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The importance of endogenous opioids in feeding behavior

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Declaration

I, hereby, solely declare that I prepared this thesis entitled “The importance of endogenous opioids in feeding behavior” entirely by myself except otherwise stated. All text passages that are literally or correspondingly taken from published or unpublished papers/writings are indicated as such. All materials or services provided by other persons are equally indicated.

Aishwarya Ghule

Abbreviations

AL	*ad libitum*
µg	microgram
AgRP	agouti-related peptide
Amy	amygdala
ANOVA	analysis of variance
ARC	arcuate nucleus
AUC	area under curve
avg	average
BG	blood glucose
BP	break point
bp	basepair
CCK	cholycytokinin
DA	dopamine
DAPI	4',6-diamidino-2-phenylindole
DIO	diet-induced obesity
dL	deciliter
DNA	deoxyribonucleic acid
DOR	delta opioid receptor
DYN	dynorphin
EDTA	ethylene diamine tetra-acetic acid
EtOH	ethanol
FR	fixed ratio
Fwd	forward
G	acceleration (unit of centrifugation)
g or gm	gram/s
GABA	γ-aminobutyric acid
GLP-1	glucagon like peptide-1
GPCR	G-protein coupled receptors
HET	Haus für Experimentelle Therapie
HFD	high-fat diet

i.p.	intraperitoneal
Kb	kilobase
kg	kilogram
KO	knockout
KOR	kappa opioid receptor
LH	lateral hypothalamus
M	molar
mg	milligram
min/s	minute/s
ml	milliliter
mM	millimolar
MOR	mu opioid receptor
MSN	medium spiny neurons
NAc	nucleus accumbens
ND	normal diet
ng	nanogram
nm	nanometer
NPY	Neuropeptide –Y
NTS	nucleus tractus solitaris
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDYN	prodynorphin
PENK	proekephalin
PFA	paraformaldehyde
pH	measure of acidity/basicity
POMC	proopiomelanocortin
PR	progressive ratio
PVN	paraventricular nucleus
rev	reverse
RNA	ribonucleic acid
RT	room temperature
SEM	standard error of mean

TE	tris EDTA
TR	time-restricted
v/w	volume/weight
VTA	ventral tegmental area
WHO	World Health Organization
WT	wild-type
wt	weight

Summary

Endogenous opioids are involved in a broadly distributed neural network regulating eating behavior. Opioid transmission has long been implicated in controlling hedonic and homeostatic feeding as well as regulating body weight and metabolism. Most evidence implicating endogenous opioids is based on studies using pharmacological, or genetic knockout of opioid receptors, which alters feeding behaviors. However, individual contribution of each opioid peptide in feeding behavior and metabolism is not entirely clear.

The present study was aimed to distinguish the role of the two major classes of endogenous opioids, namely enkephalin and dynorphin, in the motivational aspect of feeding behavior. Prodynorphin knockout and proenkephalin knockout mouse models were presented with highly palatable chocolate-flavored pellets and tested in an operant-self administration paradigm. The results suggest that the endogenous peptides dynorphin and enkephalin are involved in the modulation of hedonic control of feeding behavior. The second aim of the study was to elucidate the impact of dynorphin in modulation in the body weight and metabolism after prolonged voluntary palatable high-fat food consumption. Prodynorphin deficient and wild-type mice were maintained for 12 weeks on a high-fat diet (60% fat) or a normal chow with either a time-restricted access (8 hours) to food or an *ad libitum* access to food. The outcomes from the present study demonstrate the crucial role of endogenous opioid dynorphin in the regulation and maintenance of body weight. Blood glucose levels were modulated in high-fat diet-fed female mice, whereas, food consumption in animals was unaltered. Furthermore, prodynorphin deficient animals displayed significantly reduced levels of hypothalamic orexigenic peptides NPY and orexin-A under different feeding regimens.

The present study gives a first insight about the modulation of metabolic and endocrine changes associated with diet composition and feeding regimen by endogenous opioid dynorphin. The study also demonstrates the involvement of the opioid peptides dynorphin and enkephalin in hedonic control of feeding behavior.

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1 Introduction

1.1 Obesity

Obesity is currently one of the leading preventable diseases in the world. A number of population-based studies over the last few decades have demonstrated an increase in the percentage of overweight and obese humans. Consequently, there has been a remarkable rise in the obesity related health problems such as hypertension, diabetes, hypercholesterolemia, and liver disease (Wilborn et al., 2005). The World Health Organization (WHO) considers obesity as “one of the greatest public health challenges of the 21st century”. The body mass index (BMI), also known as the Quetelet index, is a WHO accepted unit for classifying the degree of obesity. Standards defining overweight and obesity on the basis of BMI were developed by the International Obesity Task Force of the WHO (Nakajima, 1998). Obesity is defined as having a BMI of 30 kg/m^2 or more.

The regulation of eating behaviors of palatable food is an important determinant of obesity. Three major factors that modulate body weight are metabolic factors, diet, and physical activity, each influenced by genetic traits (Weinsier, Hunter, Heini, Goran, & Sell, 1998). Although the driving causes are multifactorial, the fundamental cause of obesity and overweight is an energy imbalance between calories consumed and expended. Therefore, understanding the relation between energy balance and obesity is a challenge and a necessity to develop effective prevention programs and policies (Romieu et al., 2017). From an evolutionary perspective, eating is essential for survival and is underpinned by the fundamental physiological need to consume energy. However, we often consume excess of the basic nutrient and energy requirement needed to maintain physiological homeostasis, predominantly when there is an abundance of readily available food (Reichelt, Westbrook, & Morris, 2015). Food also differs from other addictive substances because it is legal and inexpensive. In combination with our innate preferences, these factors can be used to exploit the vulnerability in individuals and increase the probability of 'misuse' of food by overconsumption (Taylor, Curtis, & Davis, 2010). Repeated exposure to drugs of abuse can cause long-lasting effects in the neuronal circuitries and eventually induces loss of

control over its intake. However, the neural mechanisms underlying the motivation to eat and the rewarding effects of food altering the state of obesity are still largely undetermined, holding back the development of effective therapy. Therefore, it is important to understand these mechanisms by which these reward components induce adaptive changes in the neuronal circuitry responsible for addictive behaviors.

The brain reward systems plays a critical role in diet and feeding behavior of animals and humans (Lutter & Nestler, 2009; Saper, Chou, Elmquist, & Homeostasis, 2002). In general, bland tasting foods are not eaten in excess, whereas palatable foods are often consumed even after energy requirements have been met. Indeed, obtaining the pleasurable effects of palatable food is a powerful motivational force, that in certain individuals can override homeostatic signals (H. Zheng, N. Lenard, A. Shin, 2009; Shomaker et al., 2010). Most theories of feeding regulation propose that two parallel systems interact to influence food intake, the homeostatic and the hedonic system (Hommel et al., 2006).

1.2 Homeostatic and hedonic system

Hedonic and homeostatic feeding pathways are complementary and regulate intake of food. The homeostatic pathway controls energy balance by increasing the motivation to eat following depletion of energy stores. In contrast, hedonic or reward-based regulation can override homeostatic pathway during periods of energy abundance by increasing the desire to consume food that is highly palatable (Lutter & Nestler, 2009).

On one hand, the homeostatic control of feeding system comprises of hormonal regulators of hunger, satiety, and adiposity levels, such as leptin, ghrelin, and insulin, which act on hypothalamic and brainstem neuronal circuits. These peripheral signals stimulate or inhibit feeding in order to maintain appropriate levels of energy balance. Dysfunction in components of homeostatic system can result in a persistent state of positive energy balance and the development of obesity (G F Koob & Le Moal, 2008). Therefore, homeostatic feeding is controlled primarily by neuronal circuits in the hypothalamus and brainstem, which relay information from peripheral signals of various circulating hormones, nutrients and

vagal afferents. The peripheral signals from the gut are crucial for control of appetite as well as regulation of energy- and glucose-homeostasis (Page, Symonds, Peiris, Blackshaw, & Young, 2012). Under the conditions of food deprivation, metabolic and neuronal signals are activated, which increase the motivation to eat. Hunger signals, such as ghrelin in the stomach and orexigenic peptides such as neuropeptide-Y (NPY) and agouti-related peptide (AgRP) in the hypothalamus, are suppressed after intake of standard food, while satiety signals like cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), peptide PYY, insulin and leptin are raised (Erlanson-Albertsson, 2005; Lindqvist et al., 2005). Once the energy needs of the body are met, the negative feedback mechanisms consequently reduce the motivation for consumption of food.

The hedonic control of feeding, on the other hand, mainly concerns with palatability and incentive properties of food. Palatable food induces resistance to several satiety signals resulting in overeating. The reward and motivational aspects of food are controlled by neurons in cortico-limbic regions (Nora D Volkow, Wang, & Baler, 2011). There is a substantial amount of evidence pointing to the fact that overconsumption of the highly palatable food can lead to alterations in the brain rewarding circuit, due to repeated stimulation of dopamine pathway, similar to the phenotype seen with drug addicts (Smith & Robbins, 2013; N. D. Volkow, Wang, Tomasi, & Baler, 2013). Neuronal circuits activated and modified by highly palatable food and drugs of abuse overlap. The neuronal and cellular mechanisms regulating the feeding behaviors are fundamentally similar to those engaged by drugs of addiction. Rewarding effects of food are indirectly mediated by various peripheral and central signaling pathways, whereas, drugs of abuse elicit direct effects on the reward circuit (Dileone, Taylor, & Picciotto, 2013; Rada, Avena, Barson, Hoebel, & Leibowitz, 2012; N. D. Volkow et al., 2013). Due to these similarities between drug addiction and food intake, many findings from drug research can be translated to obesity.

1.3 Brain reward circuit

The mesolimbic dopaminergic pathway, also known as the brain 'reward' circuit, comprises of dopamine neurons in the ventral tegmental area (VTA) projecting to the nucleus accumbens (NAc), where it mediates reward. Ingestion of palatable food activates the reward circuit, by release of dopamine in NAc, and therefore leading to a positive emotional state (Russo & Nestler, 2013). The VTA–NAc reward circuit is crucial for the recognition of rewards in the environment and for initiating their consumption, as well as respond to aversive stimuli (Spanagel, R, Weiss, 1999). VTA dopamine neurons also innervate several other regions of the prefrontal cortex, central and basolateral amygdala as well as hippocampus (G F Koob & Le Moal, 2008; Russo & Nestler, 2013) (Fig. 1).

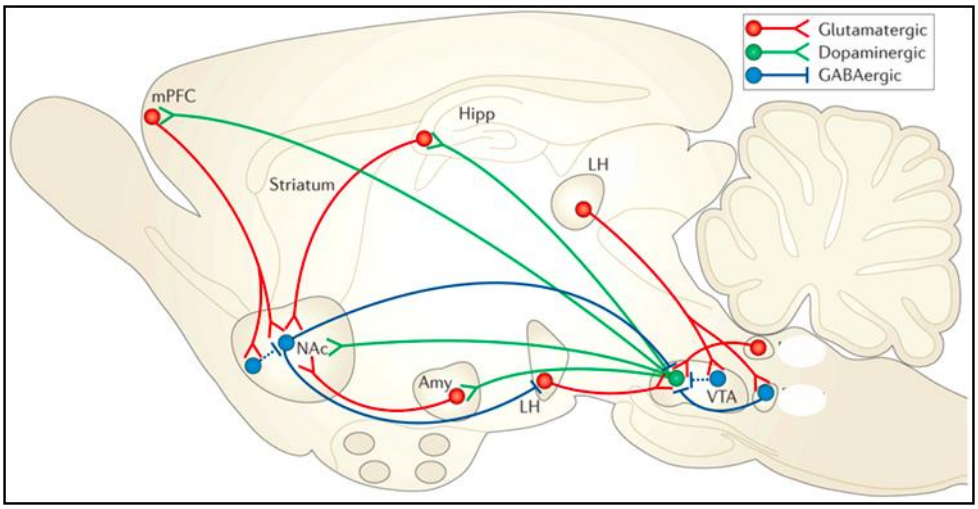

Fig. 1. VTA-NAc reward circuit (adapted from Russo & Nestler, 2013)
The mesolimbic dopaminergic pathway mediates brain reward signaling of dopamine projections (green) from the VTA to the NAc, Amy and mPFC. The dopamine release results in a positive emotional state, whereas GABAergic interneurons (blue) project their inhibitory effect. This feedback loop is functional via the participation of the endogenous opioid system. Other brain areas such as the lateral hypothalamus (LH) innervate the dopamine neurons on the VTA via glutamatergic projections (red). LH = lateral hypothalamus, NAc = nucleus accumbens, VTA = ventral tegmental area, Amy = amygdala, mPFC = medial prefrontal cortex.

The majority of neurons that populate the NAc are the GABAergic (γ-aminobutyric acid) medium spiny neurons (MSN) that receive extensive glutamatergic inputs from amygdala, hippocampus and prefrontal cortex (Bruijnzeel, 2010; Floresco, Blaha, Yang, & Phillips, 2001). The MSNs are divided into two classes - striatonigral cells containing substance P, dynorphin and D1 receptors, and striatopallidal cells containing enkephalin and D2 receptors (Gerfen et al., 1990; Robison & Nestler, 2011). The activation of both types of dopamine receptors in the NAc is strongly correlated with behavioral performance in motivation-dependent paradigms as well as their strong responses to rewards (Bromberg-Martin, Ethan S., Matsumoto & Hikosaka, 2011; Soares-Cunha et al., 2016). In the NAc, optogenetic stimulation of D1-type MSNs in mice enhanced cocaine-induced place conditioning and locomotor activation, whereas stimulation of D2-type MSNs had an opposite effect. Optogenetic stimulation of D1-type neurons in the dorsal striatum induced a persistent increase in operant lever pressing, whereas stimulation of D2-type neurons induced a transient decrease, indicating their involvement in motivational behavior with opposing functional roles (Kravitz, Tye, & Kreitzer, 2012; Lobo et al., 2011; Russo & Nestler, 2013).

Dopamine neurotransmission in the NAc is thought to mediate the motivational drive by the attribution of incentive salience (Box 1) to reward-related stimuli, thereby facilitating behaviors directed towards specific goals such as food seeking and eating.

Box 1. Incentive salience

Incentive salience is a cognitive process, which confers a 'desire' or 'wanting' attribute including a motivational component to a rewarding stimulus. The "wanting" of incentive salience differs from "liking". Liking is the pleasure that is immediately gained from the consumption of a rewarding stimulus, whereas, "wanting" of incentive salience serves a motivational quality of a rewarding stimulus that makes it a desirable and attractive goal, transforming it from a mere sensory experience into something that commands attention, induces approach, and causes it to be sought out (Berridge, 1996).

Food and food-related cues can activate different brain circuits involved in reward including NAc, hippocampus, amygdalda, prefrontal cortex and midbrain. It is believed that mesolimbic DA system promotes the learning of association between

natural rewards and the environment in which they are found (Dalley et al., 2005; Palmiter, 2007). Hence, these food-related cues promote the rapid firings of DA neurons, and facilitate behaviors directed towards acquisition of the reward (Baik, 2013). Indeed, firing of these VTA dopamine neurons has been suggested to signal the difference between expected rewards and the actual outcome, which is also known as reward-prediction error (Boender et al., 2014; Cohen & Uchida, 2012; Steinberg et al., 2014). This signaling, involving the DA release in the NAc, has also been suggested to assign increasing reward value to food cues (Roitman, 2004). Rewarding stimuli associated with 'wanting' or 'liking' (i.e. pleasure or desire) function as positive reinforcers, which can be evaluated by paradigms such as operant conditioning (Sandeep Sharma, Hryhorczuk, & Fulton, 2012) (Box 2). Operant conditioning paradigm provides one of the most effective means to evaluate changes in the motivational properties of food.

Box 2. Behavioral paradigm and terminologies

Operant conditioning is the learning process of association between behavior and its consequences, thereby strengthening or weakening the behavioral responses. It involves voluntary responses, in which the likelihood of a specific behavior increases or decreases in response to reinforcement or punishment that occurs when the behavior is exhibited, so that the subject comes to associate the behavior with the pleasure from the reinforcement or the displeasure from the punishment.

Positive reinforcement is the process by which presentation of a stimulus, usually pleasant (e.g. palatable food or drug itself) increases the probability of a behavioral responding (G F Koob & Volkow, 2010).

Besides dopamine, other neurotransmitter systems such as the endogenous opioid system and endocannabinoid system play a vital role in acute reinforcement and rewarding behavior. Endogenous opioids are postulated to be critical regulators of hedonic responses of food intake, which influence the activation of mesolimbic dopamine system (Hayward, Schaich-borg, Pintar, & Low, 2006).

1.4 Endogenous opioid system

The endogenous opioid family consists of three main classes of peptides - β-endorphins, enkephalins and dynorphins and the three opioid receptors, mu (MOR), delta (DOR), and kappa (KOR), respectively. Opioid receptor family belongs to G-protein coupled receptors (GPCR), coupled to Gi/Go protein. Endogenous opioid peptides and receptors are broadly expressed throughout peripheral and central nervous system and have been a subject of intense investigation over the last several decades. Expression of opioid receptors is found primarily in brain regions such as cortex, limbic system, and the brain stem and immunoreactivity studies have shown a large overlap of the peptide projection fibers with the localization of the receptors (Merrer, 2009; Schwarzer, 2009).

The three endogenous opioid ligands - enkephalin, dynorphin, and ß-endorphin are produced by proteolytic cleavage of large protein precursors known as preproenkephalin (PENK), preprodynorphin (PDYN), and proopiomelanocortin (POMC), respectively. PENK precursor protein is the most abundant and widely distributed, found in the thalamus, while PDYN is present in most brain structures with the highest concentration in the NAc. POMC distribution is restricted in most of the cortical structures although detected in arcuate nucleus, nucleus tractus solitarius (NTS, brain stem) and the anterior lobe of pituitary. All the opioid peptides share a common NH_2-terminal Tyr-Gly-Gly-Phe signature sequence, which interacts with opioid receptors (Mansour, Fox, Akil, & Watson, 1995; Merrer, 2009).

Endogenous opioid receptors differ in their binding affinity for each receptor. Enkephalins have a high affinity for DOR and to a lesser extent for MOR, whereas β-endorphins activate both, MOR and DOR with similar affinities. Endogenous dynorphin exhibits the highest affinity exclusively for KOR (Clarke, Zimmer, Zimmer, Hill, & Kitchen, 2003)(Fig. 2).

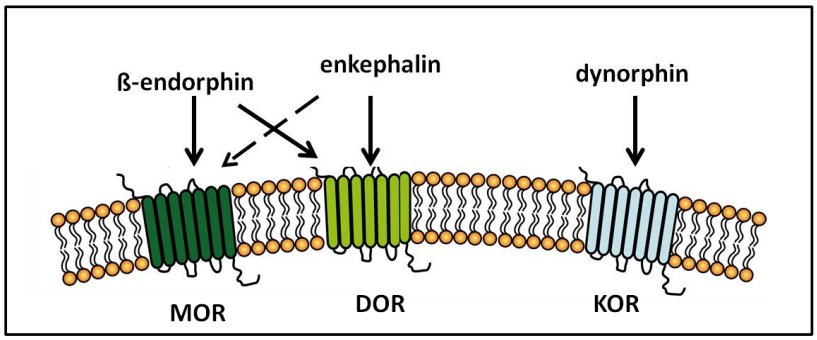

Fig. 2. Endogenous opioids peptides, receptors their binding affinity
β-endorphin activates MOR and DOR, respectively, with a high affinity. Enkephalin has a high affinity for DOR, whereas, dynorphin predominantly bind to KOR. MOR = mu opioid receptor, DOR = delta opioid receptor, KOR = kappa opioid receptor.

The endogenous opioid system modulates the rewarding circuitry through the receptors in VTA by influencing the activity of dopamine neurons. At the NAc level, it affects the dopamine release and activity of postsynaptic neurons. Opioid receptors in the dorsal striatum also contribute to hedonic mechanisms regulating feeding behaviors. Several studies have proved that pharmacological agonism or antagonism of opioid receptors, increased or decreased the food intake, respectively (Bodnar, 2004; M. Zhang, Gosnell, & Kelley, 1998). Opioid receptor antagonists such as naloxone and naltrexone attenuate both addictive drug taking and appetite for palatable food, whereas opioid agonists such as morphine or synthetic enkephalin analogues increase food consumption (Nogueiras, Maria, Novelle, López, & Diéguez, 2012). Synthetic compounds and natural rewards that activate the MOR or DOR opioid receptors generally have a positive motivational effects, whereas KOR opioid receptor agonists induce aversive effects (Shippenberg, Bals-Kubik, & Herz, 1987, 1993). These opposing pharmacological responses of opioid receptors reflect an opposite modulation of the reward circuitry (Fig. 3).

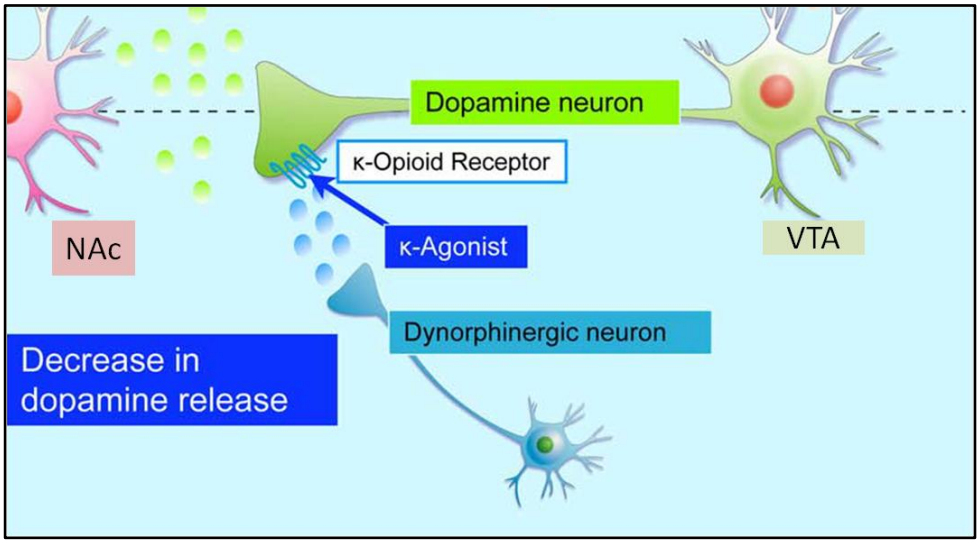

Fig. 3. Modulation of dopamine by endogenous opioid signaling (adapted and modified from Niikura et al., 2010). Endogenous opioid ß-endorphin or MOR agonist activates MOR and inhibit GABAergic neurons that normally inhibit dopaminergic neurons in the VTA. This disinhibition leads to an increase in dopamine release in the NAc. Endogenous opioid dynorphin activates the KOR in the NAc and acts as a countermodulatory system by decreasing the release of dopamine and the reward effects of MOR (bottom half: blue).

1.5 Dynorphin

Dynorphins (DYN) are a class of opioid peptides that arise from the precursor protein prodynorphin (PDYN), which on cleavage produces active components - PDYN A and PDYN B and α/β-neo endorphins (Day, Lazure, Basak, & Boudreault, 1998). Even though the dynorphin peptide is found widely distributed in the central nervous system, the highest expression is found in hypothalamus, midbrain, medulla pons and spinal cord (Goldstein & Ghazarossian, 1980). Depending upon its site of production, has increasingly been thought to play a crucial regulatory role in numerous functional pathways of the brain related to learning and memory, emotional control, stress response and pain and in modulation of reward-related processes (Goldstein & Ghazarossian, 1980; Schwarzer, 2009). Although dynorphin selectively activates the endogenous KOR, it is also known to bind to MOR and DOR with a very low affinity (S. Zhang et al., 1998). Conversely, the other opioid peptides, β-endorphin and enkephalin, poorly

interact with the KOR. Therefore, the DYN/KOR signaling pathway forms a distinct process within the opioid system (Goldstein A; Tachibana S; Lowney LI; Hunkapiller M; Hood L, 1979). The distribution of dynorphin closely matches that of KOR showing high concentrations in the NAc and hypothalamus (Fallon & Leslie, 1986; Khachaturian, 1985).

Pharmacological activation of KOR in humans evokes reports of dysphoria and anxiety similar to those detected in rodent models (Millan, 1990; Pfeiffer, Brantl, & Herz, 1985). KOR activation in the dopamine neurons in VTA have been implicated in the aversive properties of the stress response (Bruchas, Land, & Chavkin, 2010; Van't Veer & Carlezon, 2013). Pioneer studies from Di Chiara and Imperato demonstrated that KOR agonists inhibit dopamine release in the striatum, and suggested that dysphoria was a consequence of reduced dopamine tone. DYN/KOR signaling is known to negatively modulate the reward by decreasing the dopamine release in the striatum and increasing dopamine uptake (Di Chiara & Imperato, 1988; Thompson et al., 2000; Yokoo, Yamada, Yoshida, Tanaka, & Nishi, 1992). As a result, DYN/KOR signaling acts as a countermodulatory mechanism (negative feedback loop) of the mesolimbic dopaminergic reward circuit (Fig. 4).

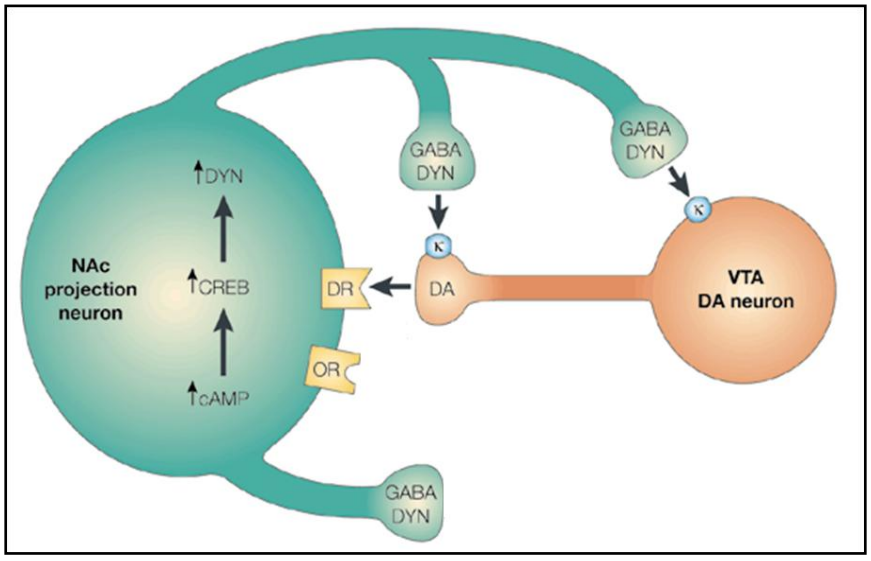

Fig. 4. DYN/KOR signaling (figure modified from Nestler, 2001)
The DYN/KOR is a negative feedback mechanism modulating the dopamine release. DYN on synthesis via the cAMP-CREB cycle, is released from the terminals of the NAc neurons,

act on the KOR located on the nerve terminals and cell bodies of dopamine (DA) neurons of VTA, to inhibit their functioning (Nestler, 2001). DYN = dynorphin, DR = dopamine receptor, OR = opioid receptor, VTA = ventral tegmental area, NAc = nucleus accumbens, cAMP = cyclic adenosine monophosphate, CREB = cAMP response element binding protein, GABA = ϒ- amino butyric acid.

Over the past few years, refinement of pharmacological tools and availability of genetic approaches in animal studies have clarified the specific role of each opioid ligand and receptor in several aspects of opioid-related behaviors (Pradhan, Befort, Nozaki, Gaveriaux-Ruff, & Kieffer, 2011). The generation of prodynorphin deficient mice has facilitated us to investigate open questions on network effects of endogenous dynorphin. So far, three research groups have generated prodynorphin deficient mouse models (Loacker, Sayyah, Wittmann, Herzog, & Schwarzer, 2007; Sharifi, Diehl, Yaswen, Brennan, & Hochgeschwender, 2001; Zimmer et al., 2001). PDYN deficient mice exhibit phenotype of moderate enhancement of anxiety related behaviors and most striking deficits in the extinction of conditioned fear responses. The effects of drugs of abuse in PDYN KO mice increased locomotor sensitization to cocaine, whereas, reduced aversive responses to cannabinoids and nicotine self-administration, while altered stress responses in long-term ethanol exposure (Galeote, Berrendero, Bura, Zimmer, & Maldonado, 2009; Mendizábal, Zimmer, & Maldonado, 2006; Rácz, Markert, Mauer, Stoffel-wagner, & Zimmer, 2012; Zimmer et al., 2001). In case of natural rewards, operant reinstatement of food-seeking behavior in mice showed a reduction in the prodynorphin mRNA levels (Martín-garcía, Burokas, Kostrzewa, & Gieryk, 2011). In humans, the PDYN polymorphism modulates neural processes associated with the anticipation of rewards (Votinov et al., 2014). However, due to the complexity of the endogenous opioid system, the exact role of dynorphin in palatable reward-driven behavior still needs to be thoroughly investigated.

1.6 Enkephalin

The opioid precursor proenkephalin (PENK) is enzymatically cleaved into leu-enkephalin, met-enkepahalin, and two-carboxyl (C)-terminally extended met-enkephalins (Weisinger, 1995). PENK is widely expressed in the brain regions involved in reward and reinforcement. PENK positive neurons have been detected in the bed nucleus of the stria terminalis, the central nucleus of the amygdala, the caudate putamen, and various hypothalamic nuclei including the paraventricular hypothalamic nucleus (Sukhov, Walker, Rance, Price, & Young III, 1995).

Studies from PENK deficient mice have demonstrated the phenotype of increased anxiety, aggressive behaviors and abnormal stress responses (Bilkei-Gorzo, Racz, Michel, Zimmer, & Klingmüller, 2004; König, Zimmer, Steiner, Holmes, & Crawley, Jacqueline, Brownstein, Michael J., Zimmer, 1996). Enkephalin has also been known to participate in the reinforcing properties of drugs of abuse. PENK deficient mice exhibit unaltered ethanol preference, no stress-induced elevation of alcohol consumption or no nicotine-conditioned place preference, as well as decreased nicotine-induced release of dopamine in NAc (Berrendero, 2005; Bilkei-Gorzo et al., 2008; Bilkei-Gorzo, Racz, Michel, Zimmer, Klingmüller, et al., 2004; König et al., 1996; Racz et al., 2009; Valverde, Maldonado, Valjent, Zimmer, & Zimmer, 2000).

Exposure to alcohol is also known to influence the endogenous opioid peptide expression and alters opioid peptide release in distinct brain regions associated with reward and reinforcement (Gianoulakis, 2004; George F. Koob, Sanna, & Bloom, 1998; R Spanagel, Herz, & Shippenberg, 1992). In case of natural rewards, PENK deficient mice with access to sucrose solution exhibited fewer bouts than WT controls, indicating diminished motivation for food. On the contrary, consumption of palatable chocolate in rats led to an upsurge in levels of enkephalin in the dorsal striatum, a region implicated in the reward-related functions (Difeliceantonio, Mabrouk, Kennedy, & Berridge, 2012; Mendez, Ostlund, Maidment, & Murphy, 2015). This effect was observed only in enkephalin levels, as dynorphin levels were unaltered. Taken together, these studies suggest a vital role of enkephalin peptide in the modulation of food reward and motivational behavior. Recently, a floxed PENK locus was generated, in our laboratory to investigate the

role of enkephalin signaling in specific neuronal cells (Dr. Britta Schürmann, PhD thesis). The floxed locus contains a lacZ gene, downstream of the enkephalin coding exon. The introduction of the loxP into the genome allows the subsequent excision of embedded region via cre-recombinase mediated recombination, which enables the tissue- or cell-specific deletion of the corresponding gene. Schematic representation of the floxed PENK locus is depicted in Fig. 5.

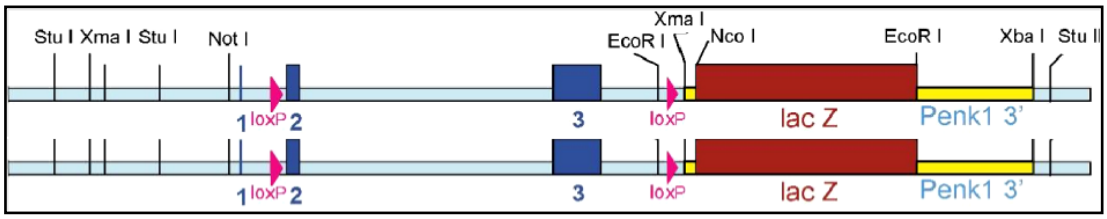

Fig. 5. Schematic representation of floxed proenkephalin gene locus
Exons 1, 2, and 3 are indicated in blue boxes, loxP sites are indicated with pink triangles and lacZ gene indicated by a brown box. Cre-mediated recombination results in the excision of exons 2 and 3 and activation of lacZ reporter gene.

1.7 Preference for food and diet-induced obesity

Endogenous opioids are thought to modulate the reward circuitry based on food preferences in humans as well as animals. In humans, opioid receptor antagonism has shown to reduce high-fat diet consumption along with decrease in preference for sucrose (Nogueiras et al., 2012). In animals, the role of the opioid system in the modulation of food preference has mostly been studied using pharmacological methods and opioid receptor knockout mice. Generally, opioid receptor agonists are known to increase food consumption, whereas opioid antagonists decrease food consumption. This idea is supported by several studies conducted by administration of opioid receptor agonists or antagonists targeted in particular brain sites. The administration of MOR agonists in NAc caused an enhanced "liking" reactions to sweet pleasure and that stimulate food intake (Pecina, 2005). Administration of opioid antagonist naltrexone into a central amygdala, a reward-related site, resulted in inhibition of intake of 'preferred diet' such as high-fat or high-sugar (and in another experiment chocolate or banana flavor), when presented alone. However, when both diets were presented together,

only preferred food intake was stimulated in animal. Therefore, opioids seem to modulate flavor-based preferences in food consumption. (Naleid, Grace, Chimukangara, Billington, & Levine, 2007; Woolley, Lee, & Fields, 2006). Furthermore, naltrexone inhibits food consumption in mice regardless of its hedonic value after injection into the hypothalamic paraventricular nucleus (Glass, Billington, & Levine, 2000; Olszewski, Alsiö, Schiöth, & Levine, 2011). Collectively, data from previous studies have also suggested that opioid regulation of palatable food intake is independent from its nutritional value.

It has been shown that the development of obesity and associated metabolic changes depends on the nutrient content of the high-fat diet, as well as the context and timing of the food presentation (Conlon & Bird, 2015). Furthermore, the gender also plays an important on feeding behaviors and physiological changes associated with the development of obesity (Meyer, Clegg, Prossnitz, & Barton, 2012). Previously opioid peptide knockouts have also demonstrated sex-specific phenotypes in case of ethanol preference (Racz et al., 2009). With regards to consumption of a high-fat diet, endogenous opioids and receptors such as MOR, has been implicated as a critical factor. Direct stimulation of the MOR by an agonist in NAc, specifically stimulated the consumption of high-fat food in rats (M. Zhang et al., 1998). Chronic consumption of palatable high-fat diet in mice has also shown to increase anxiety- and depressive-like behavior, heighten the HPA response to stress and is responsible for several biochemical modifications in brain reward circuitry (S Sharma, Fernandes, & Fulton, 2013; Singh, 2014). There are a number of animal models of obesity generated so far by genetic engineering, pharmacological treatments or different diets to induce obesity (Lutz & Woods, 2012). The mouse model of diet-induced obesity (DIO) has become one of the most important tools for understanding the interplay of high-fat Western diets and the development of obesity. The DIO model closely mimics the increasingly availability of the high-fat foods in modern society, which are main contributors to the obesity trend in human (Wang & Liao 2012).

It has been postulated that opioid signaling also contribute to binge-eating phenomenon in animals and humans (Blasio, Steardo, Sabino, & Cottone, 2013; Chamberlain et al., 2012). Research from Corwin, Avena, & Boggiano illustrated three models of binge-eating: i) a model of sugar-bingeing in which animals with

repeated intermittent access to a sugar solution developed behaviors and brain changes that were similar to the effects of some drugs of abuse, ii) model with history of dieting and stress that could perpetuate further binge-eating of palatable and non-palatable food, iii) limited access model in which non-food deprived rats with sporadic limited access to a HFD develop binge-type behaviors (Corwin, Avena, & Boggiano, 2011). Crucial differences between animal and human binge eating include the fact that subjective feelings of distress or loss of control, which have been found in some but not all studies linked prospectively to binge episodes in humans, are not easily assessed in animals (Mathes, Brownley, Mo, & Bulik, 2009).

Recent research using the limited access protocol has revealed that mice are partially protected against the metabolic effects of the HFD under a time-restricted feeding condition, showing no signs of hyperinsulinemia, hepatic steatosis, or inflammation (Hatori et al., 2012). Therefore, the limited access or restricted access to feeding regimen enables us to differentiate between the direct effects of HFD on brain functions and its indirect effects via metabolic changes. Now, the question arises whether the consumption of HFD is sufficient to change the feeding behaviors by modulating the reward circuitry? or whether metabolic changes induced by the HFD are crucial in the neuronal modulation of reward or not? To clarify these uncertainties, investigations need to be performed taking into account several factors such as diet, feeding regimen as well as sex at the same time.

1.8 Hypothalamus: homeostatic regulation of feeding

The main brain structure that controls the regulatory signals for food consumption in homeostatic control of feeding is the hypothalamus (Martín-garcía et al., 2011). Hypothalamus is involved in the regulation of feeding, satiety, and energy homeostasis. It plays a key role in the control of food intake by sensing metabolic signals from peripheral organs and modulating these feeding behaviors. On interaction with other brain areas such as the brainstem and reward-related limbic pathways, the hypothalamus mediates regulation of short-term and long-term dietary intake via synthesis of various orexigenic and anorexogenic

neuropeptides. The neuropeptides, which are present in the hypothalamus and are involved in regulating food intake, also play a key role in regulating glucose metabolism and energy expenditure (Heijboer et al., 2006).

Within the hypothalamus, there are complex interactions between many nuclei of which the arcuate nucleus (ARC) is considered one of the most important hypothalamic centers that regulate food intake. ARC acts as a feeding control centre and integrates hormonal signal for energy homeostasis. ARC encloses the third ventricle and lies directly above the median eminence, which allows the entry specifically for peripheral peptides including leptin and insulin (Arora & Anubhuti, 2006; Schwartz et al., 1992). ARC contains two distinct populations of neurons: orexigenic neuropeptide NPY, AgRP neurons and anorexigenic POMC neurons. The orexigenic neuropeptides such as NPY and orexins/hypocretins, which project to the paraventricular nucleus (PVN) and the lateral hypothalamus (LH), to act on local neurons to affect food intake and energy homeostasis (Elmquist, Elias, & Saper, 1999).

NPY exhibits a variety of biological and physiological actions including modulation of feeding, thermoregulation, locomotor activity, cardiovascular function, cognition and memory, and stress-related behaviors (Bi, 2007; Colmers and Wahlestedt, 1993; Gray and Morley, 1986,Sheng Bi 2012) It is known that dorsomedial hypothalamic NPY expression is enhanced in rodent models of obesity with chronic food restriction (Bi, Robinson, & Moran, 2003). Opioid peptide dynorphin is also important in maintaining homeostasis through appetite control (Przewłocki, Gramsch, Pasi, & Herz, 1983). Dynorphin expressing neurons in ARC also contained NPY mRNA, with a co-localization in the lateral and dorsal part of the Arc, suggesting potential overlapping functions of these two neurotransmitters in feeding type behavior (Lin et al., 2006). Previous studies in rodents have shown that food deprivation results in a significant increase of NPY, which is co-localized with PDYN mRNA, in the hypothalamus (Przewłocki, Gramsch, Pasi, & Herz, 1983), whereas in PDYN KO mice, the expression of NPY was found to be downregulated (Lin et al., 2006) (Fig. 6).

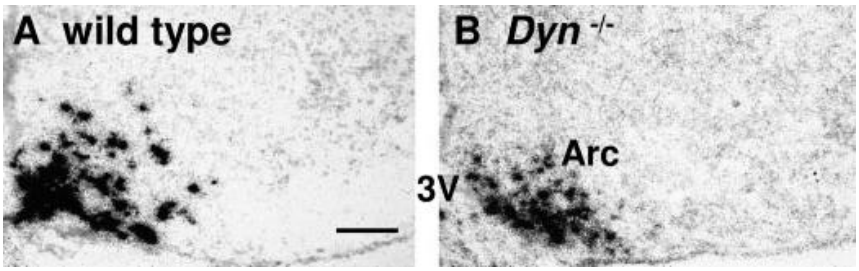

Fig. 6. Effect of dynorphin knockout on hypothalamic NPY expression. (adapted from Lin et al 2006) NPY mRNA levels in the hypothalamic arcuate nucleus of wild-type (A) *vs.* dynorphin knockout (B) mice. *Scale bar*, 40 m, *Dyn-/-* = dynorphin knockout, 3V = third cerebral ventricle; Arc = arcuate nucleus of the hypothalamus.

Another important brain structure is the lateral hypothalamus (LH), which is innervated by several orexigenic peptides such as orexin-A, orexin-B, melanin concentration hormone (MCH) and galanin. These neuropeptide cell populations are important in regulation of feeding and reward as well as metabolism through their neuronal circuitry. Orexins/ hypocretins are neuropeptides that are exclusively produced in LH, projecting to the ARC (Date et al., 1999; Horvath, Diano, & Pol, 1999). Orexins regulate appetite along with arousal and wakefulness and orexigenic neurons are regulated by peripheral metabolic cues, including ghrelin, leptin, and glucose concentration linking energy homeostasis to arousal state. Intracerebroventricular injections of orexins in rodents induce feeding behavior during the light period (Edwards et al., 1999; Haynes et al., 2000; Tsujino & Sakurai, 2013). A blockade orexin signaling with the orexin-1 receptor antagonist reduced acute HFD consumption. Acute HFD consumption thus requires orexin signaling (Valdivia, Patrone, Reynaldo, & Perello, 2014).

Orexin signaling role in reward seeking behavior is well established, where it promotes drug-induced plasticity of glutamatergic synapses onto DA neurons of VTA, involved in motivated behaviors (Harris, Wimmer, & Aston-Jones, 2005). Double-labeled *in situ* hybridization studies indicated high levels of co-localization of orexin-A neuropeptide and dynorphin in rodents. Hence, orexin facilitates reward by attenuating the anti-reward effects of co-expressed dynorphin in the VTA of mice during cocaine self-administration (Muschamp, Hollander, Thompson, Voren, & Hassinger, 2014). However, their interaction in natural reward-seeking behavior such as highly palatable food still remains unknown.

1.9 Aim of the study

The present study focuses mainly on two members of the endogenous opioid peptide family, dynorphin and enkephalin, which preferentially bind to the opioid receptors KOR and DOR, respectively. Enkephalin peptide modulates the brain rewarding circuit by activating the DOR and increasing the release of dopamine in NAc, therefore mediating a positive rewarding effect. Conversely, dynorphin on activating the KOR reduces the dopamine release and negatively modulates the rewarding effect.

One aim of the study was to elucidate the involvement of dynorphin and enkephalin peptides in the palatable diet-induced rewarding behavior. It is hypothesized that knockout of proenkephalin (PENK KO) will result in decreased rewarding effect and consequently lowered motivation in mice to obtain highly palatable food reward. In contrast, knockout of prodynorphin (PDYN KO) will result in the increased rewarding effect and therefore increased motivation responses to obtain the highly palatable food reward. To test the hypothesis, constitutive PDYN KO and PENK KO mice and the controls (wild-type and floxed mice, respectively) were evaluated for their motivational behavior in an operant conditioning paradigm.

Another aim of the study was to identify the role of dynorphin in the modulation of metabolic changes associated with diet composition and feeding regimen. Prolonged voluntary consumption of a high-fat palatable diet, leading to obesity, produces alterations in the opioid signaling and hedonic aspects of feeding behavior. Therefore, it is hypothesized that mice lacking dynorphin will show an increased feeding behavior of palatable high-fat diet and alterations in the body weight and metabolism. To test this hypothesis, PDYN KO and wild type mice were exposed to either a normal diet or a high-fat diet for a period of 12-weeks, and were enabled either a time-restricted or an *ad libitum* access to food.

Hypothalamic orexigenic peptides together with opioid signaling mechanisms orchestrate the homeostatic feeding behavior. Therefore, changes in expression of hypothalamic orexigenic peptides after prolonged voluntary palatable high-fat diet consumption were analyzed in brain samples by immunohistochemistry.

2 Material and Methods

2.1 Equipment

Table 1. List of technical equipment

Technical instrument	Identifier, Company
analytical balance	BP 121 S, Sartorius
animal tracking software	EthoVision® XT, Noldus
Bioanalyzer	Agilent 2100 bioanalyzer, Agilent Technologies
CCD camera	AxioCam MR, Zeiss
centrifuges	Biofuge fresco, Heraeus Instruments
ChemiDoc gel imaging	MP imaging systems, Bio-Rad Laboratories
Cryostat	CM3050S, Leica GmbH
glucose meter	Accu-Check Aviva, Roche Diagnostics GmbH
laser scanning microscope	SP8, DMI 6000 CS, Leica
microscopes	Eclipse TS 1000, Nikon Axiovert 200 M fluorescent microscope, Zeiss Axioscope 40, Zeiss
open-field test device	Open-field ActiMot &VideoMot, TSE-Systems
operant conditioning System	TSE Systems
PCR iCycler	Bio-Rad Laboratories
pH meter	inoLab
Skinner Box/Operant box	TSE Systems
Superfrost Plus® slides	Menzel-Gläser

2.2 Software

Table 2. List of software

Software	Company
AxioVision LE	Carl Zeiss
Ethovision	Noldus
Graphpad Prism 6	GraphPad Software, Inc
ImageJ	Wayne Rasband
Leica Application Suite	Leica
Mouse-E-Motion	Infra-E-Motion
SartoConnect	Sartorius
Statistika	StatSoft, Inc.
PhenoMaster Program	TSE Systems
VideoMot 2	TSE Systems
Ethovision XT	Nodulus

2.3 Chemicals and reagents

2.3.1 Chemicals

Table 3. List of chemicals

Chemicals	Company
Albumin bovine Fraction V, pH 7.0 standard grade, lyophil. (BSA)	Serva
DAPI Fluoromount-G®	SouthernBiotech
Ethidium bromide solution (10 mg/ml)	Sigma-Aldrich
Fluoromount-G®	SouthernBiotech
paraformaldehyde	Sigma-Aldrich
Tween20	Sigma-Aldrich

2.3.2 Buffers and reagents

If not stated otherwise all buffers and solutions were prepared with dH_2O and all chemicals were purchased from Applichem, Life Technologies, Merck, Carl Roth or Sigma-Aldrich.

Table 4. List of reagents

Buffers and Solutions	Composition	Application
mouse tail lysis buffer	100 mM Tris/HCl pH 8.0 5 mM EDTA 200 mM NaCl 0.2% (w/v) SDS	mouse tail lysis
TE buffer	10 mM Tris 1 mM EDTA, pH 8.0	DNA isolation
4% PFA	4% (w/v) paraformaldehyde	fixation of brain tissue
2x SSC	0.3 M NaCl 30 mM Na-citrate dihydrate adjusted to pH 7.0	immunohistochemistry
TBS (Tris-buffered saline)	50 mM Tris-HCl 150 mM NaCl, p.H 7.5	immunohistochemistry
PBS (Phosphate buffered saline)	1.44 g KH_2PO_4 90 g NaCl 4.21 g Na_2HPO_4	immunohistochemistry
permeabilization solution	0.5% Triton X-100 in PBS	immunohistochemistry
blocking solution 1%BSA/PBS and 10% donkey serum	1% BSA/PBS and 10% donkey serum	immunohistochemistry
anesthetic	500 µl xylariem 1000 µl ketamin 8500 µl 0.9% (w/v) NaCl	pre – perfusion

2.4 Antibodies

Table 5. List of antibodies

Antibody	Host	Dilution/s	Identifier, Company
NPY	Rabbit	1:1000	ab30914, Abcam
Orexin-A	Rabbit	1:500	PC362, Millipore
Anti-rabbit Alexa Fluor® 488	donkey	1:1000 (NPY) 1:1500 (orexin-A)	A21206, Invitrogen

2.5 Food pellets

Table 6. List of food pellets

Food pellets	Identifier, Company
standard (normal) pellets	V1534, Ssniff Spezialdiäten GmbH
high-fat diet soft pellets	#F3282, Bio-serv, Plexx, Europe
normal diet precision pellets	#5TUM, TestDiet, USA
chocolate-flavored precision pellets	#F05301, Bioserv, Plexx, Europe

2.6 Animals

In the present study, constitutive prodynorphin knockout and proenkephalin knockout (König et al., 1996; Zimmer et al., 2001), along with age-matched control littermates on a C57BL/6J genetic background were used. Wild-type and PENK floxed mice were used as controls for PDYN KO and PENK KO mice, respectively. Schematic representation of the PENK floxed mice is represented in Fig. 5. All the animals used in this study were of age group 10-12 weeks old. The mice were kept in a reverse light/dark cycle, lights off between (6.00 a.m. and 6.00 p.m.). Food and water was provided in all cages, under SFP conditions, unless otherwise specified. The housing conditions were maintained at 21 $\pm$ 1°C and 55 $\pm$ 10% relative humidity. All experimental procedures were complied with regulations for animal experimentation in Germany and approved by Landesamt für Natur, Umwelt und Verbraucherschutz in Nordrhein-Westfalen, Germany (Zimmer et al., 2001). All procedures were in compliance with national regulations and institutional guidelines.

2.7 Behavior experiments

2.7.1 Operant conditioning paradigm

The motivational responding in PDYN KO, PENK KO and the control animals was assessed in an operant conditioning paradigm. The animals were single-housed for a week before the start of the experiment. Body weight and daily food intake was monitored during the habituation week (7 days before the start of the experiment) as well as during the operant conditioning. For assessment of motivational responding, two types of pellets were presented to the animals- either standard normal precision pellets or highly palatable chocolate-flavored precision pellet. The normal precision pellet formulation consisted of 0.79 kcal/g protein, 0.345 kcal/g fat, 2.162 kcal/g carbohydrate, with a total caloric value of 3.3 kcal/g. The chocolate-flavored precision pellet, rendered more palatable to the mice, had a similar composition to the normal pellet: 0.74 kcal/g protein, 0.50 kcal/g fat, 2.36 kcal/g carbohydrate with an addition of 2% unsweetened cacao constituting a total caloric value of 3.6 kcal/g (table 2.5). Two days prior to the start of the operant conditioning, mice were habituated to the either of the two

pellets, to prevent the potential influence of food neophobia on operant performance. Food pellets were presented only during the experimental sessions. Otherwise, animals were maintained on standard normal pellets for their daily food intake.

2.7.2 Apparatus

The instrumental responding for food was conducted in the operant chambers/boxes. Each operant chamber or 'Skinner box' is a sound and light-attenuated box equipped with a food dispenser, two holes for the nose-poke task on each side of the food dispenser hole, a house light and a stimulus light. One of the nose-poke hole was randomly assigned as active hole/sensor whereas the other as inactive (Fig. 7).

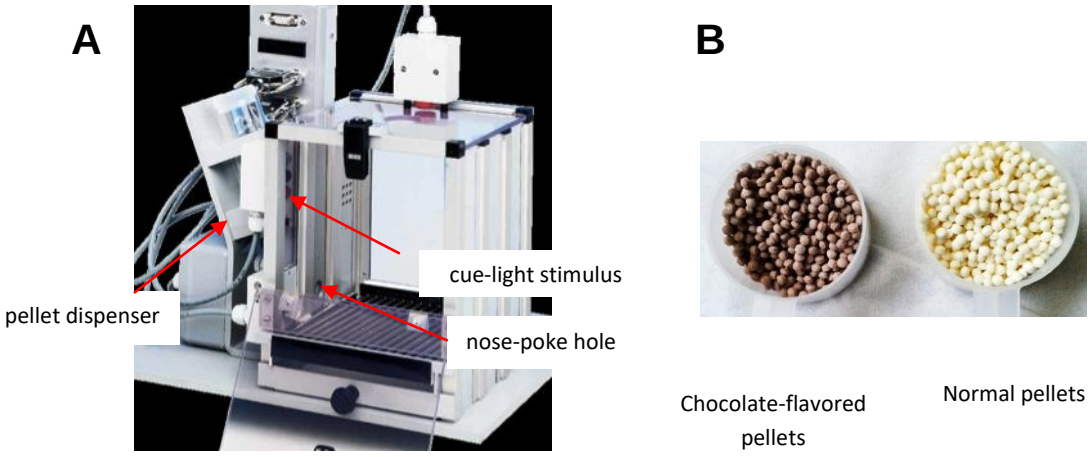

Fig.7. A. Operant conditioning system / Skinner box (modified from TSE Systems) and **B.** two types of precision pellets used for the conditioning paradigm, chocolate-flavored precision pellets (left) and normal precision pellets (right).

Mice were trained for 7 days a week in 30 minutes daily session to acquire instrumental responding for food. There were two groups of animals - one group responded only for normal precision pellets while the other group responded only for chocolate-flavored precision pellets in the operant box. Each training session started with a 2-seconds presentation of the yellow cue-light stimulus, located above the assigned active hole for nose-poke. Active poking in the correct hole resulted in delivery of a food pellet, followed by a confirmatory 1-second green

light stimulus after obtaining the food reward (Guegan et al., 2013). Nose-poke into the inactive hole had no consequences. A 10 seconds time-out interval was established after obtainment of each reinforcer, where the stimulus cue-light was off and no pellet was delivered upon sensor activation.

2.7.3 Operant training schedules

Mice were trained to respond for food under the fixed ratio (FR) schedule of reinforcement, which consisted of a fixed number of pellet deliveries obtained for each active nose-pokes. Fixed- ratio 1 (FR1) consisted of one reward per correct active nose-poke responding. To acquire the skills for correct nose-poke task, mice were deprived to 80% of their average daily food intake during acquisition/learning phase. Hence, the first training schedule is referred to as 'FR1 with food deprivation'. The criteria for acquisition of the operant conditioning schedule was achieved when mice maintained a stable responding with less than 20% deviation from the mean of the total number of food pellets earned during three consecutive sessions (80% of stability), with at least 75% responding on the active nose-pokes, and a minimum of 10 pellets per session (Barbano, Castañé, Martín-garcía, & Maldonado, 2009). Once the acquisition criteria were achieved, the animals were shifted to the subsequent fixed-ratio schedule of reinforcement. Animals that did not reach the criteria until 15 days of each training schedule were omitted from the experiment.

In the second reinforcement schedule, mice were given *ad libitum* (AL) access to food in their cages (FR1 AL). The motivational aspect of animals was assessed by instrumental responding for obtain palatable food, in satiated conditions. The successive operant schedules of reinforcements consisted of FR3 (three active response led to one food pellet delivery) followed by FR5 (five correct responses led to one food pellet delivery) to evaluate the increase in motivation of mice for obtaining palatable food, on increasing complexity in reinforcement schedules. All the animals had an AL access to food during FR3 and FR5 schedules. The mean values and standard error or mean (SEM) of nose-pokes was recorded daily.

Lastly, the animals were trained under the progressive ratio (PR) schedule of reinforcement. PR is a commonly used measure of reward strength in an operant

procedure and a well-validated unit to assess hedonic control of feeding behavior in rodents (Baldo et al., 2013; Brunzell et al., 2006; Papaleo, Kieffer, Tabarin, & Contarino, 2007). The objective of the PR is to escalate the response ratios until the animal no longer responds. The number of correct sensor responses made to achieve the last reward or response is called as the breakpoint (BP), which reflects the maximum effort the animal will expend in order to receive the food rewards (Hodos, 1961; Sandeep Sharma et al., 2012). Under PR schedule, the responses can be calculated as per Richardson and Roberts using the following formula $PR = \left[5e^{(R*0.2)}\right]-5$, where, *R* = number of food rewards already earned plus 1(Richardson & Roberts, 1996). PR is usually rounded up to the nearest integer. Thus, the number of responses required to earn a food reward follow the order: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95 and so on. Each PR session lasted for an hour. The data were analyzed using the non-parametric Mann-Whitney U test.

2.8 Monitoring body weight and metabolism

To evaluate the metabolic changes, PDYN KO and WT control mice were maintained on a 12-week long high-fat diet or normal diet regimen, with either *ad libitum* or time restricted access to food. The mice were categorized into 4 main groups of diet and feeding in both sexes (Table 6). During the 12-week feeding regimen, body weight, blood glucose levels and food intake was monitored.

Table 6. Diet and feeding schedules

Diet	Feeding schedule	Gender
Normal (ND)	time-restricted (TR)	Males
		Female
Normal	*ad libitum* (AL)	Males
		Female
High-fat (HFD)	time-restricted	Males
		Female
High-fat	*ad libitum*	Males
		Female

2.8.1 Body weight and food consumption

Body weight and food intake of mice maintained on a HFD or a ND, were monitored twice per week i.e. on the first and third day of every week. Additionally, for the TR feeding groups, the food was also weighed in the evening on these two days, at the end of their 8 hours of daily feeding regimen. The amount of food left in the cages was subtracted from the prior food value recorded, to evaluate the amount of food consumed by each cage. Pellets smaller than approximately 5 mm in size were recorded, and later removed to avoid spillage and inaccuracy in the food weight measurement. Data from male mice and female mice were calculated separately.

2.8.2 Blood glucose levels

For monitoring the blood glucose levels, mice were starved overnight before the test, to avoid nocturnal consumption and reduce the variability in baseline blood glucose(Ayala et al., 2010). Standard normal diet pellets and high-fat diet soft pellets were removed from all cages on the previous day evening, and on the following day morning, blood glucose levels were measured using a fine syringe needle (23G). The tail vein was pricked, a drop of blood was collected onto the glucose test strip and measured using portable glucose meter. Food was then added back into all the cages. Blood glucose levels were recorded on week 4, 6, 8, 10 and 12.

2.9 Molecular Analysis

2.9.1 Brain and organ dissection

Animals were anaesthetized by injecting the narcotic solution intraperitonially (i.p.) and were fixed onto a grid plate lying on their back. The thorax and abdomen were opened and transcardially perfused with 1X PBS, followed by ice-cold 4% PFA/PBS (4 ml/min). After fixation, the brain was carefully dissected and post-fixed overnight in 4% PFA/PBS at 4°C. On the following day, the PFA solution was changed with a 10% (w/v) sucrose solution for the next 24 hours, and then to 20% (w/v) sucrose solution for another 24 hours. Brains were snap-frozen in ice-cold isopentane and stored in cryovials at -80°C until further use.

2.9.2 Immunohistochemistry

Perfused, snap-frozen brains were embedded in TissueTek®. Coronal slices of 16 µm thickness were cut using a cryostat and dried at 37°C for 30 minutes on a heating plate. The glass slides were labeled and stored at -80°C until use. Brain slices were marked with a PapPen and dried at 37°C for 30 minutes on a heating plate. The slides were rinsed with 1X PBS at room temperature (RT) for 5 minutes and permeabilized with 0.5%Triton X-100 in 1X PBS for 1 hour. The slices were then washed twice for 10 minute each, with 1X PBS and blocked with 1% BSA/PBS and 10% donkey serum for 1 hour in a humid chamber. Primary antibody were applied to slices, except for the negative controls, in which case, 3% BSA/PBS was

applied, and incubated at 4°C overnight. On the following day, the slices were incubated at 37°C for 2 hours, prior to washing steps. The slices were then washed 3 times with 1X PBS for 10 minutes each. Anti-rabbit Alexa Fluor® 488 was applied as a secondary antibody and slices were incubated for 1 hour at RT in dark conditions. The slices were washed for 4 times with 1X PBS for 10 minutes each and briefly immersed for in H_2O. Subsequently, immunostained brain slices were embedded in Fluoromount-G® media and carefully covered with glass coverslips. The sides of the slides were secured by sealing with nail polish and left to dry overnight and stored at 4°C.

2.10 Image acquisition and signal intensity analysis

Images were observed under Zeiss Axiovert 200M fluorescent microscope with 10X objective lenses. For quantification of immunostaining, the region of interest was identified in accordance with the mouse brain Atlas (Paxinos G. & Franklin K.B.J., 2001). For hypothalamic peptide NPY, a threshold value was set for determining signal intensity and measured using ImageJ software (Version 1.50i, NIH, USA). In case of orexin-A expression, integrated signal density was measured using ImageJ. Hypothalamic peptide expression patterns in the arcuate nucleus (ARC) for NPY and lateral hypothalamus (LH) for orexin-A, respectively, were defined using a free-hand selection (Fig. 8).

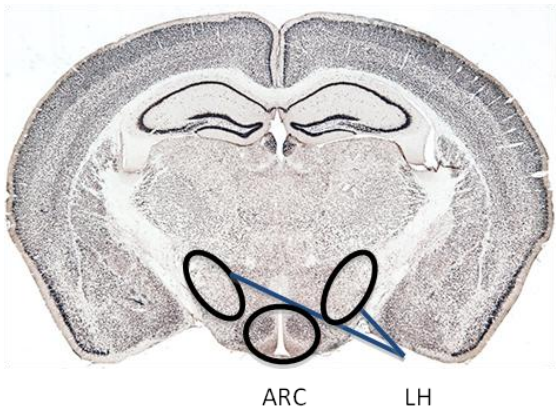

Fig. 8. Coronal section of brain representing areas of hypothalamic peptide expression. NPY is expressed in the arcuate nucleus (ARC) and neuropeptide orexin-A is expressed in the lateral hypothalamus (LH). (Fig. modified from Allen's brain Atlas)(Sunkin et al., 2013).

2.11 DNA isolation, purification, and measurement

For genotyping of PDYN KO and WT mice, a 2 mm piece of the mouse-tail tip was cut and incubated in lysis buffer with proteinase K (1 mg/ml) at 45°C on an agitating shaker (550 rpm) overnight. The samples were centrifuged at 12000 *g* for 10 minutes and the supernatant was transferred into a fresh tube. Equal volume of isopropanol was added to precipitate the DNA. The DNA pellet was washed at 12000 *g* for 3 times, 10 minutes each, with 70% ethanol and air-dried for approximately 15 minutes. The pellet was dissolved in 100µl TE buffer. The concentration of DNA was determined by using a spectrophotometer. The absorbance wavelength of DNA (A_{260}) of 1 unit was equivalent to 50 µl/ml of DNA concentration. Hence, the purity of a DNA preparation was assessed by the ratio of absorbance at 260/280 nm.

2.12 Agarose gel electrophoresis

Agarose gel electrophoresis was performed for separation of DNA fragments from PCR reactions, to confirm genotyping. One percent agarose gel in TAE buffer was used to test samples in an electrophoresis chamber filled with TAE buffer. The sample run time usually lasted 30 minutes at 120 V. To estimate the size of DNA fragments, a 1 kb Plus DNA ladder was used as a unit of measure. Agarose gel containing DNA fragments was stained with ethidium bromide by incubation in an ethidium bromide bath for 20 minutes. Stained DNA fragments were detected using ChemiDoc MP imaging system.

2.13 PCR for genotyping

For the genotyping of PDYN KO mice, a polymerase chain reaction (PCR) was performed. Each PCR was specifically optimized to the oligonucleotide temperature requirements and length of the desired product. All PCR reactions were performed using GoTaq® Green Master Mix (Promega). The components of master mix consisted of Taq polymerase, dNTPs, $MgCl_2$ and reaction buffers. The wild type allele led to a PCR product of 290 bp, whereas the knockout allele yielded a 500 bp fragment (table 7).

2.14 Statistical analysis

Statistical calculations were performed using STATISTICA software package. Datasets containing three independent variables were analyzed by a three-way ANOVA (treatment, genotype and sex) whereas dataset containing two independent variables (treatment and genotype) were analyzed by two-way analysis of variance (ANOVA). Analysis of the area under the curve (AUC) by two-way ANOVA was used to determine significant differences in body weight, and glucose levels (genotype, and feeding as main effect, whereas time as within effect). Post-hoc Tukey's test was applied with significance observed at $p < 0.05$. Data for males and females were analyzed separately. Repeated measures of ANOVA were used when the data from the same animal was collected over a period of time (within effect: time). Hypothalamic peptide expressions were analyzed by two-way ANOVA. For non-parametric datasets Mann-Whitney U test was performed. The data are presented as the mean ± standard error of the mean (SEM) for all measurements. All graphical data was prepared using GraphPad Prism (version 6, La Jolla California USA) software. A value of $p < 0.05$ is denoted with *, $p < 0.01$ is denoted with **, $p < 0.001$ is denoted with *** and $p < 0.0001$ is denoted with ****.

Table 7. Primer sequence and PCR program for PDYN KO mice

Primer sequence		
PDYN common (D25R)	CTT CAG AAT AGG TAT TGG GGT TCT CCT GGG	
PDYN KO (Neo3)	AGC GCA TCG CCT TCT ATC GCC TTC TT	
PDYN WT (D27)	CGC ACC GTC CAT TTT AAT GAG GAG GAC TTG	
PCR reaction for PDYN KO genotyping		
sterile water	16 µl	
P1: KO PCR PDYN KO fwd	1 µl	
P2: KO PCR PDYN KO rev	1 µl	
P3: KO_PCR_WT	1 µl	
GoTaq®Green Master Mix	10 µl	
mouse tail DNA (100-150 ng/µl)	1 µl	
PCR program		
1 x initial denaturation	95 °C	2 min
Denaturation	95 °C	30 s
35X annealing	60 °C	45 s
Elongation	72 °C	60 s
1 x final elongation	72 °C	5 min
Cooling	4 °C	∞

3 Results

The results section illustrates the outcomes of hedonic control of feeding behavior in endogenous opioid peptide knockout mouse models. Constitutive PDYN KO and PENK KO mice were tested for voluntary reward-related motivational responses for highly palatable 'chocolate-flavored' food rewards and normal food rewards in an operant conditioning paradigm. Next, to elucidate the role of endogenous opioid dynorphin in regulation of food intake and energy homeostasis, mice maintained on a high-caloric diet or a normal diet for 12 weeks were monitored for their metabolic changes. Furthermore, modulation in the hypothalamic peptides involved in the homeostatic feeding behavior was monitored by immunohistochemistry.

3.1 Acquisition and maintenance of operant responding

3.1.1 Operant motivational responding in PDYN KO and WT mice

PDYN KO and WT mice were tested for their motivational behavior, where animals either responded for a highly palatable chocolate-flavored pellet or a standard normal pellet in the operant conditioning paradigm.

Under the FR1 schedule of operant conditioning, under food deprivation conditions, a significant effect of pellet preference ($F_{(1, 46)} = 4.1797$, $p = 0.04$), however no sex effect and no interaction were observed. Additionally, significant interaction effects were observed with respect to time, where time x pellet ($F_{(7,322)} = 8.2112$, $p < 0.0001$), time x genotype ($F_{(7,322)} = 2.4058$, $p = 0.02$), time x sex ($F_{(7, 322)} = 3.35$, $p = 0.001$) effects were observed. Both, PDYN KO and WT control mice displayed a significantly higher preference for chocolate-flavored pellets over standard normal pellets. WT males responded to chocolate-flavored pellets significantly higher than PDYN KO males, whereas, this effect was absent in female mice (Fig. 9A & B). Under FR1 AL schedule, a significant effect of pellet preference ($F_{(1,44)} = 16.01$, $p = 0.0002$), genotype ($F_{(1,44)} = 9.02$, $p = 0.003$), sex ($F_{(1,44)} = 14.56$, $p = 0.0004$), as well as pellet x genotype interaction ($F_{(1,44)} = 9.02$, $p = 0.003$). Furthermore, significant interactions of time x pellet ($F_{(9,396)} = 2.20$, $p = 0.021$) and

time x genotype ($F_{(9,396)}$ = 2.02, p = 0.035) were observed. WT mice displayed a significantly higher preference for chocolate-flavored pellets than normal pellets. Furthermore, WT female mice also showed a significantly higher preference (motivation) compared to PDYN KO female mice to obtain the highly palatable chocolate-flavored pellets. In general, the female mice had higher operant responses as compared to male mice (Fig. 9C & D). Next, under the FR3 schedule of reinforcement, significant effects were observed in pellet ($F_{(1,42)}$ = 17.1022, p = 0.0001), genotype ($F_{(1,42)}$ = 16.048, p = 0.0002), and sex ($F_{(1,42)}$ = 8.293, p = 0.006). Significant effect of pellet x sex ($F_{(1,42)}$ = 17.1025, p = 0.019), time x pellet ($F_{(6,252)}$ = 10.38, p < 0.0001) and time x genotype ($F_{(6,252)}$ = 2.69, p = 0.0014) were also observed. Overall, PDYN KO animals had a significantly lower motivation for obtaining palatable food than the WT controls, and highly palatable chocolate-flavored pellets were preferred over normal food pellets. Over the course of time, WT female mice demonstrated a higher preference than PDYN KO female mice for chocolate-flavored pellets (Fig. 9E & F). Subsequently, under the FR5 schedule of reinforcement, significant effects of pellet ($F_{(1,43)}$ =17.54, p = 0.0001), genotype ($F_{(1,43)}$ = 6.835, p = 0.012) and sex ($F_{(1,43)}$ = 20.747, p < 0.0001) were observed (Fig. 9G & H). Similar to outcomes from the FR3 schedule, a highly significant preference for palatable chocolate-flavored pellets was revealed. WT mice activated the sensor significantly more compared to PDYN KO mice. This was observed particularly in female mice over the course of time ($F_{(6,258)}$ = 3.021, p = 0.0071).

Under the progressive ratio (PR) schedule, significant sex effect (p = 0.0058) was observed, however no significant genotype effect or interaction was seen. The breakpoint (BP) responding in female PDYN KO mice for chocolate-flavored pellets was significantly higher than normal pellets (p= 0.048) (Fig. 10A). In male mice, BP responding was similar in all groups (Fig. 10B). Furthermore, PDYN KO females displayed a significantly higher motivation compared to the male littermates for the palatable chocolate-flavored pellets (p = 0.005) (Fig. 10C).

The time to reach the criteria for the change of the operant conditioning schedule to the successive schedule is represented in table 8. It was observed during the experiment that male and female mice from the same group required similar number of days to acquire the task of operant conditioning. Hence the data from both the sexes was pooled together. Under the FR1 food deprivation schedule, the

mice reached the criteria in between 8 to 10 days for acquisition of the operant nose-poke task. There was no significant difference observed between the groups, during the acquisition phase. Next, in the FR1 schedule with AL access to food, mice required longer to achieve the criteria i.e. 10 to 16 days, particularly PDYN KO mice responding for palatable chocolate pellets. Under the AL access schedule, mice responded with their motivation (hedonic feeding) rather than hunger (homeostatic feeding). Therefore, the time required to achieve the stable responding for was longer than in the FR1 deprivation schedule. Consecutively, in the FR3 and FR5 schedules, mice reached the criteria in the duration of a week, and between 7 to 8 days, respectively.

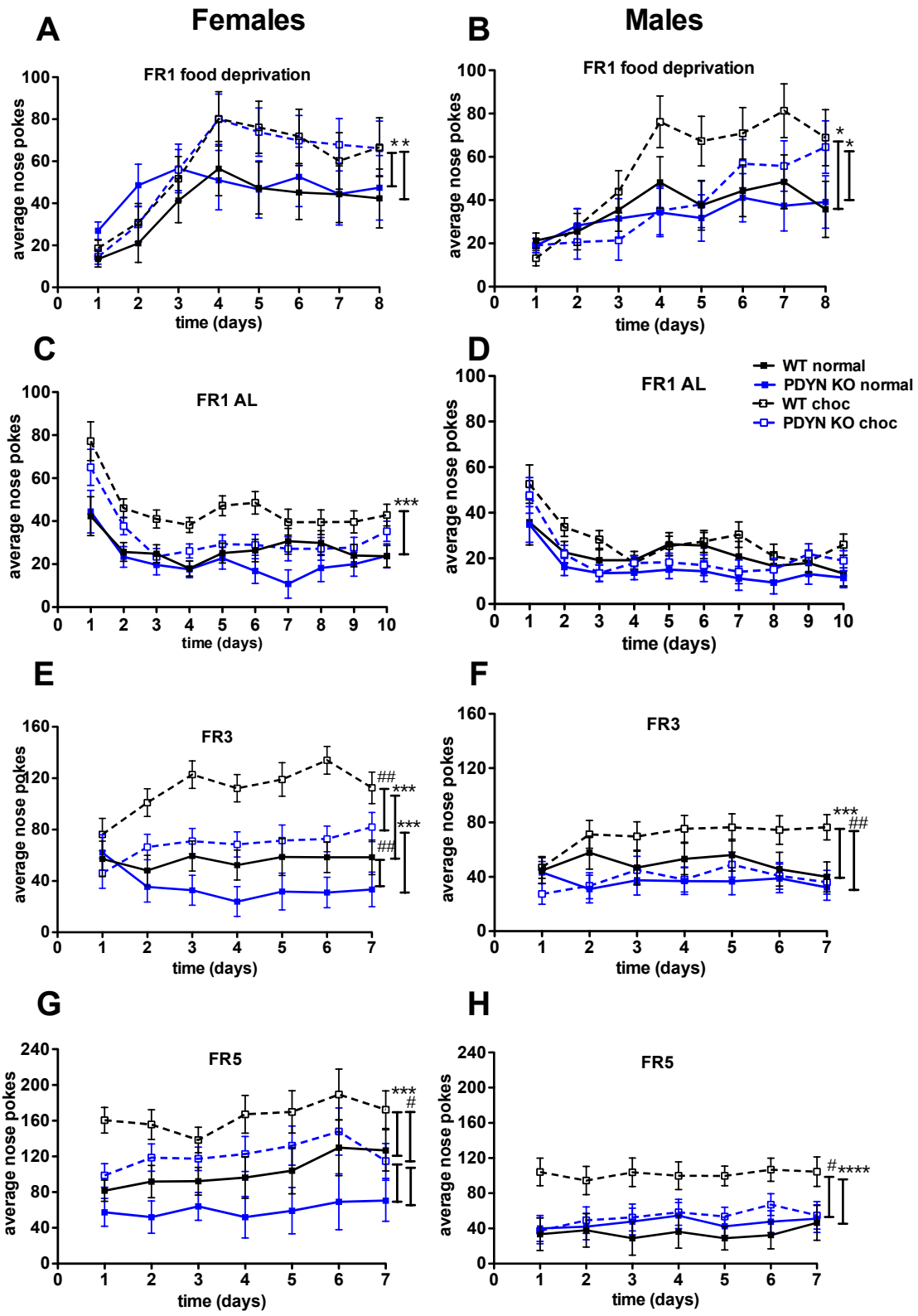

Fig. 9. Operant motivational responding in PDYN KO and WT mice

Panel A and B represent data from the average nose-poke responses in female and male mice under FR1 food deprivation schedule, panel C and D represent operant responses under FR1 AL schedule, panel E and F represents responses under FR3 schedule and panel G and H represents responses from the FR5 schedule of reinforcement. Females: A,

C, E, male: B, D, F, n = 7- 8/group. *= pellet effect, # = genotype effect, */# p= 0.05, **/## p < 0.005, ***p < 0.0001, ****p<<0.0001.

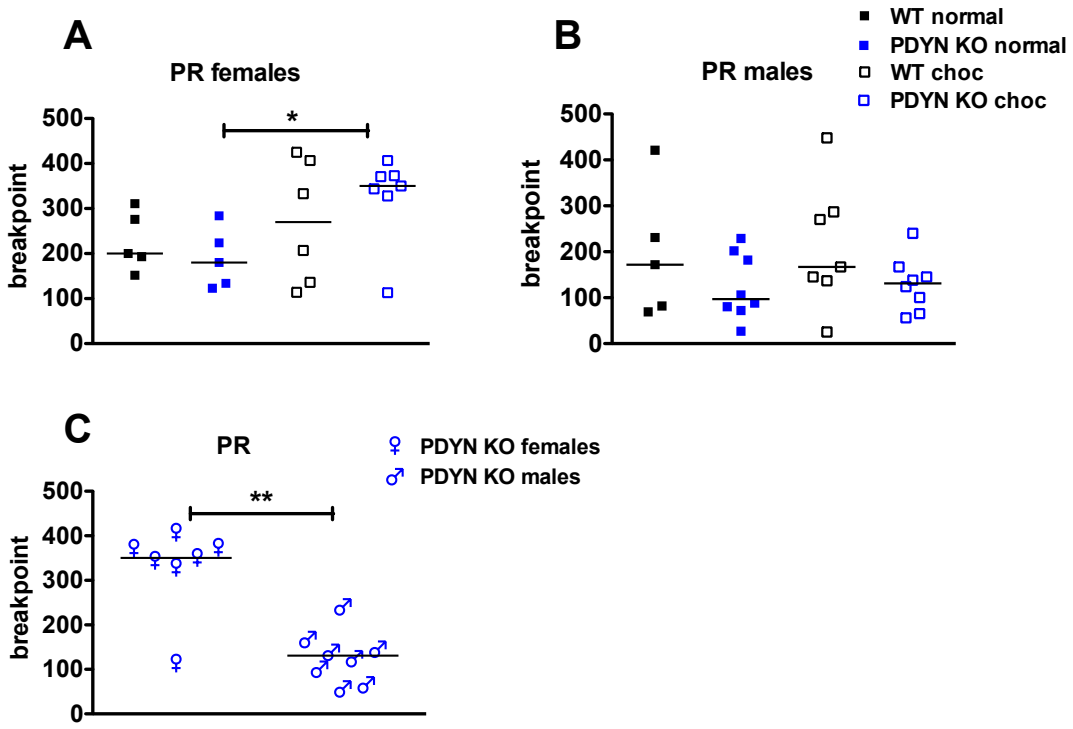

Fig. 10. Breakpoint under PR schedule in PDYN KO and WT mice

In PDYN KO groups, female mice had a significantly higher number of BP responding for palatable chocolate-flavored pellets compared to normal pellets (A), whereas, male mice had similar BP responding in all groups (B). Between sexes comparisons showed that PDYN KO females had significantly higher breakpoint than PDYN KO males for obtaining palatable chocolate-flavored pellets (C). PR = progressive ratio, BP = breakpoint, PDYN KO = prodynorphin knockout, WT = wild type, choc =chocolate-flavored pellets, n=5-8/group, *p < 0.05, **p ≤ 0.005.

Table 8. Time to reach criteria for operant schedules in PDYN KO and WT mice

Operant schedules	**Time to reach criteria (days)**			
Genotype	**PDYN KO**	**WT**	**PDYN KO**	**WT**
pellet type	**normal**	**normal**	**choc**	**choc**
FR1 food deprivation	8	8	10	10
FR1 AL	10	10	16	11
FR3	7	7	7	7
FR5	7	7	8	7

3.1.2 Operant motivational responding in PENK KO and PENK floxed mice

PENK KO and PENK floxed (control) mice were tested for their motivational responses in an operant conditioning paradigm. Under the FR1 schedule of conditioning a significant effect of pellet preference ($F_{(10,41)} = 2.68$, $p = 0.012$), but no genotype effect was observed. Sex-separated analysis showed a significant effect of pellet preference ($F_{(10,10)} = 3.217$, $p = 0.039$) in female and ($F_{(10,22)} = 2.371$, $p = 0.043$) in males, respectively (Fig. 11A & B). Both, PENK KO and PENK floxed mice, demonstrated a higher motivation to obtain highly palatable chocolate-flavored pellets over normal pellets.

Under the FR1 AL schedule of reinforcement, a significant effect of pellet preference ($F_{(8,43)} = 4.011$, $p = 0.0012$) and pellet x genotype x sex ($F_{(8,43)} = 4.684$, $p=0.00035$) interaction were observed. In female mice, a significant preference for pellet ($F_{(8,12)}= 2.849$, $p = 0.049$) was observed, where female mice of both genotypes preferred palatable chocolate-flavored pellets as compared to normal pellets. A significant genotype x pellet interaction was observed in females ($F_{(8,12)} = 6.375$, $p=0.0023$) as well as males ($F_{(8,24)} = 5.71$, $p = 0.0004$) respectively (Fig. 11C & D). Overall, PENK KO mice preferred chocolate pellets than normal pellets.

Next, under the FR3 schedule, significant effect of pellet ($F_{(6,45)} = 7.519$, $p = 0.00001$), and strain x sex x pellet ($F_{(6,45)} = 2.332$, $p = 0.047$) interaction were observed. Overall, significant effect of pellet was observed in female mice ($F_{(6,14)} = 3.678$, $p = 0.020$), and in male mice ($F_{(6,26)} = 4.85$, $p = 0.009$), respectively (Fig. 11E & F). Both the sexes preferred palatable chocolate flavored-pellets to normal pellets, irrespective of their genotype.

Successively, under the FR5 schedule, significant effect of pellet ($F_{(5,46)} = 4.695$, $p = 0.0015$), but no genotype or sex effect was observed. In sex-separated analysis, there was a significant pellet preference in females ($F_{(5,12)} = 2.495$, $p = 0.0077$) and in males ($F_{(5,27)} = 4.041$, $p = 0.007$), respectively (Fig. 11G &H). PENK KO and PENK floxed mice preferred palatable chocolate-flavored pellets to normal pellets.

Data from the progressive ratio schedule revealed a significant pellet effect ($p = 0.00003$) with a preference for the chocolate-flavored pellets over normal pellets

in all mice. Significant pellet preference was observed in female mice ($p = 0.00018$) and male mice ($p = 0.0057$), respectively (Fig. 12A & B). PENK KO and PENK floxed mice significantly showed higher motivational responding to obtain palatable chocolate-flavored pellets compared to the groups responding for normal pellets. There was no significant effect of genotype or sex observed.

The time to reach criteria for FR schedules of reinforcement is represented for female and male mice, respectively (table 9). All the groups under the FR1 schedule of deprivation required similar number of days, irrespective of the genotype or pellet. Female mice responding for chocolate-flavored pellets (10 days) achieved the acquisition task faster than females responding for normal pellets (12 days). In case of males, PENK KO mice responding for chocolate-flavored groups required longer (13 days) for acquisition of the schedule compared to the other groups. Consecutively, under the FR5 schedule, PENK KO female and male mice responding for chocolate-flavored pellets required more number of days (10 days and 7 days, respectively) to achieve the criteria compared to other groups.

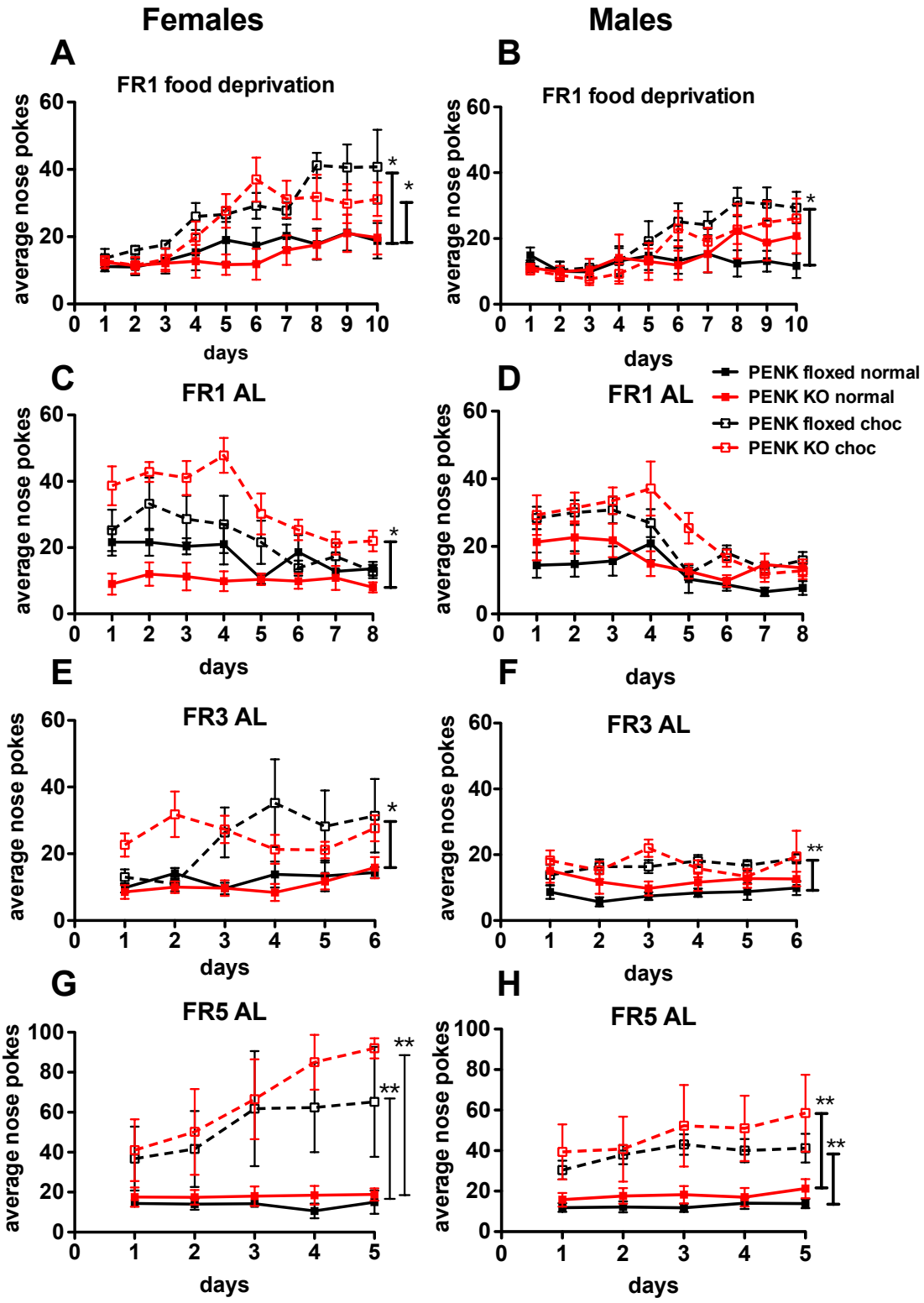

Fig. 11. Motivational responses under fixed ratio schedules in PENK KO and WT mice. Panel A and B represent data from the average nose-poke responses in female and male mice under FR1 food deprivation schedule, panel C and D represent operant responses under FR1 AL schedule, panel E and F represents responses under FR3 schedule and panel G and H represents responses from the FR5 schedule of reinforcement. females: A, C, E, G; male: B, D, F, H; n = 5-10/group, *p < 0.05, **p < 0.001.

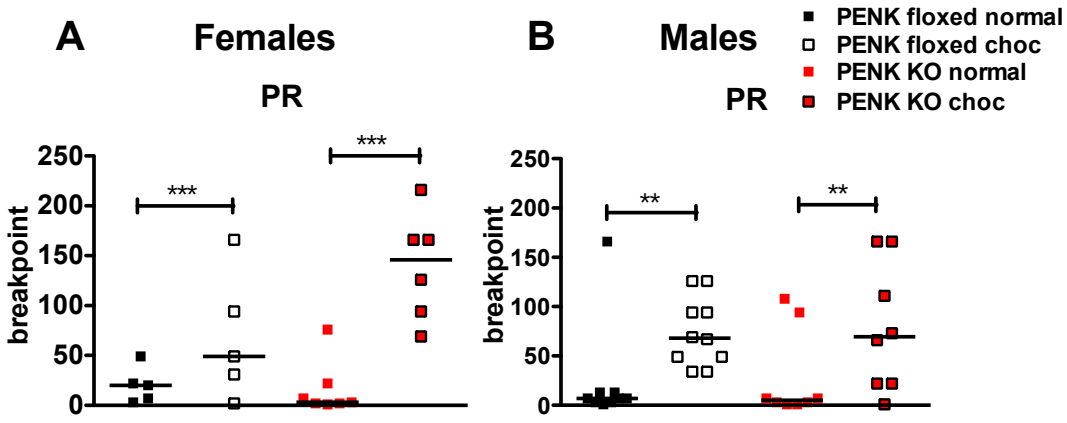

Fig. 12. Breakpoint under progressive ratio schedule in PDYN KO and WT mice
Under the PR, the BP responding was significantly higher for chocolate-flavored pellets than normal pellets in PENK KO and PENK floxed females (A) and males (B), respectively. Time required to reach criteria for the different schedules in operant conditioning schedules were similar in all groups in female mice (C) as well as male mice (D). PR = progressive ratio, BP = breakpoint, PENK KO = proenkephalin knockout, FR = fixed ratio, choc = chocolate-flavored pellets, n=5-8/group, *p < 0.05.

Table 9. Time to reach criteria in PENK KO and floxed mice.

Operant schedules	**gender**	PENK KO **normal**	PENK floxed **normal**	PENK KO **choc**	PENK floxed **Choc**
FR1 food deprivation	females	12	12	10	10
	males	10	11	10	13
FR1 AL	females	9	8	12	9
	males	8	9	11	11
FR3	females	6	8	7	9
	males	6	5	6	9
FR5	females	8	5	5	10
	males	6	5	5	7

3.2 Effects of diet, feeding, and metabolism

The effects of prolonged voluntary consumption of high-fat diet compared to normal diet in PDYN KO and WT mice, on the development of body weight regulation and metabolic changes was investigated. Furthermore, the groups were subjected to two different feeding regimens and assessed for the impact of time-restricted feeding compared to *ad libitum* access in mice. Therefore, the results obtained are demonstrated in comparison of three factors i.e. genotype, diet and feeding regimen in both sexes.

3.2.1 Effects of normal diet on PDYN KO and WT mice

First, the change in the body weight of PDYN KO and WT mice maintained on a normal diet, with *ad libitum* or intermittent access to food was determined. Analysis of the accumulated data (AUC) by 2-way ANOVA revealed main effects for the feeding schedule ($F_{(1, 29)} = 12.83$, $p = 0.0012$) and genotype ($F_{(1, 29)} = 7.882$, $p = 0.0088$), but no interaction ($F_{(1, 29)} = 1,859$, $p = 0.1833$) in females. In males, only a feeding effect ($F_{(1, 28)} = 7.219$, $p = 0.0120$), but no effect of genotype and no interaction was observed. Post hoc analysis revealed a significantly higher body weight in the AL feeding groups as compared to TR feeding in WT animals. This was observed in both sexes (Fig. 13A, B). However, this effect was absent in PDYN KO mice. Furthermore, PDYN KO females on a TR schedule had a generally higher body weight than WT females (Fig. 13A).

Analysis of blood glucose levels showed a significant effect of feeding ($F_{(1, 30)} = 5.582$, $p = 0.0248$) in females, but no genotype effect and no interaction. Post hoc analysis revealed that blood glucose levels were significantly higher in PDYN KO females of the AL group, when compared to WT controls (Fig. 13C). In case of males, there were no significant differences (Fig. 13D).

As shown in Fig. 13 E & F, mice on AL feeding consumed a similar amount of food as TR feeding groups, although the variance was higher in the AL feeding groups.

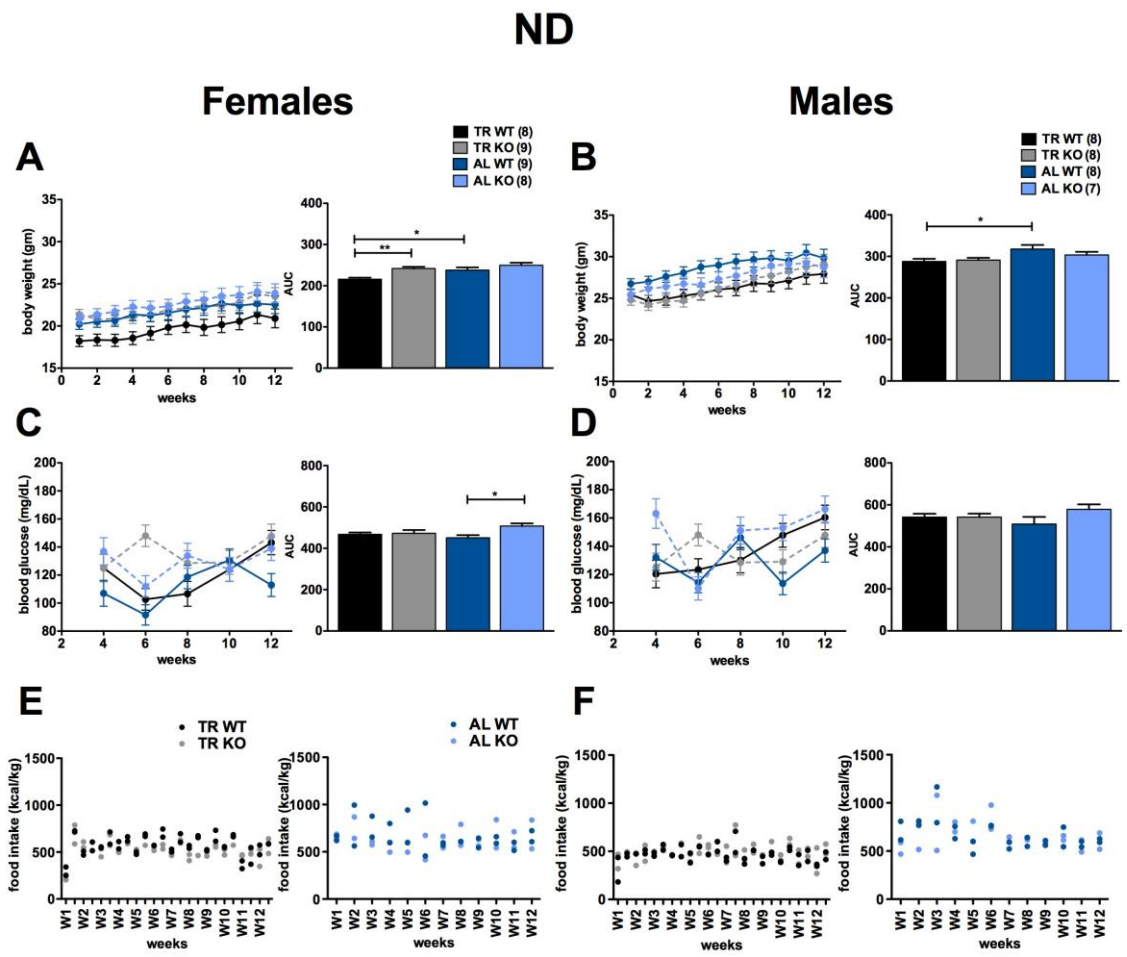

Fig. 13. Effects of normal diet in PDYN KO and WT mice

The PDYN KO and WT animals maintained on a 12-week of normal diet during AL or TR feeding schedules are depicted in Fig.13. A-F. Line diagrams show the mean values of individual measurements, whereas bar graphs represent the cumulative (AUC) data. Scatter plot represents the food intake every week. (Panel A, C, E: female mice, Panel B, D, F: male mice, n = 7-12/group). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. TR = time restricted feeding, AL = *ad libitum* feeding, WT = wild type mice, KO = PDYN KO /prodynorphin knockout mice.

3.2.2 Effects of high-fat diet on PDYN KO and WT mice

Analysis of body weight of WT and PDYN KO animals maintained on a high-fat diet showed a main effect of genotype ($F_{(1, 30)} = 186.9$, $p < 0,0001$), feeding schedule ($F_{(1, 30)} = 11.66$, $p = 0.0019$) and an interaction effect ($F_{(1, 30)} = 4.647$, $p = 0.0392$) in female mice. Similarly, in male mice, a main effect was observed in genotype ($F_{(1, 26)} = 36.55$, $p < 0.0001$) and feeding ($F_{(1, 26)} = 18.65$, $p = 0.0002$), but no interaction ($F_{(1, 26)} = 0.7026$, $p = 0.4095$). Post hoc analysis revealed a significantly higher body weight in the AL feeding groups as compared to TR feeding in WT animals. This was observed in both sexes (Fig. 14A, B). Furthermore, PDYN KO females on a TR schedule displayed higher body weights than WT females (Fig. 14A), whereas, PDYN KO males on an AL schedule had higher body weights as compared to WT male mice (Fig. 14B).

Blood glucose analysis revealed a main effect of genotype in females ($F_{(1, 30)} = 73.48$, $p < 0.0001$) and in males ($F_{(1, 26)} = 18.85$, $p = 0.0002$), as well as an effect of feeding schedule in females ($F_{(1, 30)} = 7.778$, $p = 0.0091$) and in males ($F_{(1, 26)} = 4.915$, $p = 0.0356$), respectively. However, no interaction was observed in both sexes. A post hoc test revealed generally higher blood glucose levels in the AL compared to the TR schedule in WT animals (Fig. 14C, D). PDYN KO females on the AL schedule showed higher blood glucose levels than WT females (Fig. 14C), whereas in males, this effect was absent (Fig. 14D). Additionally, PDYN KO females on a TR schedule showed higher blood glucose levels compared to WT (Fig. 14C).

Food consumption was initially higher in the AL feeding groups (Fig. 14E and F), but eventually decreased to the same level as the TR feeding groups.

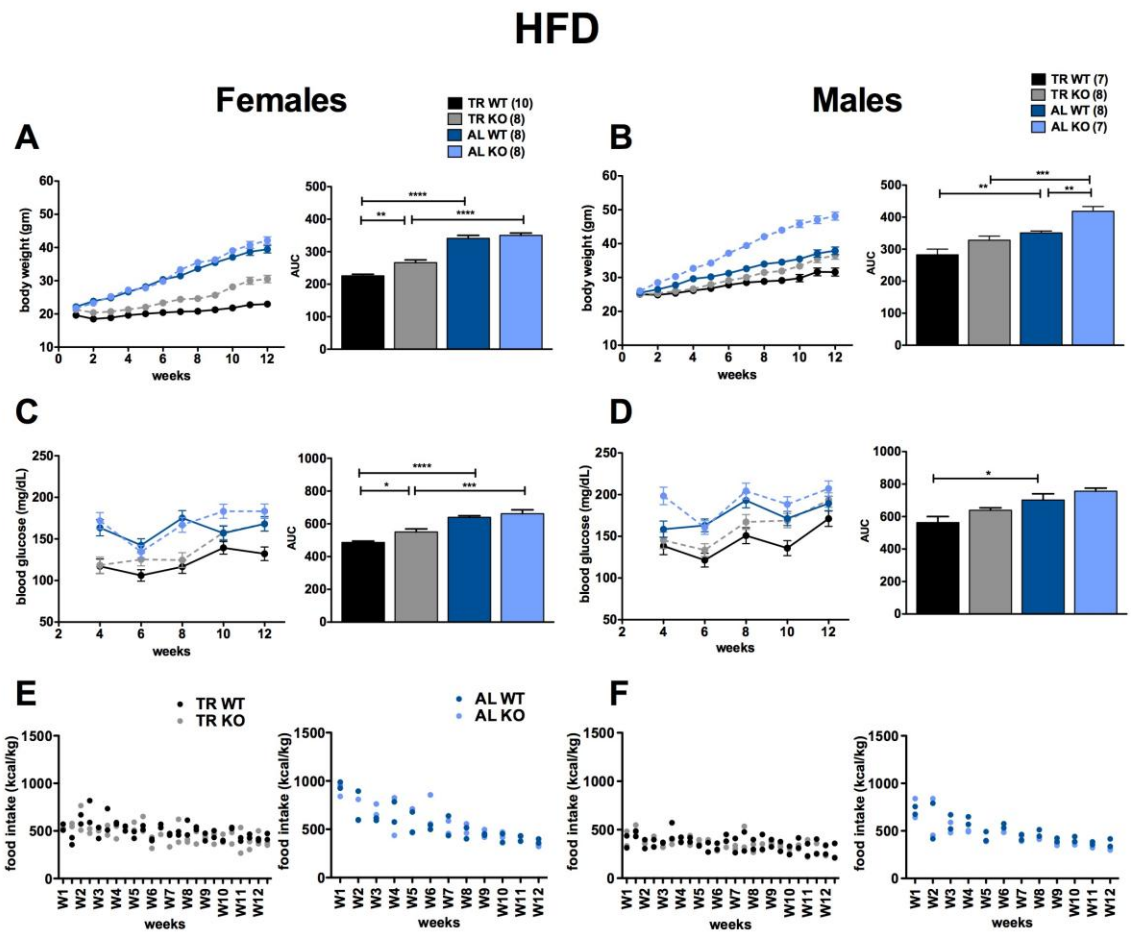

Fig. 14. Effects of HFD in PDYN KO and WT mice

The PDYN KO and WT animals maintained on 12-weeks of high-fat diet during AL or TR feeding schedules are depicted in Fig. 14. A-F. Line diagrams show the mean values of individual measurements, whereas bar graphs represent the cumulative (AUC) data. Scatter plot represents the food intake for every week. Panel A, C, E: females, panel B, D, F: males, n = 7-12/group. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. TR = time restricted feeding, AL = *ad libitum* feeding, WT = wild type mice, KO = PDYN KO/prodynorphin knockout mice.

3.3 Genotyping of PDYN KO mice

To assess the genotype of the mice, DNA analysis was performed using tail biopsies. After isolation of the DNA and polymerase chain reaction (PCR), was visualized using agarose gel electrophoresis. Bands of sizes 290 bp for WT animals and 500 bp for PDYN KO were produced (Fig. 15).

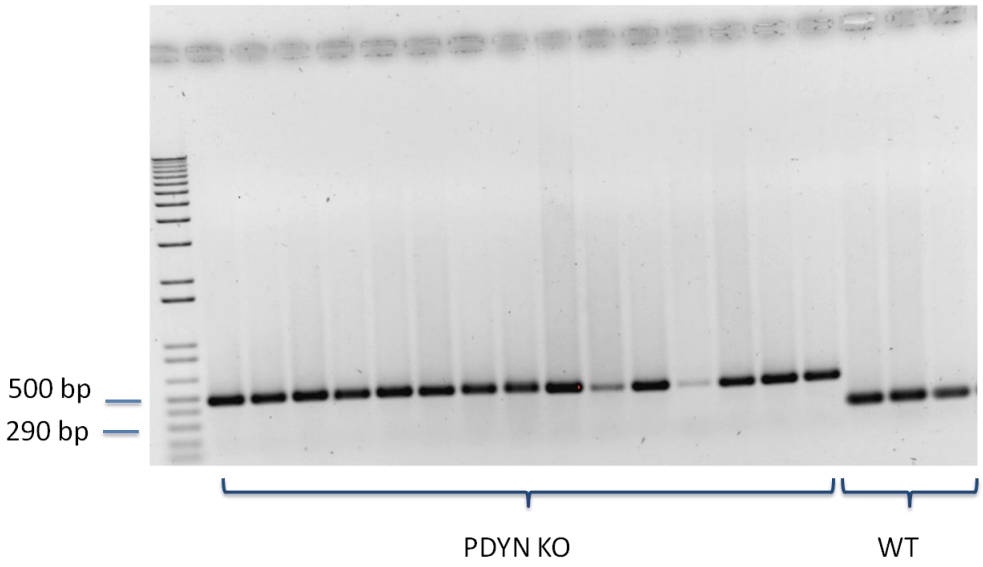

Fig. 15. Genotyping of the PDYN KO and WT animals by PCR analysis
Band size 290 bp depicted the WT allele and 500 bp depicted the PDYN KO allele.

3.4 Molecular analysis of hypothalamic peptides

Changes in the expression levels of hypothalamic NPY and orexin-A peptides in PDYN KO and WT mice were investigated by immunohistochemistry. The hypothalamic peptides levels of NPY were monitored in the ARC region whereas neuropeptide orexin-A levels were monitored in the LH region of the brain, respectively. The outcomes from the molecular analysis reflect the changes in the expression levels occurring after a high-fat diet or normal diet consumption.

3.4.1 Neuropeptide-Y

Analysis of neuropeptide-Y (NPY) peptide signal intensity in females maintained on a normal diet revealed main effects for genotype ($F_{(1, 8)} = 26.20$, $p = 0.0009$) and feeding ($F_{(1, 8)} = 11.54$, $p = 0.0094$), but no interaction. In males, the only main effect was observed for genotype ($F_{(1, 8)} = 6.359$, $p = 0.0357$). In WT and PDYN KO animals on HFD, a main effect for genotype ($F_{(1, 7)} = 21.19$, $p = 0.0025$) was seen in females, whereas in males, significant effects of genotype ($F_{(1, 8)} = 204$, $p < 0.0001$) and feeding ($F_{(1, 8)} = 56.97$, $p < 0.0001$) were calculated. No significant interaction was observed in both sexes.

Post hoc analysis showed that PDYN KO females maintained on ND had generally lower NPY expression levels in the *ad libitum* feeding groups as compared to time-restricted feeding (Fig. 16A). This effect was absent in males (Fig. 16B). Similarly, PDYN KO females on HFD showed lower NPY expression after AL feeding as compared to the TR feeding schedule (Fig. 16C). This effect was also observed in HFD-fed males, irrespective of the genotype (Fig. 16D). Additionally, PDYN KO males showed lesser NPY signal intensity as compared to WT males, in TR feeding as well as AL feeding schedule (Fig. 16D).

3.4.2 Orexin-A

Analysis of the hypothalamic peptide orexin-A in the lateral hypothalamus of female mice maintained on a ND showed main effects for genotype ($F_{(1, 28)}$ = 23.40, $p < 0.0001$), feeding ($F_{(1, 28)}$ = 69.62, $p < 0.0001$) and interaction ($F_{(1, 28)}$ = 68.83, $p < 0.0001$). In males, only an interaction effect ($F_{(1, 22)}$ = 7.292, p = 0.0131), but no genotype or feeding effect, was observed. In WT and PDYN KO mice maintained on a HFD, a significant effect of feeding ($F_{(1, 27)}$ = 25.23, $P < 0.0001$) was observed in females. In males, in addition to a main feeding effect ($F_{(1, 22)}$ = 1.640, p = 0.2136), there was a genotype ($F_{(1, 22)}$ = 0.003081, p = 0.9562) and interaction effect ($F_{(1, 22)}$ = 7.292, p = 0.0131). Post hoc test of mice maintained on a normal diet revealed that WT females had generally higher orexin-A expression levels in *ad libitum* feeding group as compared to KO females (Fig. 17A). Additionally, AL-fed WT females had a higher signal density as compared to TR-fed females. In the TR feeding schedule, ND-fed male PDYN KO mice showed higher levels of orexin-A as compared to WT males (Fig. 17B). PDYN KO animals on HFD on AL feeding regimen generally showed lower orexin-A expression compared to WT mice. This effect was seen in both sexes (Fig. 17C, D). Furthermore, in PDYN KO males on AL feeding schedule showed lower signal density levels of orexin-A peptide as compared to the TR feeding group (Fig. 17D).

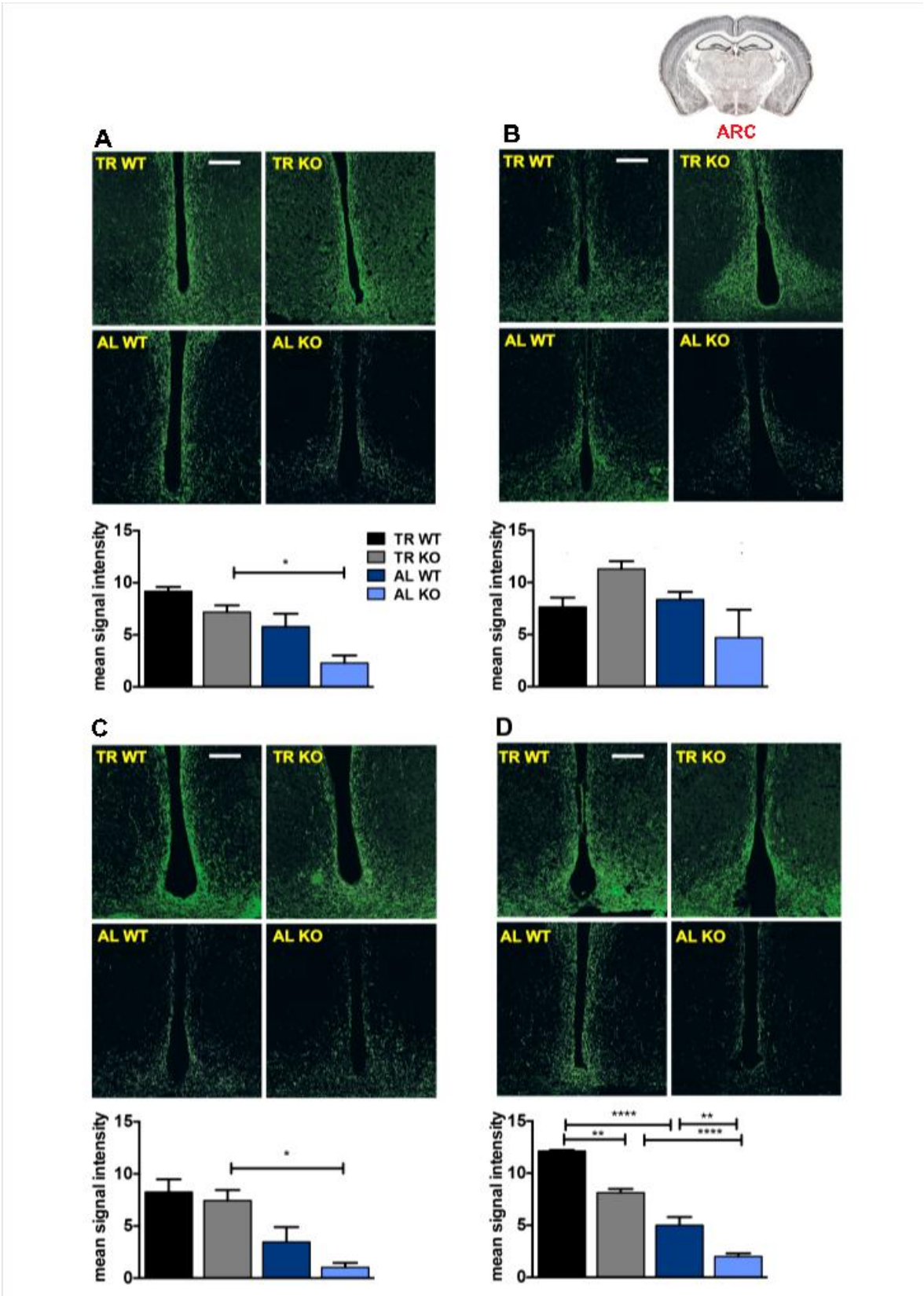

Fig. 16. Signal intensity of hypothalamic peptide NPY was analyzed in females and males maintained on a normal diet (A, B) and females and males on a high-fat diet (C, D), respectively. n= 3-5/ group. Females: A, C; Males B, D. WT = wild-type, KO = PDYN KO / prodynorphin knockout, TR = time restricted, AL = *ad libitum*, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

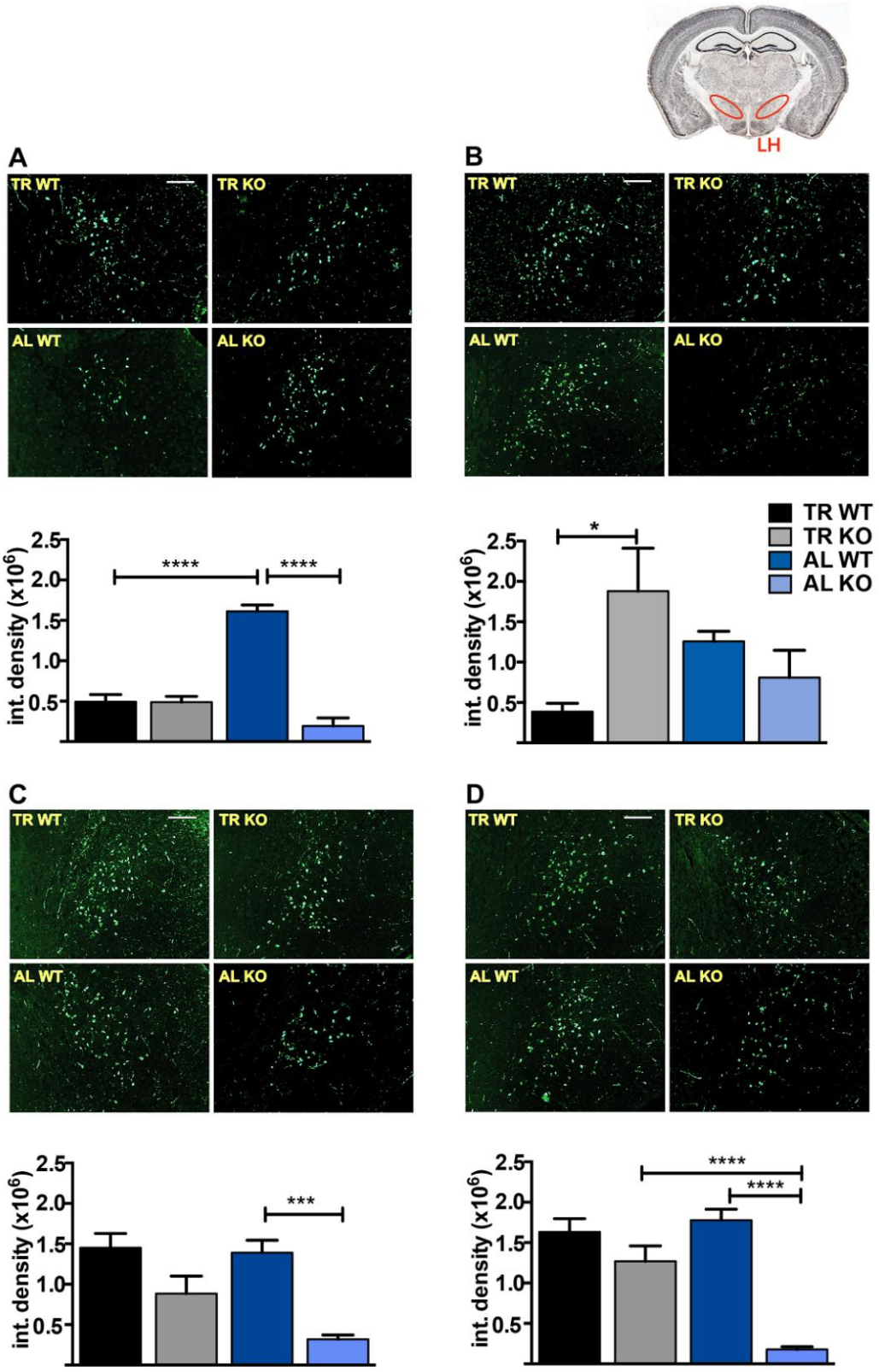

Fig. 17. Integrated signal density of hypothalamic peptide orexin-A analyzed in females and males maintained on normal diet (A, B) and on a high-fat diet (C, D), respectively. n= 3-5/ group. Females: A, C; Males B, D. WT = wild type, KO = PDYN KO / prodynorphin knockout, TR = time restricted, AL = *ad libitum*, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

4 Discussion

In the present study the contribution of the two major classes of endogenous opioids, namely enkephalin and dynorphin in the modulation of hedonic control of feeding behavior was investigated. This behavior was assessed using palatable food pellets and standard normal food pellets in an operant conditioning task. Therefore, at first, the learning and motivation aspects of palatable reward-induced feeding behavior in dynorphin deficient and enkephalin deficient mice will be discussed. Another aim of the study focused on the modulation of feeding behavior and metabolism, where dynorphin deficient mice were exposed to prolonged voluntary high-fat diet consumption. The results demonstrating the impact of feeding behavior and diet regimen will be discussed. Lastly, the outcomes from the molecular changes of hypothalamic peptide expression following the different diet and feeding regimen, will be discussed in the later part of this section.

4.1 Learning ability in an operant conditioning task

The present study demonstrates the role of endogenous opioids dynorphin and enkephalin in learning and motivation of palatable food rewards in an operant conditioning paradigm. The use of knockout mouse models in this study facilitates to explore the contribution of individual opioid ligand in natural reward-related reinforcement behaviors. Under the operant training schedule of FR1 food deprivation (acquisition phase) all groups of mice (PDYN KO, PENK KO, WT and PENK floxed), learnt the paradigm equally well, indicating intact learning abilities. The results were similar to a previous study showing intact learning abilities of dynorphin deficient mice, when trained under operant bar pressing responses for sucrose reinforcers (Hayward et al., 2006). Dynorphin has been suggested to play a modulatory role in cognitive acquisition and appear to enhance memory retention in negative reinforcement tasks, whereas, inhibitory effects on memory and learning in tasks based on positive stimuli (Kuzmin, Madjid, Terenius, Ogren, & Bakalkin, 2006; Wall & Messier, 2000). PENK KO mice also displayed similar learning abilities as the PENK floxed controls, during the operant learning phase, suggesting intact learning ability. In line with the current results, earlier research

on enkephalin deficient mice also showed an intact learning ability during a ethanol-reinforced operant conditioning task (Hayward, Hansen, Pintar, & Low, 2004). Therefore, it can be concluded that the deficiency of endogeonous peptide enkephalin or dynorphin doesn't affect the learning behavior in an operant conditioning experiment.

4.2 Motivational responding to obtain highly palatable food rewards

An important focus of this study was to evaluate the motivational responding for natural rewards in endogenous opioid knockout mice of either dynorphin or enkephalin, based on their palatability preferences. Animals were food-deprived during the first training phase to facilitate learning of nose-poke responses. However, an *ad libitum* access to food was provided during the remaining schedules of the experiment, in order to return the animals to normal physiological situation. Absence of food deprivation during the acquisition phase could have possibly led to decreased probability of food seeking-behavior in mice. Animals from all four groups i.e. PDYN KO, PENK KO, WT and PENK floxed, respectively, exhibited a prominent preference for the palatable chocolate-flavored pellets over normal pellets under all FR schedules. As both the food rewards were isocaloric in nature, the reinforcing property of chocolate pellets was its flavor rather than its caloric content. Similarly, previous studies on rats have shown a strong preference for flavor-based reinforcers such as saccharine or caffeine over unflavored reinforcers (Fedorchak, Mesita, Plater, & Brougham, 2002; Lockie & Andrews, 2013). PDYN KO male and female mice under FR1 *ad libitum* access schedule, showed a steady tendency of decreased motivation for palatable chocolate-flavored reinforcers as compared to their WT controls, however a prominent difference in the genotype was observed only in later FR3 and FR5 schedules. Furthermore, this phenotype was also present in PDYN KO mice responding for standard normal pellets. Surprisingly, under the progressive ratio schedule of reinforcement, the genotype difference in PDYN KO and WT mice was lost. The PDYN KO mice, particularly females, sustained the higher responding rate for palatable chocolate-flavored pellets over normal pellets. PDYN KO females also

displayed higher number breakpoint responding than the male counterparts, suggesting that female mice are more vulnerable to hedonic signaling mechanisms than male mice. Dynorphin has been known to decrease the rewarding properties of drugs by lowering dopamine levels in the NAc (Di Chiara & Imperato, 1988; Rainer Spanagel, Herz, & Shippenberg, 1990). This suggests that knockout of dynorphin would result in increased DA levels and increased motivation for food rewards in PDYN KO compared to WT, as hypothesized in the present study. However, contrary to the proposed hypothesis, the results showed decreased motivation in PDYN KO mice during the operant nose-poke task for palatable food rewards. The plausible reason for the decreased motivation in the PDYN KO could be that the deletion of dynorphin resulted in a compensatory increase in the number of endogenous KOR. Hence, in the absence of dynorphin, endogenous peptides such as met-enkephalin or ß- endorphin activate the KOR, resulting in the decrease of dopamine levels in the rewarding circuit. This might have resulted in the decreased motivation in the knockout animals. Supporting the present results, an earlier study in PDYN KO mice also demonstrated a strong reduction in the preference for saccharin compared to control mice. On the contrary, PDYN KO male mice showed enhanced ethanol preference in a two-bottled paradigm test (Blednov, Walker, Martinez, & Harris, 2006; Rácz et al., 2012). Together these data suggest that dynorphin plays a critical role in the differential modulation in reward-related behavior, however, depending upon the type of reward.

Lastly, under the progressive ratio schedule, PDYN KO mice did not differ than their controls in their motivational responses for obtaining the highly palatable food rewards. Therefore a genotype effect was missing. A prominent sex effect was observed under the progressive ratio schedule, where the PDYN KO females had higher number of operant responses compared to their male counterparts. Studies on operant-self administration of ethanol consumption also show difference in the responses in males and females in C57Bl/6 mice (Hayward et al., 2004; Middaugh & Kelley, 1999). Therefore, we can conclude that endogenous dynorphin is involved, but is not critically essential in the modulation of hedonic control of feeding behavior.

Assessment of the PENK KO mice in the operant conditioning paradigm, showed a considerable increase in the motivation to obtain palatable food rewards

compared to the PENK floxed control mice. A tendency of increased motivational behavior responses was noted, especially in female PENK KO mice, during the later FR schedules i.e. FR3 and FR5, respectively. PENK floxed mice used in the study are known to have reduced levels of PENK mRNA compared to WT littermates (Britta Schürmann PhD thesis). Therefore, this could be one of the possible explanations for the absence of a genotype effect in the operant assessed modulation of the hedonic control of palatable food rewards. Furthermore, under progressive ratio schedule, the tendency of genotype difference that was observed during the fixed ratio schedules disappeared. Both, PENK KO male and female mice showed similar motivational responses for the highly palatable food rewards. The results from the PENK KO experiment as well contradicted the proposed hypothesis of the study.

These outcomes from the PDYN KO and PENK KO mice subjected to operant conditioning confirm the paradoxical finding to the proposed hypothesis. Both, PDYN KO and PENK KO mice failed to increase and decrease the motivation for palatable reward intake, respectively as compared to their controls. Furthermore, control animals, WT and PENK floxed mice showed a similar phenotypic behavior in the preference for palatable food rewards as the opioid peptide deficient mouse models. While the current data does not necessarily contradict the extensive pharmacological research findings suggesting opioids modulate the hedonic behavior, they do support a conclusion that neither of these peptides is essential in the operant self-administration behavior of palatable food intake. A possible explanation for the lack of any dramatic effect might be that other non-opioid pathways have compensated for the lack of enkephalin and dynorphin, resulting in the unpredictable phenotype.

In summary, the results present here support the hypothesis that the endogenous opioids can modulate palatable food intake, however, are not absolutely necessary for the hedonic aspect of feeding behavior. In line with the current results, opioid peptide knockout studies have previously showed that enkephalin and dynorphin modulate sucrose preference but are not necessary to support sucrose consumption (Hayward et al., 2006), therefore suggesting that these endogenous opioids may contribute to food and drug reinforcement via different neuronal pathways.

4.3 Effects of diet in feeding regimen in PDYN KO mice

In second part of this study based on metabolic changes induced by feeding behavior, PDYN KO and WT control animals were exposed to HFD or ND, under two different feeding regimens, TR or AL. AL feeding resulted in a more pronounced increase in body weight than TR feeding, although animals on AL consumed less food. The difference in body weight was particularly prominent in animals that were maintained on a HFD. PDYN KO mice were significantly heavier that wild type controls. This resulted in a weight pattern ranging from low to high in the following order WT TR, KO TR, WT AL and KO AL, respectively. This pattern was inversely correlated to the level of hypothalamic NPY expression. Differences between the sexes were observed.

The body weight data revealed that the constitutive deletion of dynorphin has a prominent impact on the body weight when maintained on a HFD. The ability of HFD formulation can increase body weight and disrupt cognition is linked to brain inflammation(Bruce-Keller et al., 2011). The exact mechanism how obesity detrimentally affects health is still unclear, and deregulation in metabolism is one of the key physiological factors of obesity (Pistell et al., 2010). The current results contradict the findings of a previous study, which found neither an effect of diet composition nor the dynorphin deletion on body weight (Sainsbury et al., 2010). These discrepancies may be due to the differences in HFD composition (46% fat, 4.72 kcal/g versus 59% fat, 5.49 kcal/g) and the age of animals (7 weeks versus 12 weeks). The body weight gain of the mice were in good agreement with other studies, where animals were exposed to similar diets and feeding conditions (Hatori et al., 2012). In line with the current data from food intake, previous studies on mice maintained on a high fat-diet during TR feeding also showed that animals consumed the same amount of calories as AL-fed animals, protecting them from weight gain and obesity. It is important to note that feeding episodes rather than the size of the meal, or total energy intake contributes to weight gain and development of obesity (Chaix, Zarrinpar, Miu, & Panda, 2014; Hatori et al., 2012; Murphy & Mercer, 2014). In agreement with this theory, the results showed that mice maintained on TR feeding regimen displayed lower body weights as compared to the AL feeding group. Furthermore, this effect was observed in all

animals irrespective of the type of diet consumed. However, in case of PDYN KO animals on HFD, the TR feeding groups had significantly higher body weights compared to WT controls. This implies that the loss of dynorphin contributes to a deregulation in metabolism inducing disturbed weight gain. Therefore, dynorphin peptide is a crucial factor involved in the regulation of the body weight of animals, when subjected to similar feeding conditions with a normal as well as with a high caloric diet.

Overweight human individuals restricted to eat only within a self-selected 10–11 hour period on every other day showed outcomes where participants lost 4% of body weight in 16 weeks and retained this weight loss for up to 1 year (Gill & Panda, 2015). During 6-month intermittent feeding, overweight human participants demonstrated effects such as lost abdominal fat, improved insulin sensitivity, as well as reduced blood pressure (Harvie et al., 2011). Interestingly, in normal weight men and women, three weeks of alternate day fasting also resulted in are similar to outcomes as animal studies showing that intermittent feeding can improve glucose metabolism even with little or no weight change (Anson et al., 2003; Halberg et al., 2005). Therefore, time-restricted feeding seems to be a promising strategy in regulation of body weight and metabolism.

As the time of accessibility of food has a direct effect on body weight regulation, in the present study, this effect was seen to be independent on the type of diet consumed. Nocturnal rodents, such as mice and rats feed primarily during the dark hours and characteristically eat their largest meal shortly after the dark phase (lights off) initiates (Erickson, Clegg, & Palmiter, 1996; Green, Wilkinson, & Woods, 1992) To distinguish between consumption and response to a palatable stimulus, mice are usually provided with limited access to a highly palatable stimulus at a time different than their normal consumption period – e.g., during their active phase (lights on). This approach has been used for comparison of palatable responses in wild type and mutant mice (Blednov et al., 2006; Sindelar, Palmiter, Woods, & Schwartz, 2005).

The effect of HFD consumption on the levels of blood glucose in PDYN KO female mice showed significant elevation as compared to the WT controls after 12 weeks, whereas in males similar levels were found in all feeding groups. In general, we also observed significantly higher body weights in PDYN KO male mice compared

to female mice, in the HFD feeding regimen. Furthermore, studies on sex-specific effects in body weight regulation have reported that HFD-fed males were more susceptible to weight gain compared to females, with similar energy intake. These alterations in body weight gain could either be a cause of a reduced metabolism or anti-obese effects of female estrogen that is mediated through the estrogen receptor α (Gao et al., 2007; Hwang et al., 2010; Winzell & Ahre, 2004).

It was observed that the feeding regimen or the timing of meal consumption had a crucial impact on the average food intake in mice. In general, animals fed on a TR feeding regimen devoured similar amount of food as mice on AL access. As this observation was made in all mice irrespective of the genotype, we can say that the constitutive absence of dynorphin does not play a role in the alteration of feeding behavior during different time schedules. The possible explanation for the higher food intake in TR groups is the binge-eating effect, which is an addiction-like phenomenon characterized by excessive food consumption within discrete periods of time (Avena, Rada, & Hoebel, 2008; Blasio, Angelo, Steardo, Luca, Sabino, Valentina, and Cottone, 2012; Corwin, 2008). The motivation to obtain highly-palatable food increases during dietary restriction (Rossetti, Spena, Halfon, & Boutrel, 2014). Similar observations have been made in rodent models where shifting the normal feeding time de-synchronizes circadian rhythms and results in metabolic disorders and weight gain (Arble, Ramsey, & Ph, 2011). Human studies suggest that meal timings influence energy homeostasis and circadian-driven behavior (Murphy & Mercer, 2014). To conclude, lack of dynorphin causes a dysregulation of body weight and food intake in animals when maintained on a high-fat diet. These outcomes suggest that dynorphin modulates metabolic changes associated with diet composition and feeding regimen.

4.4 Hypothalamic peptides in feeding behavior

The endogenous opioid system is involved in the homeostatic control mechanism by regulating the release of orexigenic hypothalamic peptides such as NPY and orexin-A. The activities of these hypothalamic peptides are dependent on opioid signaling, and it is known that dynorphin and NPY are important players in modulation of energy homeostasis and neuroendocrine regulation. Previous

research has shown that intracerebrovascular injections of NPY or dynorphin results in marked increase in food intake in fasted animals, demonstrating relatable role in central control of feeding behavior. Consequently, NPY produces significant effects on behavior and other functions, its most noticeable effect is the stimulation of feeding after central administration, and therefore, it is mainly involved in the regulation of feeding (Wisialowski et al., 2010).

The results from the present study showed that NPY expression levels in the ARC region of mice maintained on AL food were prominently lower than TR feeding. This outcome was observed in all PDYN KO animals, irrespective of the diet type. Hence, diet did not play a direct role in the hypothalamic peptide expression levels. Therefore, the data suggests that the feeding regimen, especially the time of food consumption, plays a crucial role in the regulation of NPY levels. The results are in line with a study that demonstrated the co-localization of NPY and dynorphin mRNA with a downregulation of NPY mRNA in PDYN KO mice (Lin et al., 2006). Neurons expressing the orexin-A peptide are well known to populate the lateral parts of the hypothalamus (Date et al., 1999; Yamamoto, Ueta, Date, Nakazato, & Hara, 1999), Furthermore, orexigenic neurons in the lateral hypothalamus play a role in arousal, reward-seeking and feeding behavior (Harris et al., 2005). Therefore, orexin peptides are considered as appetite-stimulating neuropeptides that regulate body weight homeostasis (Sakurai, 2014). The results from immunostaining showed that orexin-A peptide levels in PDYN KO mice during the AL feeding regimen were significantly lower compared to the TR feeding group. These results are similar to the NPY outcome, indicating that both orexigenic peptides show upregulation in TR-fed mice. Therefore, the impact of TR feeding regimen is prominent in case of modulation of hypothalamic orexigenic peptides as well.

In summary, this work has revealed for the first time a significant role of endogenous dynorphin in modulation of metabolic changes occurring after a prolonged consumption of different diets, under different feeding considering both the male and female sexes of mice. Comparison of these several factors at once in PDYN KO and WT mice was crucial to elucidate and specifically identify the main components influencing the alteration of diet-induced metabolism.

5 Conclusion

The endogenous opioid system is known to be involved in the modulation of hedonic and homeostatic control of feeding behaviors. So far, several studies have elucidated the involvement of the opioid signaling in the brain reward circuitry in the context of natural reward and feeding behaviors. Most evidence implicating endogenous opioids is based on studies using pharmacological or genetic knockout of opioid receptors, which alters feeding behaviors. However, individual contribution of each opioid peptide in feeding behavior and metabolism is not entirely clear. With the use of genetically modified mouse models of endogenous peptides, the present study assessed the impact of palatable-reward induced feeding behavior as well as metabolic changes after exposure of the animals to different diet conditions.

This study aimed to distinguish the role of the two major classes of endogenous opioids, namely enkephalin and dynorphin, in the motivational aspect of feeding behavior. Prodynorphin knockout and proenkephalin knockout mice were presented with a highly palatable chocolate-flavored pellet or a normal pellet and tested in an operant-self administration paradigm. It was hypothesized that the knockout of dynorphin will increase the rewarding behavior in mice towards high-palatable food, whereas, knockout of enkephalin will decrease the rewarding behavior. The proenkephalin knockout and prodynorphin mice showed a higher preference for the palatable chocolate-flavored pellets compared to the normal food pellets. However, no significant genotype effect was absent under the progressive ratio schedule assessment. These findings suggest that the endogenous opioids enkephalin and dynorphin are involved in the modulation of the reward-induced motivation but play no essential role in the hedonic aspects of feeding behavior.

The present findings evidently support the assumption that a dysregulation in the endogenous opioid system causes modulation in the reward mechanism in mice. Deficiency of dynorphin seems to negatively affects the body weight and metabolism, after high caloric diet consumption. Prodynorphin deficient and wild type mice were maintained for twelve weeks on a high-fat diet or a normal chow

with either a time-restricted access or an *ad libitum* access to food. The results show that prodynorphin deficient mice gained significantly higher body weight compared to the wild-type controls, and this effect was prominently observed in male mice. Food intake in both genotypes was found to be similar. Furthermore, an immunohistochemical analysis of hypothalamic peptides involved in homeostatic feeding behavior, demonstrated that prodynorphin deficient animals displayed significantly reduced levels of hypothalamic orexigenic peptides NPY and orexin-A under different feeding regimens. Furthermore, prodynorphin knockout mice maintained on an *ad libitum* high-fat diet feeding had lower levels of orexin-A expressions compared to wild-type controls. These results suggest that the modulation in hypothalamic peptide expression is an effect of the feeding regimen, than the effect of diet itself.

Taken together, the results from the study suggest that endogenous opioid peptide dynorphin not only contributes to the modulation motivated-feeding behavior but also is crucial in the homeostatic control of feeding behavior.

6 Appendix

6.1 List of figures

6.2 List of tables

7 References

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