365

Pedragosa-Badia, X., Stichel, J., & Beck-Sickinger, A. G. (2013). Neuropeptide y receptors: How to get subtype selectivity. *Frontiers in Endocrinology*, *4*(FEB), 1–14. https://doi.org/10.3389/fendo.2013.00005

Pittner, R. A., Moore, C. X., Bhavsar, S. P., Gedulin, B. R., Smith, P. A., Jodka, C. M., Parkes, D. G., Paterniti, J. R., Srivastava, V. P., & Young, A. A. (2004). Effects of PYY[3-36] in rodent models of diabetes and obesity. *International Journal of Obesity*, *28*(8), 963–971. https://doi.org/10.1038/sj.ijo.0802696

Rangwala, S. M., D'Aquino, K., Zhang, Y. M., Bader, L., Edwards, W., Zheng, S., Eckardt, A., Lacombe, A., Pick, R., Moreno, V., Kang, L., Jian, W., Arnoult, E., Case, M., Jenkinson, C., Chi, E., Swanson, R. V., Kievit, P., Grove, K., … Leonard, J. N. (2019). A Long-Acting PYY 3–36 Analog Mediates Robust Anorectic Efficacy with Minimal Emesis in Nonhuman Primates. *Cell Metabolism*, *29*(4), 837-843.e5. https://doi.org/10.1016/j.cmet.2019.01.017

Russell, F. A., King, R., Smillie, S. J., Kodji, X., & Brain, S. D. (2014). Calcitonin gene-related peptide: physiology and pathophysiology. *Physiological reviews*. https://doi.org/10.1152/physrev.00034.2013

Schank, J. R., Ryabinin, A. E., Giardino, W. J., Ciccocioppo, R., & Heilig, M. (2012). Stress-Related Neuropeptides and Addictive Behaviors: Beyond the Usual Suspects. *Neuron*. https://doi.org/10.1016/j.neuron.2012.09.026

Sloth, B., Holst, J. J., Flint, A., Gregersen, N. T., & Astrup, A. (2007). Effects of PYY1-36 and PYY3-36 on appetite, energy intake, energy expenditure, glucose and fat metabolism in obese and lean subjects. *American Journal of Physiology - Endocrinology and Metabolism*, *292*(4), 1062–1069. https://doi.org/10.1152/ajpendo.00450.2006

Toräng, S., Veedfald, S., Rosenkilde, M. M., Hartmann, B., & Holst, J. J. (2015). The anorexic hormone peptide yy3-36 is rapidly metabolized to inactive peptide yy3-34 in vivo. *Physiological Reports*, *3*(7), e12455. https://doi.org/10.14814/phy2.12455

Tyndall, J., & Sandilya, R. (2005). GPCR Agonists and Antagonists in the Clinic. *Medicinal Chemistry*, *1*(4), 405–421. https://doi.org/10.2174/1573406054368675

van den Pol, A. N. (2012). Neuropeptide Transmission in Brain Circuits. *Neuron*. https://doi.org/10.1016/j.neuron.2012.09.014

Wang, P., Mariman, E., Renes, J., & Keijer, J. (2008). The secretory function of adipocytes in the physiology of white adipose tissue. *Journal of Cellular Physiology*, *216*(1), 3–13. https://doi.org/10.1002/jcp.21386