Final Project Report for KKP-126998

Our overall goal was to understand the biological underpinning of the beneficial effects of calorie restriction on integrative physiology, including complex behaviors and peripheral tissue functions. We specifically hypothesized that hypothalamic neurons (the agouti-related peptide, AgRP- and proopiomelanocortin, POMC-expressing cells) responsible for appetite and feeding control play crucial roles in mediating the effects of calorie restriction on behaviors, brain development, peripheral tissue metabolism, and overall health- and life-span. In the first year of the project, we set up a fully equipped animal house, where breeding of the mouse strains used in the experiments and studies on feeding, behavior and lifespan are (also currently) being carried out. We performed a set of studies where we directly and forcefully interrogate these novel concepts using animal models. Our studies shed new light on integrative physiology and molecular principles of calorie restriction-induced alterations in health- and lifespan. Beyond this specific outcome, our studies delivered novel principles for integrative physiology where the hypothalamus plays an organizational role to align behavior with appropriate peripheral functions.

Our results:

I. Cellular organization of AgRP and POMC neurons during aging

We examined the intracellular parameters and the structural properties of AgRP cells at different ages in wild-type animals and we compared these with those of POMC neurons. AgRP and POMC expressing cells form two distinct populations in the hypothalamus and are involved in energy and food-intake regulation with opposite effect. Our results suggest that the cellular dimensions of satiety active POMC neurons change with age, but the size of hunger-promoting AgRP neurons remains constant. In addition, AgRP neurons show a lower expression of the residual bodies' characteristic of aging in the cytoplasm, suggesting that AgRP neurons are more resistant to changes in the cytoplasm over time (*Figure 1.*) Furthermore, we also examined the cellular changes of high fat diet in the AgRP and POMC neuronal population. Animals were maintained on either a standard diet (SD) or a high fat-(calorie-) diet (HFD) for 12 weeks. This high calorie diet has been shown to promote the development of age-related chronic diseases in various organisms, including mice (Rockenfeller & Madeo, 2010; Wali *et al.*, 2020). We found that in young mice (6 months) in POMC cells (but not AgRP cells) in animals on a high calorie diet, similar to ageing, the number of residual granules was significantly increased (*Figure 2.).* (*manuscript in preparation*).

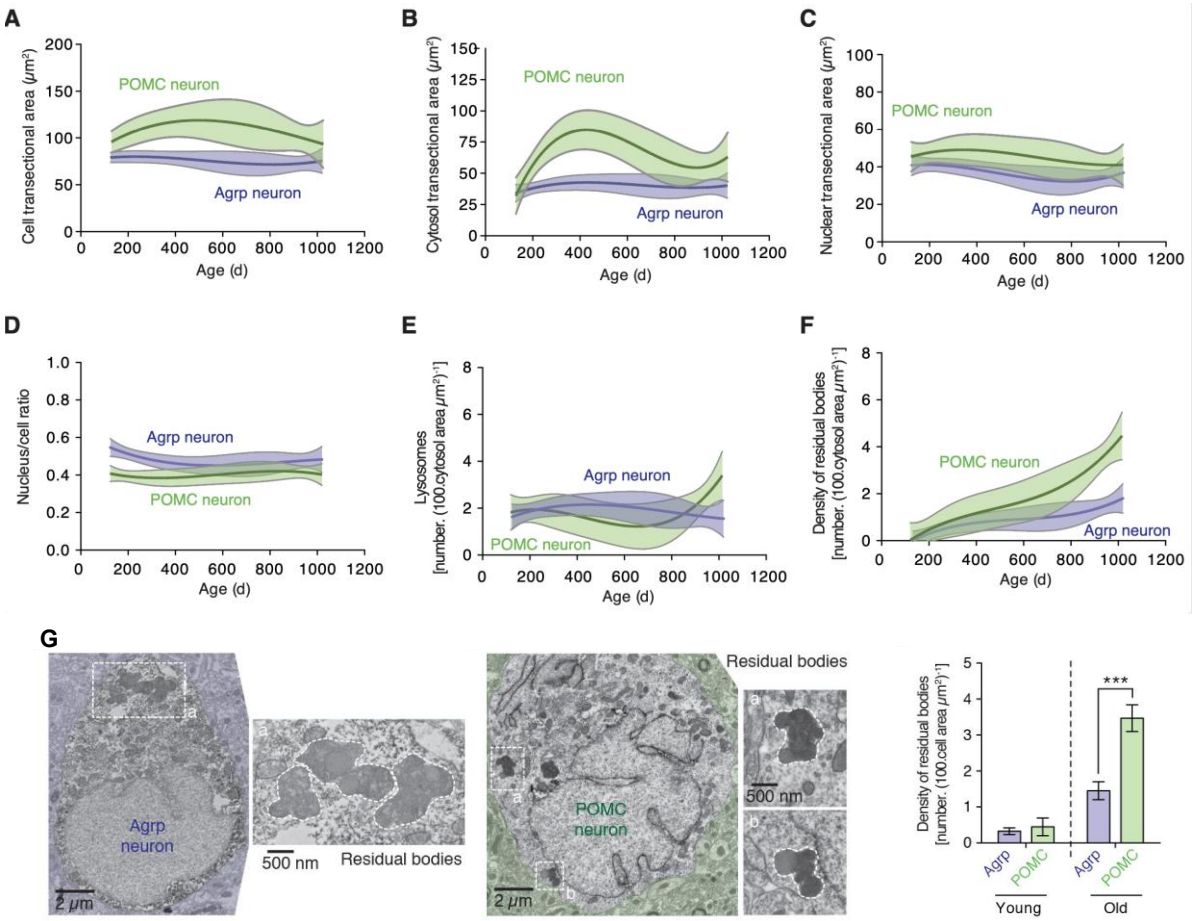


Figure 1. Cellular signs of aging in POMC and AgRP neurons. Graphs show the diameter of the cell body (A), cytoplasm (B) and nucleus (C) for POMC and AgRp cells with age. These data show that these parameters of AgRP cells are stable, whereas the cytoplasmic diameter of POMC cells increases dramatically between juvenile and middle age, then decreases and increases again in the final phase of aging. Examination of the aforementioned data (A-C➡D) shows and confirms that AgRP neurons are smaller, mainly due to the smaller size of the cytoplasm. E: Further electron microscopy analysis showed that while the number of lysosomes is stable in AgRP cells, in POMC cells these data decrease until mid-age and then increase in the last phase of aging in POMC cells. F, top: The greatest variation in the number of so-called residual or stress granules during aging is observed in AgRP and POMC cells. While in POMC cells the number of these granules increased steadily, quasi exponentially, during aging, the same parameters increased much less in AgRP cells. G: Representative electron micrographs show residual granules in AgRP (left) and POMC (right) cells. Bottom, bar graph shows a decomposition of young and old data, respectively.

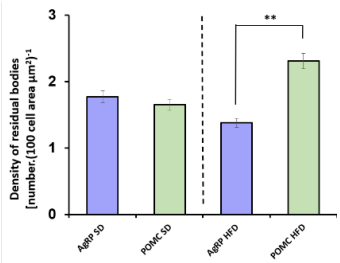


Figure 2. High calolrie diet induced cellular changes in POMC cells but not in AgRP cells. It is striking that in POMC cells of young mice, a high-fat diet causes the intracellular changes observed during aging, whereas AgRP cells are marginally affected.

II. Organizational role of AgRP neurons in compulsive behaviour

AgRp/NPY neurons have a critical role in driving food intake, but also in modulating complex, non-feeding behaviors. We interrogated whether AgRP neurons are relevant to the emergence of anorexia nervosa symptomatology using anactivity-based anorexia (ABA) model. We showed a rapid inhibition of AgRP neuronal activity following voluntary cessation of running. Thus, we used loss- and gain-of-function mouse models to examine the effects of AgRP activity on anorexia. Interestingly, all AgRP neuron-ablated (via neonatal diphtheria toxin injection in AgRP-DTR mice), food-restricted mice die within 72 h of compulsive running, while daily activation of AgRP neurons using a chemogenetic tool (via transient receptor potential cation channel subfamily V member 1, TRPV1) increases voluntary running with no lethality of food-restricted animals. Animals with impaired AgRP neuronal circuits are unable to properly mobilize fuels during food-restriction-associated exercise; however, when provided with elevated fat content through diet, their death is completely prevented. Elevated fat content in the diet also prevents the long-term behavioural impact of food-restricted fit mice with elevated exercise volume. We postulate that, in individuals with vulnerability to develop anorexia nervosa, AgRP neurons may respond to negative energy balance cues in an exacerbated manner leading to repetitive and compulsive behaviours (Halmi *et al.*, 2003) These observations shed light on a previously unsuspected organizational role of AgRP neurons, via the mediation of the periphery, in the regulation of compulsive exercise and its related lethality with possible implications for psychiatric conditions, such as anorexia nervosa.

We published our results in *Nature Metabolism* (2020 2(11):1204-1211) (Miletta *et al.*, 2020)

III. Circulating LPAs directly influence AgRP neuron's control on cortical excitability

In parallel with behavioral testing and aging studies we were also interested in whether changes in peripheral metabolism affect brain lipid levels and cortical excitability (Figure). We showed that levels of lysophosphatidic acid (LPA) species in the blood and cerebrospinal fluid are elevated after overnight fasting and lead to higher cortical excitability. LPA is a short-lived, but potent, signaling molecule5 that acts via specific G-protein-coupled receptors, LPA-R1–6 (Yung *et al.*, 2015). LPA levels are tightly regulated by specific phosphatases (LPP1–3 (Tang *et al.*, 2015)), suggesting that LPA synthesis and action are locally restricted. LPA is also locally synthesized at the synaptic cleft of glutamatergic synapses by autotaxin (ATX/Enpp2, (Moolenaar, 2002), which is expressed by astrocytic processes covering cortical glutamatergic synapses (Thalman *et al.*, 2018). Thus, LPA is a powerful modulator of presynaptic glutamate release by activation of presynaptic LPA2 receptors, which regulate glutamate release probabilities and thereby neuronal excitability (Trimbuch *et al.*, 2009). Because food restriction rapidly depletes glycogen stores and induces lipolysis, thereby altering body lipid levels, and AgRP neurons have been shown to control peripheral lipid metabolism and complex behaviours beyond feeding (Miletta *et al.*, 2020), we interrogated the effect of fasting and AgRP circuit integrity on brain phospholipid levels, as well as its impact on both cortical excitability and food intake control. LPA-related cortical excitability increases fasting-induced hyperphagia and is decreased following inhibition of LPA synthesis (Figure 3). We further showed that the effects of LPA following fasting are under the control of hypothalamic agouti-related peptide (AgRP) neurons. Depletion of AgRP-expressing cells in adult mice decreases fasting-induced elevation of circulating LPAs, as well as cortical

excitability while blunting hyperphagia. These findings reveal a direct influence of circulating LPAs under the control of hypothalamic AgRP neurons on cortical excitability, unmasking an alternative non-neuronal route by which the hypothalamus can exert a robust impact on the cortex and thereby affect food intake.

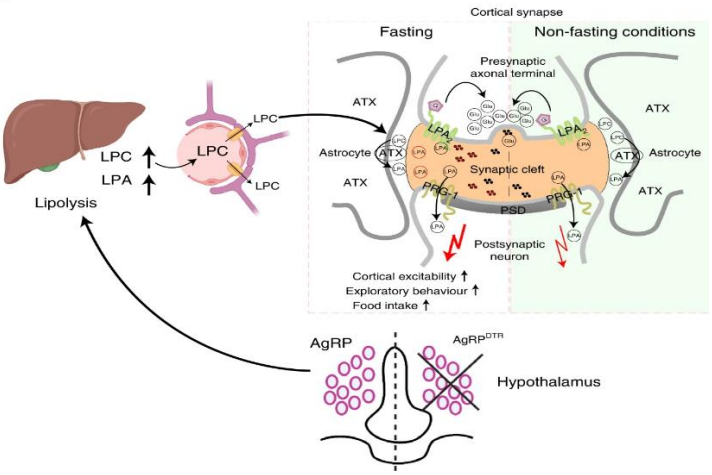


Figure 3. Fasting-induced increase in cortical excitability by peripherally produced LPA precursors. Following overnight fasting, glycogen stores in the liver are depleted and lipolysis is increased leading to LPC release in the blood, causing increased LPA levels after overnight fasting via ATX-dependent synthesis in the blood. Our data suggest that this first step in metabolic adaptation to fasting conditions is regulated by AgRP neurons in the hypothalamus. However, following peripheral release upon fasting, LPC is selectively transported across the blood–brain barrier and is metabolized by astrocytic ATX at glutamatergic synapses to generate local LPA, which stimulates presynaptic LPA2 receptors leading to fasting-induced increase in glutamatergic transmission and cortical network excitability. In turn, fasting-induced cortical excitability drives fasting-induced hyperphagia, as shown by the present data.

We published these results in *Nature Metabolism* 2022;4(6):683-692. (Endle *et al.*, 2022)

IV. OPA1 and mitochondrial Ca^{2+} dynamics in POMC neurons influence metabolic health

Homeostatic regulation of energy balance is achieved by the orchestration of adaptive behavioral, autonomic, and endocrine responses via multiple and distributed neuronal networks. A crucial feature to properly modulate such effector mechanisms is the ability of these neural circuits to sense and integrate diverse signals reflecting nutritional status (Timper & Bruning, 2017). In this scenario, POMC neurons in the arcuate nucleus of the hypothalamus (ARC) have emerged as key nutrient sensors implicated in the regulation of metabolism(Toda *et al.*, 2017).

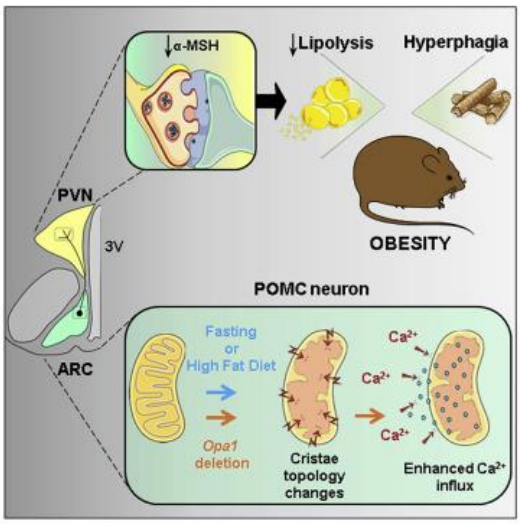


Figure 4. Mitochondrial cristae-remodeling protein OPA1 in POMC neurons couples Ca^{2+} homeostasis with adipose tissue lipolysis Nutritional state shapes mitochondrial cristae and OPA1 expression in POMC neurons. OPA1 deletion in POMC neurons alters mitochondrial Ca^{2+} handling and α-MSH release. POMC OPA1-deficient mice show impaired fat lipolysis and metabolic health. Pharmacological restoration of Ca^{2+} recovered molecular and metabolic alterations.

However, the precise molecular mechanisms of POMC neuron nutrient sensing remain incompletely understood. The connection between mitochondrial OPA1, cristae structure, and Oxidative phosphorylation activity independent of mitochondrial fusion (Patten *et al.*, 2014) suggests that cristae topology readjustments also play a critical role in energy balance control. Thus, we reasoned that such a convoluted process could represent a core mechanism in POMC neurons to sense and adapt to metabolic needs. We revealed an unexpected link between OPA1 and mitochondrial Ca^{2+} dynamics in POMC neurons, influencing adipose tissue triglyceride mobilization and metabolic health (Figure 4).

We published these results in *Cell Metabolism* 2021,33(9): 1820-1835.e9 (Gomez-Valades *et al.*, 2021)

V. AgRP neurons regulate non-feeding behavior

We also made progress in our understanding of the reasons why AgRP circuit-impaired animals have significantly altered behavior. Using two AgRP-impaired mouse-line (AgRP $Sirt1^{-/-}$ (NAD+-dependent class III deacetylase sirtuin-1 (Sirt1) KO) (Nogueiras *et al.*, 2012; Kim *et al.*, 2019) and AgRP $DTR^{+/-}$ (Luquet *et al.*, 2005)) we showed that calorie restriction (CR) promotes the activity of NPY/AgRP neurons in the brain and that disrupting the Sirt1 or reducing the number of AgRP neurons in neonates leads to impaired behavioral but not metabolic responses to CR. Interestingly we also found, that the different AgRP impairments show different behavioral responses to CR (*Figure 5 and 6*).

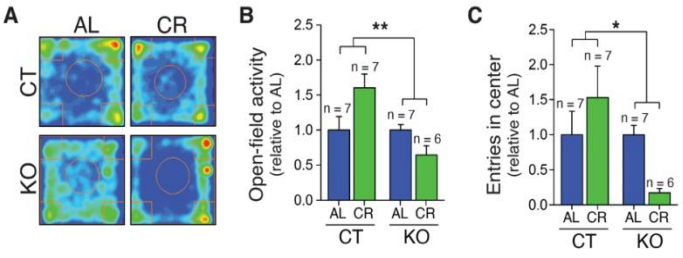


Figure 5. Behavioral response to impaired AgRp functionin the Sirt-1 KO animal. CR wild-type mice moved more and spent more time in the center of the open field compared to their ad libitum-fed controls (A-C), AgRP-Sirt1KO animals moved significantly less and effectively never touched the center (A-C).

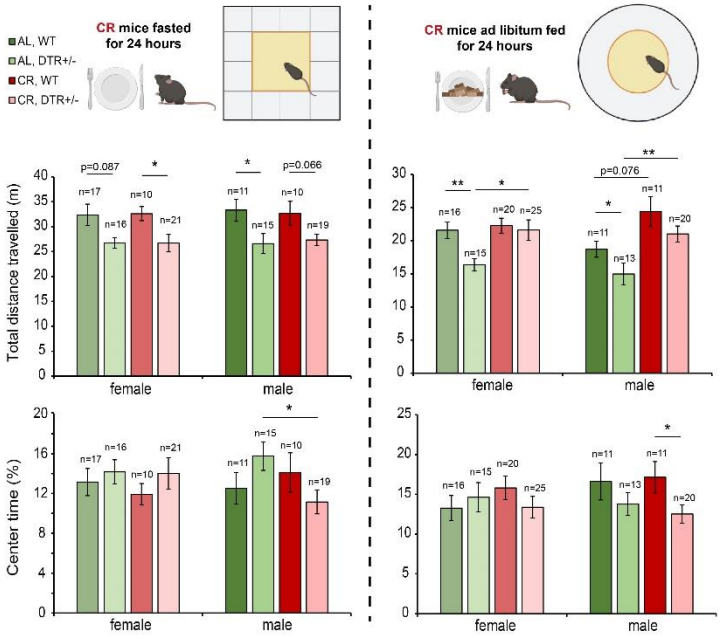


Figure 6. Open field tests of 3 months old AgRP$^{DTR+/-}$male and female, ad libitum fed or calorie restricted mice. Both male and female ad libitum fed AgRP$^{DTR+/-}$ mice show decreased locomotor activity (upper bar graphs), with no significant difference in the time spent in center area (lower bar graphs). The same holds true for calorie restricted AgRP$^{DTR+/-}$ mice on fasting days, while on feeding days AgRP$^{DTR+/-}$ mice moved significantly more than the *ad libitum* fed animals, and the difference between WT and AgRP$^{DTR+/-}$ mice decreased.

These findings highlight the pivotal role of the NPY/AgRP neurons during CR in the regulation of complex behaviors and warrant further studies to dissect the precise role of AgRP neurons in complex behaviors.

Our results focusing on these aspects of the AgRP neurons have been presented as a poster presentation at the *2023 HNS-ANA Meeting* and the *2023 SfN Meeting* and *manuscripts are under preparation.*

VI. AgRP neurons control development of the mPFC

As mentioned above, AgRP/NPY-expressing neurons have a critical role not only in feeding but also in non-feeding behaviors of newborn, adolescent, and adult mice, like reward-seeking, stereotypic and compulsive behaviors, learning, and memory processes suggesting their broad modulatory impact on brain functions. Many of these behaviors are strongly impacted by cortical developmental actions. *How AgRP/NPY neurons affect complex behaviors involving cortical areas remains poorly understood*. As reported, we found that constitutive impairment of AgRP/NPY neurons or their peripubertal DREADD inhibition resulted in both a numerical and functional reduction in the medial prefrontal cortical (mPFC) pyramidal neurons of mice. These changes were accompanied by an alteration of oscillatory network activity in mPFC, impaired sensorimotor gating, and altered ambulatory behavior that could be reversed by the administration of clozapine, a non-selective dopamine receptors antagonist. Observed AgRP/NPY effects are transduced to mPFC in part via dopaminergic neurons in the ventral tegmental area and by medial thalamic neurons. Our results revealed a previously unrecognized control role for hypothalamic AgRP/NPY neurons

in the neuronal pathways that regulate higher-order brain functions during development and in adulthood.

We published these results in *Molecular Psychiatry* 2022 27(10):3951-3960.(Stutz *et al.*, 2022)

VII. Genetic identity and laminar distribution of pyramidal cells in the mPFC

Our parallel investigation revealed that AgRP neurons have strong influence over medial prefrontal cortical (mPFC) development and functions. However, our knowledge of the organization of the mPFC, namely the genetic identity and laminar distribution of its main cell type, the pyramidal cells, have been rather obscure. Thus, we used transgenic animals, viral vectors, and various immunochemical stainings along with confocal microscopy to clarify these issues. We found using the mesolimbic system that the laminar organization of the mPFC differs from other, primary cortical regions. Pyramidal cells express distinct genetic markers in a layer- and projection-specific manner. These datasets provide fundamental anatomical and molecular information which could be incorporated into any future studies focusing on the cellular-level analysis of mPFC functions.

Related publication in *eLife* 2022, 2022 Sep 5;11:e78813. (Babiczky & Matyas, 2022)

VIII. AgRP neurons control body weight and lifespan in a sex-dependent manner

Our project also focused on age-related mechanisms and life-span. These studies take time, and the primary results of our lifespan studies will occur in the coming years. This is the nature of chronic studies. In several cases, the effects of AgRP manipulations on weight gain, life span, behaviour are already available (*Figure 7-10*). Our results point in the clear direction that underactivation of AgRP neurons induces more significant changes in male mice compared to females. These results are novel and predict sex-specific differences in the function of hypothalamic AgRP neurons.

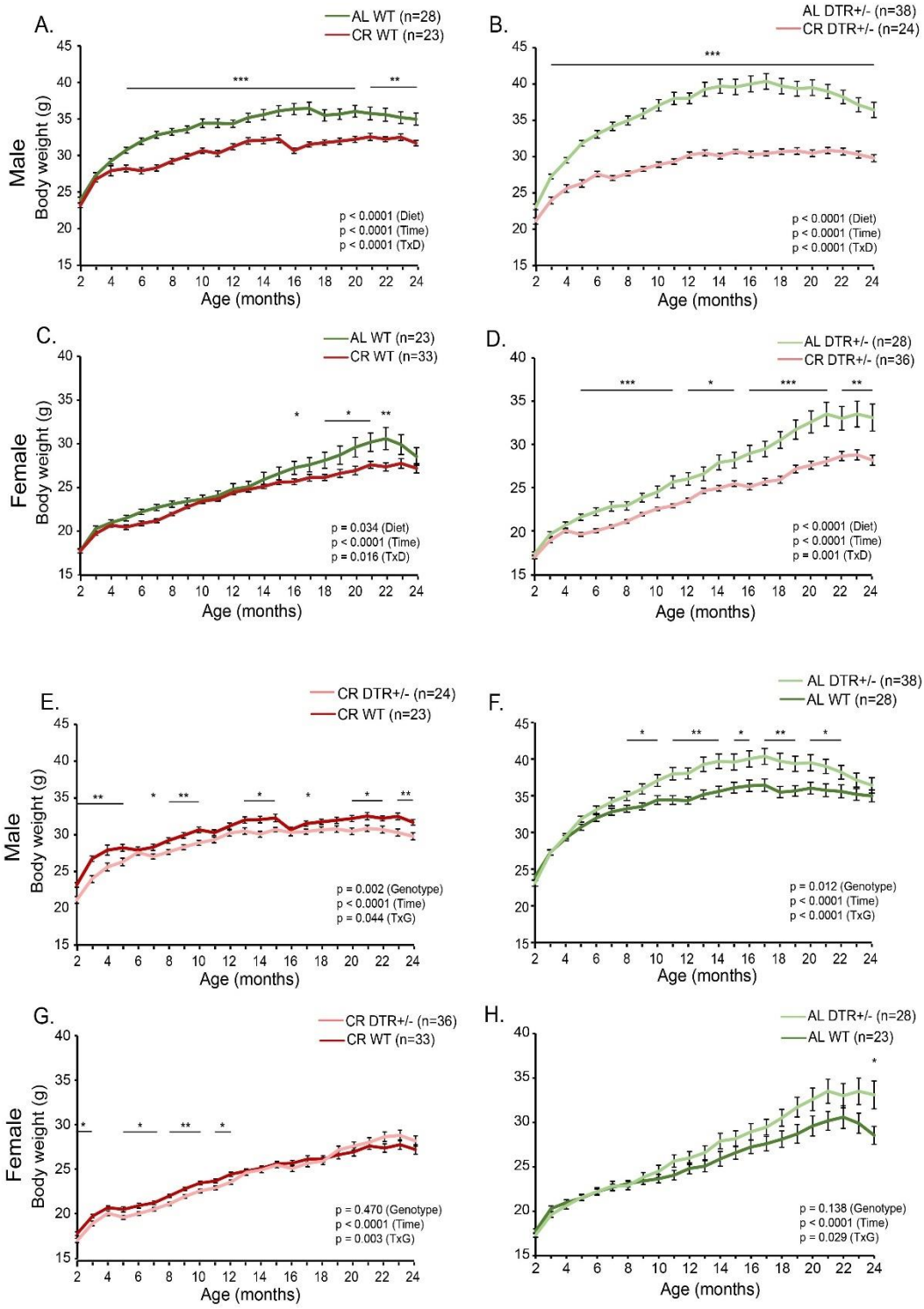
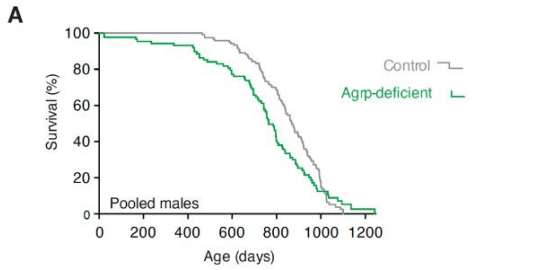
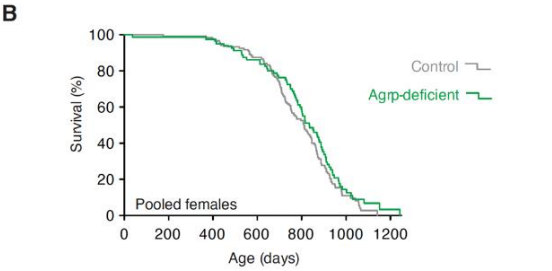


Figure 7. The AgPRDTR mouse model – effect of genotype, diet, and sex on body weight of mice up to 24 months of age. (A, C) CR diet in WT mice has its well-documented effect – reduced bodyweight is more pronounced in males. However, AgPRDTR+/- males and females responded differently to AL and CR diets, AL males gained significant weight (F), whereas this was less pronounced in females (H). Repeated measurements ANOVA with Bonferroni correction * p<0.05 ** p<0.01 *** p<0.001

These figures show the effect of AgRP downregulation on *survival* in male and female mice in three different models we investigated. The AgRP$^{DTR+/-}$ mice were also fed the CR diet, so that the effect of calorie restriction could also be investigated. Our results clearly show a sex-specific effect of the impairment of AgRP neurons.

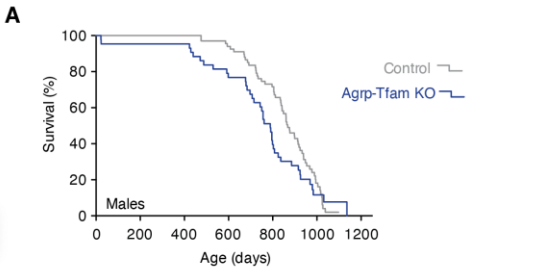


	Control	Agrp-deficient	Effect size (%)	Statistics
n =	119	88	-	Weighted Log-Rank Test
Censored	14	8	-	
Mean	853.29	752.20	13.4	*Chi2 = 12.058*
Std. Error	13.73	26.50	-	***P < 0.001***
95% CI	826 ~ 880	700 ~ 804	-	
Age in days at % mortality:				Fisher's Exact Test
25%	744	655	13.6	*P = 0.011*
50%	869	765	13.6	***P < 0.001***
75%	980	916	6.9	*P = 0.071*
90%	1024	1035	1.0	*P = 0.472*
95% CI	837 ~ 910	743 ~ 799		

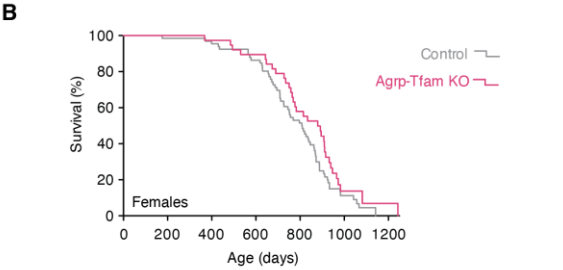


	Control	Agrp-deficient	Effect size (%)	Statistics
n =	120	80	-	Weighted Log-Rank Test
Censored	10	10	-	
Mean	791.80	813.41	2.7	*Chi2 = 1.137*
Std. Error	16.32	23.49	-	*P = 0.286*
95% CI	759 ~ 823	767 ~ 859	-	
Age in days at % mortality:				Fisher's Exact Test
25%	695	734	5.6	*P = 0.732*
50%	812	834	2.7	*P = 0.562*
75%	912	939	2.9	*P = 0.215*
90%	1020	1031	1.0	*P = 0.711*
95% CI	752 ~ 846	793 ~ 887		

Figure 8 Kaplan-Meier survival curves for overall mortality ofthe AgRP deficient Sirt-1 KO mice. - effect of genotype, and sex on longevity.



	Control	Agrp-Tfam KO	Effect size (%)	Statistics
n =	67	43	-	Weighted Log-Rank Test
Censored	7	4	-	
Mean	856.54	749.69	14.3	*Chi2 = 7.311*
Std. Error	17.55	37.59	-	***P = 0.006***
95% CI	822 ~ 890	676 ~ 823	-	
Age in days at % mortality:				Fisher's Exact Test
25%	749	677	10.6	*P = 0.062*
50%	868	789	10.0	***P = 0.001***
75%	978	924	5.8	*P = 0.145*
90%	1024	1031	0.7	*P = 0.451*
95% CI	838 ~ 915	715 ~ 803		



	Control	Agrp-Tfam KO	Effect size (%)	Statistics
n =	66	37	-	Weighted Log-Rank Test
Censored	5	5	-	
Mean	783.32	833.77	6.4	*Chi2 = 2.039*
Std. Error	23.44	31.89	-	*P = 0.153*
95% CI	737 ~ 829	771 ~ 896	-	
Age in days at % mortality:				Fisher's Exact Test
25%	672	735	9.4	*P = 0.476*
50%	810	880	8.6	*P = 0.682*
75%	888	939	5.7	*P = 0.074*
90%	1042	1082	3.8	*P = 0.791*
95% CI	727 ~ 856	764 ~ 910		

Figure 9. Kaplan-Meier survival curves for overall mortality ofthe **AgRP Tfam KO** mice. - effect of genotype, and sex on longevity.

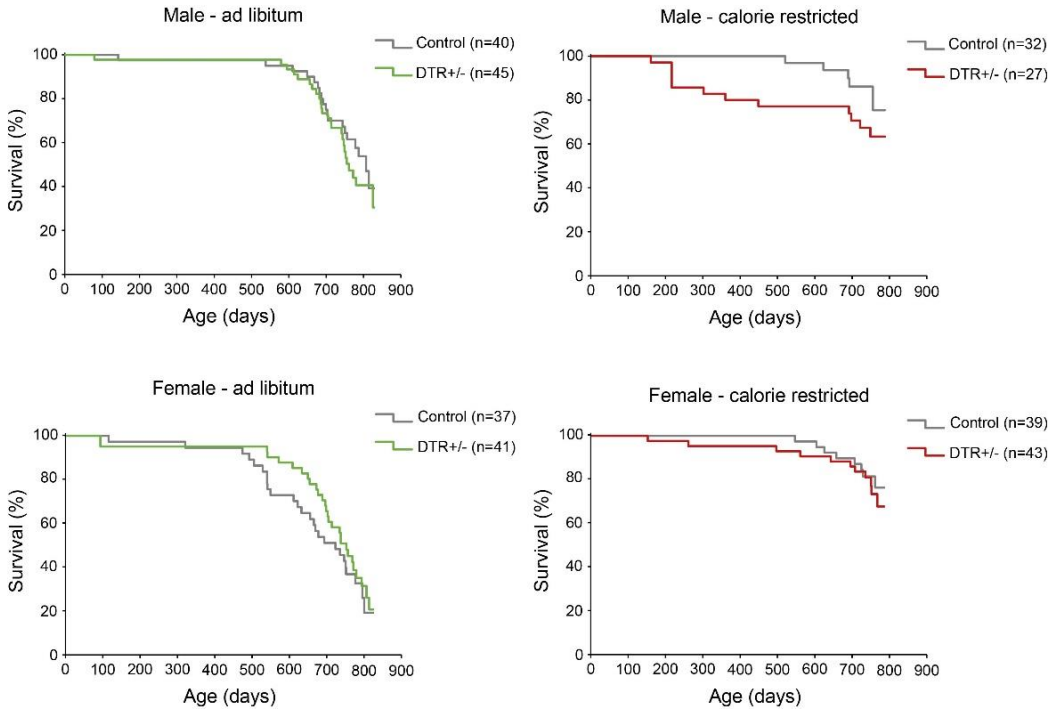


Figure 11. Survival curves for overall mortality of the AgRPDTR mice – effect of genotype and sex on longevity. At the cut-off date of te present study the neonatal ablation of AgRP neurons show little effect on the survival rates of ad libitum fed female and male mice (A, C). In contrast, when fed a calorie restricted diet male AgRPDTR+/- mice show a tendency to die at younger ages compared to WT animals fed the same diet (B) – an effect that we couldn't detect in females (D). *This experiment is not completed yet, it is still ongoing.*

In the meantime, we used cohorts of aging mice to perform various experiments with direct relevance to the understanding of the function of AgRP system. We have been executing open field and elevated plus maze test with control and AgRP-depleted mice at various ages (3, 6, 12 18- and 24-months old cohorts; data of the 3 months old mice can be seen in Figure 6) to analyze their locomotor, anxiety behavior. As all the behavioral data as well as the survival diagrams will be completed approximately for 2024 summer-autumn (based on the maximal age of laboratory mice), our final publication(s) can be expected afterwards. Our unexpected sex-, age- and AgRP-dependent results have already fueled additional experiments which we have started and will continue in the coming years. For example, RNA-single cell sequencing will be utilized to see transcriptome changes driven by CR and/or AgRP-depletion. Furthermore, CR-, AgRP- and sex-dependent effects on natural behavior and learning will be investigated using Intellicage system (TSE). The first, preliminary results of Intellicage experiments along with the life span data (Figure 8-11) have been presented at the *International Neuroscience Conference 2024* (Pécs, Hungary).

We believe that this KKP project yielded exciting results, also producing much more in the coming years, altogether more than we anticipated in the beginning, as it is now clear from the already published material. We are grateful for the support of the NKFIH and for the fact that this grant has made it possible to establish a state-of-the-art research core-facility at the University of Veterinary Medicine and has also enabled us to achieve significant and novel results in understanding the molecular mechanisms underlying nutrition and health and ageing.

PhD Students working on the project:

Mátyás Kapiller (expected PhD in 2025)

Lilla Dénes (2023)

TDK students working on the project and their TDK presentations:

Balkó Eszter Fruzsina - 2023 *The effect of impaired AgRP neuronal function on body weight, lifespan, and behavior in calorie restricted mice – 1st Prize*

Kovács Dóra - 2023 *Effect of hypothalamic hunger-regulating AgRP cells on learning in rodents - Pumukli Veterinary Clinic Prize*

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