UNIVERSITÀ DI PARMA

UNIVERSITÀ DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN
"NEUROSCIENZE"

CICLO XXXI

HISTOLOGICAL AND BEHAVIOURAL STUDY OF GPR83 KO MICE

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Anni 2015/2018

INDEX

ABBREVIATIONS

7-TM	Seven-Transmembrane
ChAT	Choline Acetyltransferase
AD	Alzheimer's Disease
AgRP	Agouti-Related Protein
ANOVA	Analysis of Variance
AP	Anteroposterior
cAMP	Cyclic Adenosine Monophosphate
CHO	Chinese Hamster Ovary
CPE	Carboxy-Peptidase E
CPu	Caudate-Putamen
DAT	Dopamine Transporter
DV	Dorsoventral
eNDo	Extracellular N-Terminal Domain
EPM	Elevated Plus Maze

ERK1/2	Extracellular Signal–Regulated Kinases 1 And 2
FR	Fixed Ratio
GH	Growth Hormone
GIR	Glucocorticoid-Induced Receptor
GPCR	G Protein Coupled Receptor
HEK	Human Embryonic Kidney
HPA	Hypothalamic-Pituitary-Adrenal Axis
hPEN	human PEN
IP3	Inositol Trisphosphate 3
Kd	Dissociation Constant
KO	Knock Out
LM	Lateromedial
LP	Lever Pressing
MAPK	Mitogen-activated protein kinase
mPEN	mouse PEN
NAc	Nucleus Accumbens

NET	Noradrenaline Transporter
NPY	Neuropeptide Y
PC	Prohormone Convertase
PLC	Phospholipase C
POA	Preoptic Area
POMC	Proopiomelanocortin
PP	Pancreatic Polypeptide
PR	Progressive Ratio
qRTPCR	quantitative Real-Time PCR
RIA	Radioimmunoassay
RP	Repeated Measure
rPEN	rat PEN
sEPSC	spontaneous Excitatory Postsynaptic Current
SERT	Serotonin Transporter
SPT	Sucrose Preference Test
TAN	Tonically Active Neuron

VTA	Ventral Tegmental Area
WSN	Warm Sensitive Neurons
WT	Wild Type

INTRODUCTION

The discovery of a new orphan GPCR: the GPR83

GPCRs: an overview

G protein-coupled receptors (GPCRs), also known as seven-transmembrane (7-TM) receptors, are the largest superfamily and most diverse group of mammalian transmembrane proteins. All GPCRs consist of 7-TM domains, connected by extracellular and intracellular loops (Fig. 1).

They are involved in different functions and mediate most cellular responses by binding different ligands, including hormones, neurotransmitters and neuromodulators. For these reasons, GPCRs are responsible for a variety of physiological functions and pathologies as well as targets for therapeutic approaches. Approximately 36% of currently marketed drugs target human GPCRs (Drews 2000) (Rask-Andersen, Almén, and Schiöth 2011) (Hopkins and Groom 2002).

GPCRs can be grouped into five main families on the basis of sequence, structural similarity and phylogenetic criteria: rhodopsin (family A), secretin (family B), glutamate (family C), adhesion and Frizzled/Taste 2. The families can be subdivided into groups on the basis of ligands that the receptor recognizes (Rosenbaum, Rasmussen, and Kobilka 2009).

For many of the receptors identified in the human genome, the biological function is still unclear and for this reason they are called “orphan GPCRs”. In order to understand the biological function of orphan receptors, there has been a large effort to identify endogenous receptor ligands and some of these researches have been successful (Civelli et al. 2013).

It is estimated that of the ~720 genes that encode GPCRs in the human genome, 150 genes encode orphan receptors. This number decreases with time because the “deorphanization” has become a challenge for many research groups (Wise, Jupe, and Rees 2004).

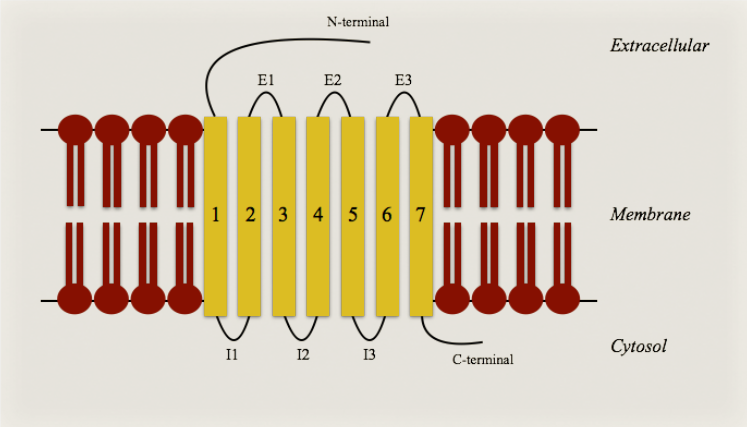

Fig. 1. *GPCR structure with the 7-TM domains. E, extracellular loop; I, intracellular loop.*

Discovery of GPR83

GPR83, also known as JP05, GPCR 72 or glucocorticoid-induced receptor (GIR), is an orphan GPCR belonging to the rhodopsin-like class A family (Alexander, Mathie, and Peters 2011).

In 1991 Harrigan and colleagues identified the GPR83 cDNA sequence in murine T-lymphocytes. They isolated four different isoforms, that arise from alternative splicing. The most abundant form of GPR83 mRNA was the isoform-1 which encodes a protein of 423 amino acids with a signal peptide of 17 amino acids. No known function has been attributed to isoform-2 and -3, whereas isoform-4 was shown to be involved in the expression of regulatory T-cells.

The receptor was categorized as a GPCR considering the presence of seven hydrophobic regions that they found in this study and the similarity to the GPCR class A family, in particular to the neuropeptide Y (NPY) Y2 receptor, and the tachykinin receptors NK-1, NK-2 and NK-3. High levels of GPR83 mRNA after stimulation with glucocorticoids or forskolin were found in the murine T-cell line WEHI-7TG and normal thymocytes, and for this reason it was identified as a stress-responsive transcript (Harrigan, Campbell, and Bourgeois 1991).

The DNA analyses in humans showed a 85% homology with mouse GPR83, and also an overall identity of 36% to the human Y2 receptor subtype. The chromosomal localization of the human GPR83 was found in chromosome 11q21.1. In the mouse the mapping results indicated that GPR83 is located in the proximal region of chromosome 9 (Parker et al. 2000).

The seven putative transmembrane alpha-helices consist of a continuous stretch of 17-23 uncharged amino acid residues, except transmembrane domains II, III and IV that contain Asp116, His155, Asp167

and His203 respectively. In the hydrophobic N-terminal region there is a potential signal sequence and signal peptidase cleavage sites located at Val115 and Ala17.
The rat GPR83 shares very high sequence identity with mouse (97%) and human (88%) GPR83. The rat GPR83 has several structural characteristics common to the members of the GPCR superfamily including four potential N-linked glycosylation sites, three of them located in the N-terminus and one in the first extracellular loop. The third intracellular loop and C terminus present sites for possible phosphorylation by protein kinase A and C. In comparison with the mouse GPR83, the rat GPR83 has a deletion involving one amino acid residue at position 40 in the N-terminus (Wang et al. 2001).

After the discovery of GPR83 as a new orphan GPCR, the researchers showed its localization also in brain regions such as the hypothalamus, amygdala, hippocampus and striatum. More recently, various works identified the specific ligand of GPR83, the PEN peptide which originates from proSAAS pro-peptide.

Distribution of GPR83 mRNA in the central nervous system

GPR83 mRNA was assayed in several rodent and human brain regions (Brézillon et al. 2001a).
In mouse brain, the most intense labeling, analyzed with in situ hybridization, was found in limbic cortex (cingulate, retrosplenial and entorhinal), paleocortex (piriform) and archicortex (subiculum). Positive cells were found also in the dorsal and ventral septal nuclei.
A strong labelling was found in ventral striatum, including nucleus accumbens (NAc) and the olfactory tubercle. The dorsal striatum as well contained a considerable population of neurons with high levels of GPR83 mRNA.
Within the hypothalamus, the highest densities of positive cells were found in the mammillary body, and another group of positive cells was localized in the arcuate nucleus. Lower densities of labelled cells were found in the ventromedial hypothalamic nucleus and in the preoptic area (POA).
Strong signal was found also in limbic areas, such as the hippocampus and basolateral amygdala.
In the mesencephalon, high levels of GPR83 mRNA were seen in the Edinger-Westphal nucleus and in the ventral tegmental area (VTA). A considerable portion of neurons appeared to be positive also in the nucleus ruber, magnocellular part, in the parvocellular reticular nucleus and, with a low intensity, in the paramedian raphe nucleus.

Several well-defined groups of positive cells were found in the rhombencephalon, within the laterodorsal tegmental nucleus and in the ventrolateral part of the gigantocellular reticular nucleus (Pesini et al. 1998) (Fig. 2)

A similar pattern of GPR83 expression was found in rat and human brain, with an important difference in the striatum and in the thalamic nuclei.

Within ventral striatum in rat and human brain the expression was limited to scattered cells located throughout the rostrocaudal extent of these structures.

Thalamic region of mouse brain did not express significant levels of GPR83, while in rat and human this brain area contains a high number of positive cells (Sah et al. 2005) (Brézillon et al. 2001a).

These differences in term of GPR83 mRNA expression may derive from species-specific differences in the regulation and structure of GPR83 gene; in fact, splice variant forms of GPR83 have been reported in the mouse (De Moerlooze et al. 2000) (Kawasawa et al. 2003).

GPR83 mRNA distribution in the brain indicates a potential role of this receptor in the control of feeding and metabolism, regulation of stress and emotional behavior, learning and memory, and drug reinforcement and reward.

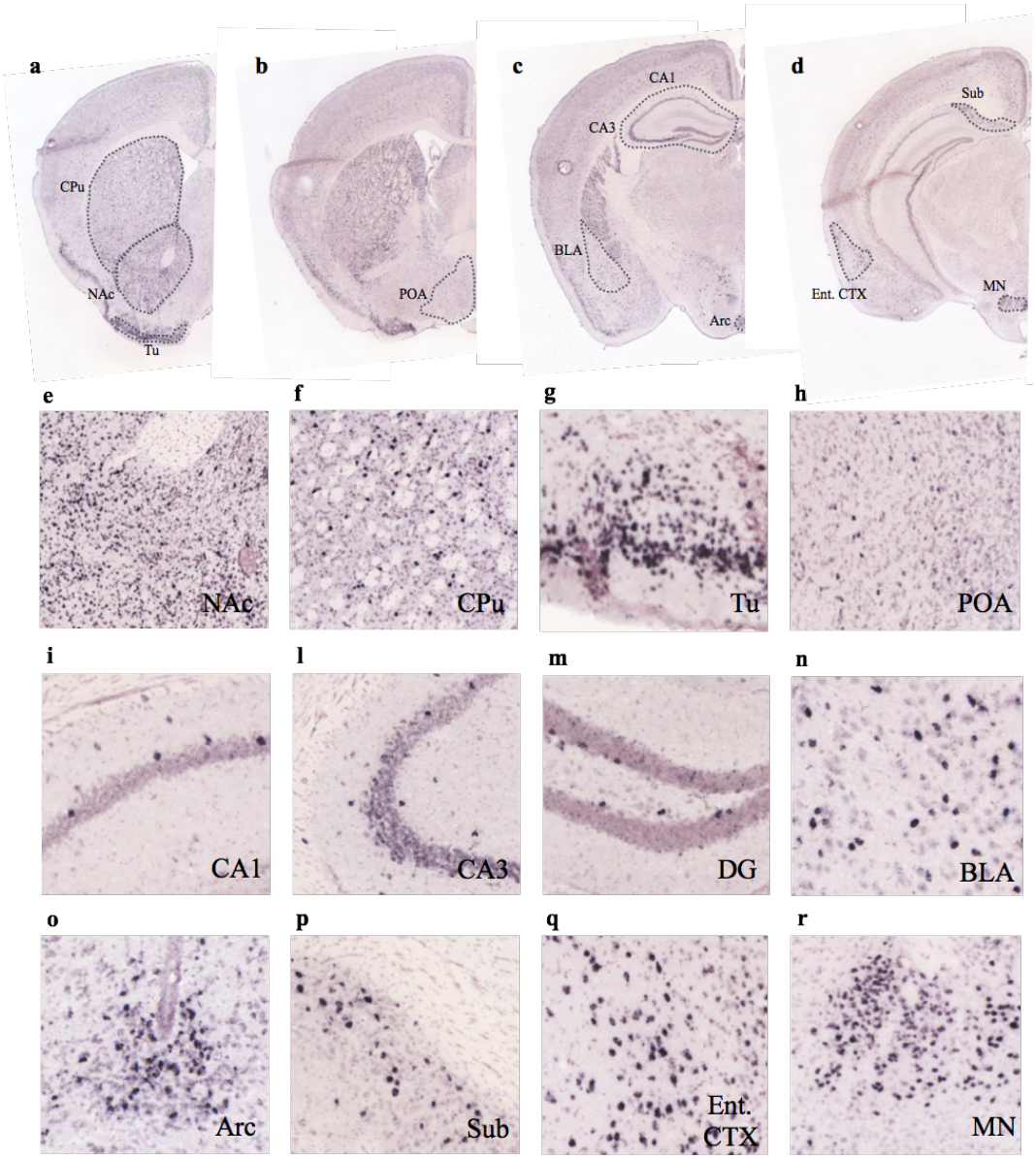

Fig. 2. *GPR83 mRNA expression from the Allen Mouse Brain Atlas. Images were obtained at http://mouse.brain-map.org/experiment/show?id=72338696. (a-d) Images from whole mouse brains probed for GPR83. Larger images of specific subregions are displayed in (e-r). NAc, nucleus accumbens; CPu, caudate-putamen; Tu, olfactory tubercle; POA, preoptic area; CA1, cornu ammonis field 1; CA3, cornu ammonis field 3; DG, dentate gyrus; BLA, basolateral amygdala; Arc, arcuate nucleus; Sub, subiculum; Ent. CTX, entorhinal cortex; MN, mammillary nuclei.*

Signal mechanisms of GPR83

GPCRs convey the majority (80%) of signal transduction across cell membranes (Millar and Newton 2010).

Recent studies aimed to establish new molecular details of mouse GPR83 (mGPR83) signaling in order to contribute further insights into the physiological and pharmacological characteristics of the receptor.

It was identified a constitutive activity of GPR83, transiently expressed in chinese hamster ovary (CHO) -K1 cells, measured by luciferase reporter assay; the receptor exhibited significant constitutive inhibition of CRE-mediated gene expression under both baseline and forskolin stimulated conditions (Martin, Steurer, and Aronstam 2015).

mGPR83 is characterized by a basal level of Gq/11 activity that mediates inositol trisphosphate 3 (IP_3) synthesis (Anne Müller et al. 2013).

Deletion constructs of the extracellular N-terminal domain (eNDo) were designed and functionally characterized in order to explore its contribution to GPR83 signaling. Interestingly, the entire deletion of eNDO induces a constitutive activity of Gq/11-signaling by GPR83, but Gs, Gi or G12/13-related pathways or MAPK-signaling were not activated. On the basis of these results, the N-terminus of GPR83 seems to be involved in the stabilization of an inactive receptor conformation and thus serves as an intramolecular inverse agonist. The other two shorter deletions analyzed showed only a weak activity or a lack of constitutive activity (Müller et al. 2014)(Anne Müller et al. 2013).

In addition, the same Authors found similarity in hGPR83 which exhibits a high basal activity for IP3 formation, most likely mediated by Gq/11 activation (Müller et al. 2016).

Regulation of GPR83 expression in brain

Since the discovery of GPR83 it has been demonstrated that the expression of this orphan receptor can be modulated by different factors, suggesting a regulation of GPR83 by stress, reward, energy metabolism and learning.

The regulation of GPR83 expression under stress was demonstrated after exposure to dexamethasone. An acute treatment of this glucocorticoid agonist decreases GPR83 expression in mouse ventral and dorsal striatum, hippocampus and different hypothalamic nuclei (Adams et al. 2003). Dexamethasone is implied in the activation of hypothalamic-pituitary-adrenal axis (HPA); this could mean that GPR83 plays a role in stress response.

Consistent with the expression of GPR83 in the hypothalamus, the role in energy metabolism was demonstrated as GPR83 levels increase after feeding and decrease after fasting (Timo D. Müller et al. 2013).

As concerns reward, chronic amphetamine treatment induced the expression of GPR83 in prefrontal cortex of rat brain. This increase persists for at least 7 days after amphetamine withdrawal, suggesting that GPR83 expression may be involved in the stable neuroadaptation to drugs of abuse (Wang et al. 2001).

Recently, it was also found an increase of GPR83 in hippocampal cultures after exposure to dopamine (Galloway et al. 2018). These results suggested that GPR83 may modulate the interaction between reward and learning. To confirm a role in learning, it was shown that depletion of the long chain fatty acid docosahexaenoic acid, which results in a decrease in the rate of learning of an olfactory discrimination task, decreases GPR83 expression in the olfactory bulb (Hichami et al. 2007).

These results lead the researchers to explore the physiological and behavioral role of GPR83.

Potential physiological roles of GPR83

Recently, different Authors published works to define the physiological roles of GPR83. The localization in the brain and the regulation of the expression of GPR83 mRNA suggest an hypothetical role of GPR83 in feeding behavior, locomotion, reward, memory and learning.

GPR83 is widely distributed in hypothalamic nuclei governing energy metabolism. Specifically, a colocalization between GPR83, ghrelin receptor (Ghsr1a) and agouti-related protein (AgRP) was found in the arcuate nucleus. Ghrelin, known as the “hunger hormone”, is an hormone involved in food intake, fat deposition but also glucose and energy homeostasis (Chacko et al. 2012) (Sato et al. 2012) (Zakhari et al., 2012) (Brézillon et al., 2001). In vitro analysis with GPR83 and the ghrelin receptor Ghsr1a showed an interaction between these receptors, and more specifically a heterodimerization which could reduce the activation of Ghsr1a and so the function of ghrelin. To confirm the role of GPR83 in metabolism, the same authors analyzed the metabolic phenotype of GPR83 KO mice. These mice have normal body weight, food intake, glucose tolerance and insulin sensitivity when fed a regular chow diet. However, KO mice showed a decrease of body fat mass and plasma leptin levels. For these reasons, it has been proposed that GPR83 under normal feeding condition may be involved in regulating lipolysis.

The functional interaction of GPR83 and Ghsr1a is supported by in vivo analysis. Acute and chronic treatment with ghrelin in GPR83 KO mice showed an increase of food intake and a similar response in ghrelin-induced growth hormone (GH) secretion compared with wild type (WT) mice (Müller et al. 2013).
The warm sensitive neurons (WSN) of the preoptic area of the anterior hypothalamus (POA), which express GPR83, are important regulators of temperature (Eberwine and Bartfai 2011). A shRNA viral mediated knockdown of GPR83 in POA significantly reduced core body temperature during the dark cycle of the day but not during the light cycle, and increased body weight in mice with no change in food intake (Dubins et al. 2012).
Taken together these data confirm the role of GPR83 in hypothalamic functions.

To characterize the involvement of GPR83 in stress responses, Vollmer and colleagues found a behavioral insensitivity to stress in GPR83 KO mice. Effects of GPR83 KO on anxiety-like behaviors and motor activity were not observed in the absence of prior stress exposure. These results suggested that GPR83 is not recruited in emotional behaviors under basal no-stress conditions. In addition, the involvement of GPR83 in stress is not under control of the HPA axis, since the plasma levels of corticosterone did not change in the transgenic mice following restraint test (Vollmer et al., 2013).

GPR83 is highly expressed in areas such as hippocampus, prefrontal cortex and amygdala, which are important for leaning. In Morris water maze, a test for spatial learning and memory in rodents, GPR83 KO mice showed a different acquisition curve during the training, but no difference in the latency to reach the platform in the testing days. Additionally, in a contextual fear conditioning paradigm GPR83 was not necessary in the fear-associated response (Vollmer et al., 2013). These results could suggest the absence of severe learning deficits in these mice.

Anhedonia is the decreased ability to experience pleasure and represents one of the symptoms of depression (Hamilton 1967) (Klein 1974). The sucrose preference test (SPT) is a reward-based test, used as indicator of anhedonia. Rodents are normally attracted by sweet foods or solutions and the reduced preference for sweet solution in SPT represents anhedonia and possibly depression (Katz 1981) (Willner et al. 1987). GPR83 KO mice showed a stronger preference for a 1% sucrose solution suggesting an increased basal sensitivity to hedonic rewards (Vollmer et al., 2013). The primary targets of the mesolimbic and nigrostriatal pathways are NAc, olfactory tubercle and extended amygdala, i.e., the

anatomical continuation of the ventral striatum into the amygdala. These are areas with a high expression of GPR83 (Pesini et al. 1998), implying a potential association of GPR83 with hedonic and reward-related behavior.

The deorphanization of GPR83

Endogenous ligands of GPR83

GPR83 shows high homology to the NPY Y_2 receptor (38-40%), with higher identity in TM domains 5, 6 and 7 (50-55%), which are important for binding of agonists and antagonists (Harrigan, Campbell, and Bourgeois 1991). This similarity between GPR83 and NPY Y_2-receptor sequence and the presence of Y_2 specific residues in GPR83 led Sah et al. to hypothesize that NPY ligands, particularly the Y_2 selective compounds, may interact with GPR83.

Competition experiments with various NPY ligands revealed a preference of rat GPR83 (rGPR83) for NPY, PYY_{3-36} and C-terminus fragments of NPY. Additionally, the poor affinity of Y_1, Y_4, Y_5 selective compounds, and pancreatic polypeptide (PP) for rGPR83, further supports the "Y_2-like" nature of rGPR83. Similar to what has been observed in the binding assay, an activation of GTP was shown after binding by NPY, PYY or their C-terminus fragments. On the other hand, Y_1, Y_4 and Y_5 preferring compounds did not show significant GTP-γS binding to rGPR83 (Sah et al. 2007).

The question arises whether NPY can bind rGPR83 under physiological conditions; in fact, a physiological interaction between NPY and rGPR83 appears possible based on the availability of sufficient phasic NPY levels.

For these reasons GPR83 can be classified as a distinct "Y_2-like" receptor that shows preference for C-terminus fragments of NPY.

Zinc(II) ions seem to be also important for the signal activity of GPR83. Zinc(II) ions are stored in glutamatergic synaptic vesicles and are co-released with neurotransmitters into the synaptic cleft where Zinc(II) concentrations up to 300 μM can be obtained (Assaf and Chung 1984). Different studies show how Zinc(II) ions could activate (Rosenkilde et al. 1998)(Swaminath, Lee, and Kobilka 2003) or inhibit the GPCRs (Schetz, Chu, and Sibley 1999). GPR83 is activated by 100 nM Zinc(II) and 100 μM Zinc(II), suggesting two binding sites for the Zinc(II) ions (Müller et al. 2013).

After almost 10 years new binding studies identified a different short neuropeptide, called PEN, as specific ligand of GPR83.

Discovery of proSAAS

ProSAAS is a neuropeptide expressed in neurons throughout the brain at high levels (Lanoue and Day 2001)(Morgan et al. 2005). Neuropeptides play important roles in cell-cell signaling, and many neuropeptide receptors are potential therapeutic targets.

The precursor protein proSAAS produces the peptides SAAS, LEN and PEN which are so named because of the presence or these amino acids in their sequences; they are among the most abundant peptides present in mouse hypothalamus (Fricker et al. 2000). It has been identified only a single PEN peptide, whereas peptides containing the SAAS and LEN sequences are produces as big and little (longer and shorter) peptides. mPEN and rPEN only differ by one residue at the N-terminal end, whereas human PEN (hPEN) is more divergent and has the sequence PEG instead of PEN (Fig. 3). The cleavage of proSAAS into SAAS, PEN, LEN (bigLEN and littleLEN) and other peptides is under the control of prohormone convertases (PC) and carboxy-peptidase E (CPE), which are the same enzymes that produce most neuropeptides (Wardman et al. 2010). The peptides derived from proSAAS are differentially sorted to distinct vesicular populations (Wardman and Fricker 2014), suggesting that these vesicles could release the peptides in distinct brain regions with different physiological activities.

Furthermore, differential cleavage by various peptidases leads to a range of big and little forms of the proSAAS-derived peptides, many of which may be functional neuropeptides (Fricker 2012)(Mzhavia et al. 2001).

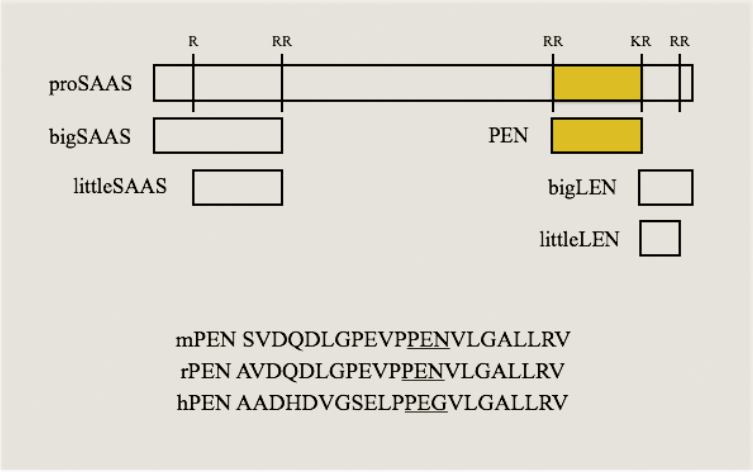

Fig. 3. *Schematic of proSAAS and proSAAS-derived peptides and PEN sequences of mouse (mPEN), rat (rPEN) and human (hPEN).*

ProSAAS was identified for the first time in mice lacking CPE activity (Cpe^{fat}/Cpe^{fat}).

CPE, the major peptide processing carboxypeptidase, is an enzyme found in all neuroendocrine tissues and cleaves many C-terminally extended peptides to generate the mature bioactive forms (Fricker 1988). The Cpe^{fat} mutation, called fat mutation, is a spontaneous point mutation in CPE that causes the peptidase to be inactive (Naggert et al. 1995). Cpe^{fat}/Cpe^{fat} mice show an accumulation of the peptide containing basic residues on their C-terminal and a reduction in levels of fully processed peptides (Fricker and Leiter 1999). An affinity chromatography technique was used to isolate these neuropeptide intermediates, and identified intermediates of many previously characterized neuroendocrine peptides as well the proSAAS peptides (Fricker et al. 2000).

The enzymes involved in the cleavage of proSAAS can be subdivided into Golgi and secretory vesicle enzymes. The cleavage in the Golgi apparatus by furin and carboxypeptidase D, generates big SAAS, an intermediate peptide representing GAV and the mid portion of proSAAS, and PEN-LEN. Furin can also cleavage PEN-LEN into PEN and bigLEN. Within secretory vesicles the cleavage is under control of PC1/3 and PC2, and CPE, generating KEP, little SAAS, PEN, bigLEN and littleLEN. In mouse brain lower levels of smaller forms of little SAAS, GAV and PEN are also present (Fricker and Leiter 1999).

ProSAAS has some characteristics in common with the granin family, including chromogranin A and B, secretogranin II and 7B2 (Ozawa and Takata 1995) (Fortenberry et al. 2002).

It was demonstrated that this neuropeptide has an inhibitory activity against proprotein convertase 1 (PC1) (Fricker et al. 2000) (Lee, Prodhomme, and Lindberg 2004); in fact, proSAAS distribution is similar to PC1 (Seidah et al. 1991).

The region of proSAAS involved in the inhibitory activity was identified near the junction of PEN and LEN; while the fully processed form of PEN is not inhibitory, but only the Lys-Arg extended PEN peptide inhibits PC1 activity (Qian et al. 2000)(Cameron, Fortenberry, and Lindberg 2000).

Consistent with this, Cpe^{fat}/Cpe^{fat} mice showed an accumulation of PEN-Lys-Arg (Fricker et al. 2000) and maturation and activity of PC1 are decreased in this animal model (Berman et al. 2001).

In the mouse brain substantial amounts of little SAAS, PEN and big LEN, but not big SAAS, big PEN/LEN or larger forms of these peptides were found. The finding that the major forms in WT mouse brain and pituitary are the smaller non-inhibitory forms suggests that PC1 is not tonically inhibited by the proSAAS peptides (Mzhavia et al. 2001).

ProSAAS distribution in the brain and physiological function

Semi quantitative in situ hybridization analysis of proSAAS mRNA in the Allen Mouse Brain Atlas shows highest levels in the amygdala, hypothalamus, hippocampus, mammillary bodies and other limbic regions.

These results are consistent with mRNA quantification by quantitative real-time PCR (qRTPCR), showing a high expression in the hypothalamus (Wardman et al. 2011).

In the hypothalamus, proSAAS mRNA is specifically concentrated in the arcuate nucleus, which is also an area expressing NPY and proopiomelanocortin (POMC). In fact, a colocalization of PEN/LEN and NPY was found in the arcuate nucleus, indicating that some cells in the hypothalamus produce both proSAAS-derived peptides and NPY (Wardman et al. 2011) (Wardman and Fricker 2014) (Fig. 4). These cells also contain AgRP and function in the stimulation of feeding (Luquet et al. 2005) (Krashes et al. 2011). Consistent with a role in feeding and body weight regulation, transgenic mice overexpressing PCSK1N (the gene encoding proSAAS) are slightly overweight (Wei et al. 2004), and mice with a disruption in the PCSK1N gene, which eliminates the production of proSAAS, are underweight (Morgan et al. 2010). In addition, intracerebroventricular injection of antibodies to either bigLEN or PEN blocks feeding (Wardman et al. 2011), suggesting that these peptides stimulate feeding. Food deprivation for 48h in Cpe^{fat}/Cpe^{fat} mice showed an increased level of many proSAAS derived peptides in hypothalamus, but proSAAS mRNA expression in the same area are not altered. These results suggest that proSAAS-derived peptides are regulated and processed differently extent in the hypothalamus by food deprivation (Che et al. 2005)

Taken together, these results are consistent with a role for proSAAS-derived peptides as neuropeptides that influence body weight independently of their function as inhibitors of PC1.

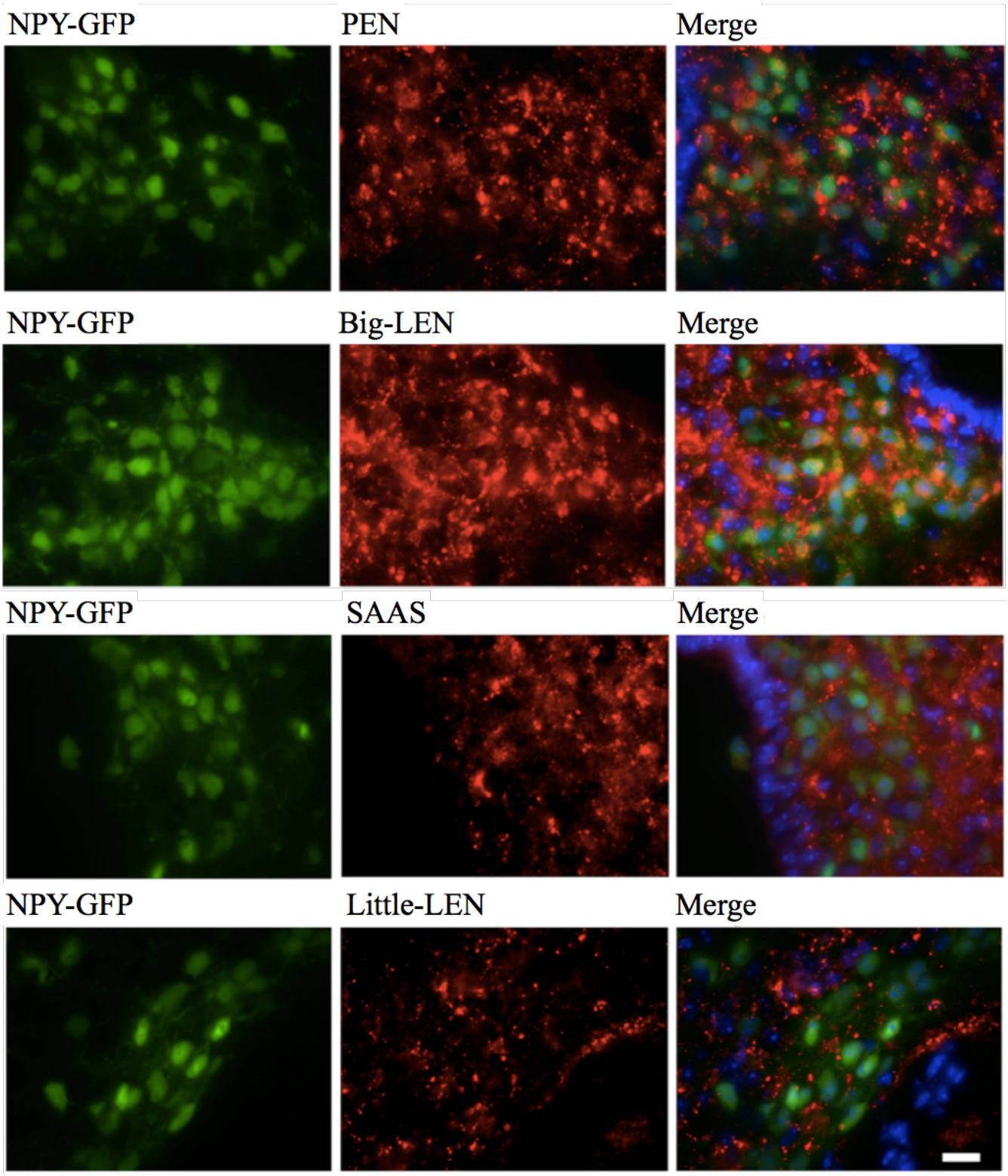

Fig. 4. *Colocalization of ProSAAS derived peptides and NPY-expressing cells in the arcuate nucleus (Wardman and Fricker 2014).*

PEN levels analysed by radioimmunoassay (RIA) showed a high expression within the cortex, pons and medulla, hippocampus and striatum, but only low expression in hypothalamus (Fig. 5). To confirm the involvement of this peptide in hypothalamic function, mice exposed to a chronic cold stress for 7 days increased the level of PEN only in female mice compared with female control, but not in male mice. Different expression of PEN was found also in hippocampus after a high fat diet. Hippocampus could be part of an extra-hypothalamic regulation by peptides that are involved in the control of feeding and energy balance (Pages et al. 1993), so that the increase of PEN peptide in hippocampus is unusual but not without precedents (Chakraborty et al. 2006).

As for feeding and metabolism, it was suggested an implication of proSAAS in anxiety-related behavior and reward. Specifically, the absence of proSAAS in mice induces an anxiety-like behavior in a novel environment, with an increase of the locomotor activity compared with WT mice (Morgan et al. 2010).
It is interesting also the fact that cocaine treatment induces an altered expression of proSAAS peptides in mouse brain. Cocaine is a psychostimulant which increases the release of dopamine in NAc. Peptidomics analyses showed a decrease of PEN and littleSAAS in the NAc and VTA. proSAAS KO mice also showed lower locomotor activity compared with WT mice after cocaine acute treatment and are less susceptible to behavioral sensitization (Berezniuk et al. 2017). These results suggest also that proSAAS is linked to the reward system.

ProSAAS may play a role in the pathogenesis of Alzheimer's disease (AD), Pick's disease, parkinsonism-dementia-complex. In human AD and mouse AD-like brain a co-localization of proSAAS with Aβ plaques was found and it was demonstrated that proSAAS inhibits the aggregation of insoluble $A\beta_{1-42}$ (Kikuchi et al. 2003)(Wada et al. 2004)(Hoshino et al. 2014)(Wang et al. 2016).
In addition, cerebrospinal fluid proteome analysis of human patients with frontotemporal dementia showed increased levels of proSAAS (Davidsson et al. 2002).

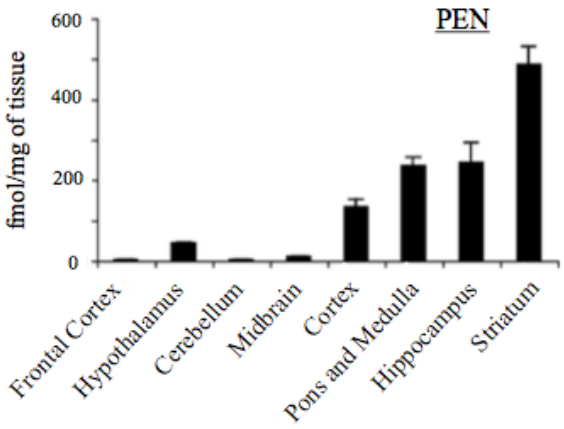

Fig. 5. *Level of PEN peptide in different brain regions analysed by RIA (Chakraborty et al. 2006).*

PEN binds and activates a GPCR in the brain

Ligand binding studies with PEN revealed the presence of a specific receptor for this peptide. A saturable radioligand binding assay revealed in mouse hypothalamus and hippocampus, a high affinity site and a low affinity side. The ability of PEN to activate GPCRs was confirmed by the increase of GTP-γS binding, whereas concentrations above 1 nM were less effective, implying desensitization of the response. In particular, the hypothalamic receptor for PEN is a Gαq-coupled GPCR that activates the phospholipase C (PLC)-mediated signaling cascade.

In contrast to the hypothalamic response, PEN had a monophasic dose-response curve with no desensitization at high concentrations in the hippocampal membranes. Indeed, in the hippocampus mPEN inhibited adenylyl cyclase activity, whereas there was no effect on PLC activity. Together these results indicated that PEN receptors are coupled to different G proteins in different regions, Gαq in the hypothalamus and Gαi in the hippocampus (Gomes et al. 2016).

In the same study they detected the mPEN binding in several brain regions, but the highest amount of binding was in the striatum.

Additionally, electrophysiological characterization of the effects of mPEN in slice preparations of rat paraventricular hypothalamic nucleus, showed a decrease of the spontaneous excitatory postsynaptic current (sEPSC) frequency without changing the amplitude, suggesting a presynaptic effect on glutamate release. mPEN induced an increase in paired-pulse ratio, which is another indication of a presynaptic effect (Gomes et al. 2016).

The presence of a PEN receptor was also confirmed in the mouse neuroblastoma cell line Neuro2A, by studying receptor activation-mediated neurite outgrowth that represents a functional outcome of GPCR activation in this cell line (Fricker et al. 2005)(Gomes et al. 2009). Exposing Neuro2A cells to mPEN significantly increased the number of neurites, suggesting the presence of receptors for PEN in these cells. Ligand-binding analysis revealed a high- and a low-affinity binding site. As with the hippocampal and hypothalamic samples, exposure of the Neuro2A cells to mPEN led to, respectively, dose-dependent inhibition of adenylyl cyclase activity, with a concomitant decrease in intracellular cyclic adenosine monophosphate (cAMP), and dose-dependent increase in PLC activity and increase in intracellular Ca^{2+}. In addition, mPEN activates mitogen-activated protein kinase (MAPK) pathways, as evidenced by the increased phosphorylation of extracellular signal–regulated kinases 1 and 2 (ERK1/2). Together, these studies demonstrated that Neuro2A cells have one or more receptors for PEN with properties similar to those detected for PEN receptors in the brain (Gomes et al. 2016).

GPR83 is a PEN receptor

To confirm that PEN is the agonist of GPR83, Gomes et al. (2016) used heterologous expression of the human and mouse GPCRs in CHO cells and human embryonic kidney (HEK) cells. Similar to what observed in mouse hypothalamus, hGPR83 revealed high-affinity binding for PEN with a dissociation constant (K_d) of 9.4 nM. The radioligand binding assays revealed a high and a low affinity site, as observed in hypothalamus and hippocampus membranes. Additionally, NPY only at concentration 10 μM exhibited a small and not significant displacement. Similar to what was observed in NeuroA2 cells, mPEN in hGPR83 expressing cells reduced cAMP and increased PLC activity, intracellular IP_3 and Ca^{2+} signals, with a desensitization of the receptor at high PEN concentrations. These results were confirmed using hypothalamic membranes from GPR83 KO mice that had no detectable PEN binding and absent stimulation of G protein activity.

Analysis of the amount of receptor at the surface over time showed that the addition of either mPEN or hPEN resulted in rapid and robust internalization of mGPR83. These results support the hypothesis that GPR83 functions as a receptor for PEN.

AIM OF THE STUDY

Recent studies discovered a new orphan receptor called GPR83, which is a receptor highest expressed in the rodent and human brain. The localization and the physiological roles of GPR83 suggest an involvement in brain systems involved in metabolism and feeding, locomotion, stress, anxiety, memory and reward. Recently, the PEN peptide, derived from proSAAS, was identified as the specific ligand of GPR83.

The aim of this study was to confirm the expression of GPR83 in mouse brain and identify new behavioral effect of GPR83, using mice lacking the receptor. We focused the experiments on behavioral test, about locomotor activity, stress response, anxiety-like behavior and motivation for food. In addition, we decided to test the behavioral effects of intracerebral injection of the specific ligand PEN.

MATERIALS & METHODS

Construction of the targeting vector and generation of GPR83 KO mice

A 9 kb cassette containing a promoterless tau-lacZ fusion gene, a PGK-Neo gene and a HSV-TK gene, (kindly provided by P. Mombaerts) was flanked in 5' by a 3.4 kb *EcoRI-XbaI* fragment ligated to a 2 kb PCR product of a GPR83 genomic clone and in 3' by a 5 kb *EcoRI-XhoI* GPR83 gene fragments. In the final construct, the cassette replaced the first 130 codons of the GPR83 gene, encoding transmembrane segments 1 to 2. The natural ATG of GPR83 was replaced by the tau-lacZ ATG, expressing the enzyme β-galactosidase.

Homologous recombination was carried out in the R1 ES cell line. G418 (400 µg/mL)-resistant clones were collected after 7 days of selection, and screened by Southern blotting following *XbaI* digestion. The 1.1 kb *XhoI-XbaI* probe is located downstream of the 3' end of the targeting construct (Fig. 6). 32 clones out of 480 were found recombined at the GPR83 locus. Additional Southern blotting experiments were performed in order to confirm homologous recombination, by testing both the left and right arms of the construct, and the use of a Neo probe allowed to exclude the presence of additional integration sites. The recombinant clone was aggregated with CD1 eight-cell stage embryos as described (Chung et al. 2006). We obtained 23 chimeras and 2 of these transmitted the mutant allele. Heterozygous mutants were bred for 12 generations on a CD1 background before generating the WT and KO mice used in this study. Genotyping was realized by PCR using the following primers:

- Fw: TGGAAATGCCACCCCAGAGC;
- Rv1: AGACGGAGATGGGAGCATGC;
- Rv2: TGTGAGCACACACTCCCTGG.

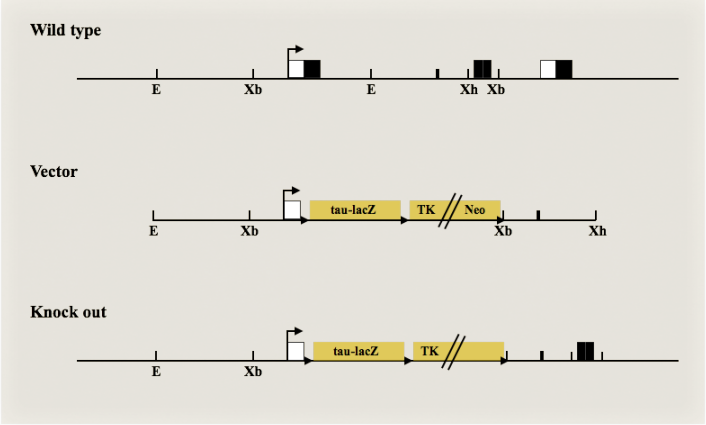

Fig. 6. *Structure of the WT GPR83 locus, the targeting vector and the locus resulting from homologous recombination (KO).*

Lac-Z staining

Mice were fixed by intracardiac perfusion with cold LacZ fixative solution (0.2% glutaraldehyde, 10% PBS, 5 mM EGTA, 100 mM $MgCl_2$, pH 7.3). Vibratome brain sections were post-fixed in LacZ fixative solution for 30 min at 4°C. Samples were then washed twice in PBS and LacZ-buffer (5 mM $K_3Fe(CN)_6$, 5 mM K4Fe$(CN)_6$, 2 mM $MgCl_2$ in PBS) for 10 min at 4°C in the dark prior to incubation at 37°C in LacZ-buffer containing 0.5 mg/ml X-gal (bromo-chloro-indolyl-beta-D-galactopyranoside). Reaction was stopped by washes in PBS and overnight post-fixation in 4% paraformaldehyde.

Immunochemistry

The method for immunohistochemistry has been described previously (Kanemaru et al. 2014). The following antibodies were used: rabbit anti-βGAL (eBioscience) and goat anti-ChAT (Merck). For fluorescence microscopy, sections were treated with a mixture of species-specific secondary antibodies conjugated to AlexaFluor-488, or -546 (Invitrogen) for 2 h at room temperature. Fluorescent images were obtained with a confocal microscope (LSM710, Zeiss).

Animals

4-6 old mice month (35-45 g) were housed four per cage in a temperature (21 ± 1°C) and humidity (55 ± 10%) controlled room with 12:12 h inverted cycle (light off at 8 am) or normal cycle (light on at 8 am).

Food and water were available *ad libitum*, except as described in the food restriction protocol for the operant behavior. For the behavioral studies, mice were moved in the behavioral room one hour before the beginning of the tests, and then exposed to the tests between 9 am – 4 pm.
All procedures were performed according to Institutional Animal Care Committee guidelines at the University of Bruxelles and were approved by the local ethics committees. All efforts were made to minimize the number of animals used and their suffering.

Open field test

The open field was a rectangular arena (40 x 40 x 40 cm). Between each the tests the open field arena was cleaned with 70% ethanol solution and allowed to dry completely. The tests were run during the dark phase or during the light phase (brightly illuminated from the top).
We analyzed the total distance traveled, time spent in internal and external zone of the arena. The duration of the test was 30' and the analysis was subdivided into 5' segments.

Elevated plus maze

The elevated plus maze (EPM) as run during the light phase. The apparatus consists of two open arms (50 x 10 cm x 40 cm) and two closed arms (50 x 10 x 40 cm) with open tops arranged such that the two open arms were opposite to each other. The maze was elevated 30 cm above the ground and illuminated from the top (100 lux). Mice were placed in the middle of the maze facing one of the open arms. Between the tests the EPM apparatus was cleaned with 70% ethanol solution and allowed to dry completely.
The cumulative time spent in the open arms, in the closed arms and in the inside zone were measured for 10' and the analysis was subdivided into 5' segments.

Cocaine stimulated hyperlocomotion

Cocaine-induced locomotion was tested in the open field arena in dark cycle. Mice were placed individually into the open field and baseline locomotion was monitored over 30 min. Animals were removed, injected intraperitoneally (i.p.) with vehicle (sterile saline, 4 mL/kg), or 20 mg/ kg cocaine (Sigma), and returned immediately to the open field for 60 min. were detected The distance traveled (cm/5 min) was detected during baseline and post-injection periods. The next day, mice were

administered 20 mg/kg cocaine using the same procedure as described above, for the test of behavioral sensitization.

Progressive ratio task

The mice received food restriction with 2.5-3.5 g of normal food chow per day, until each animals lost 15% of the original weight. Thereafter their body weights were maintained at 85% of original weight.

The task was conducted in four identical automated operant chambers (Imetronic), each set in a ventilated, sound-isolated cubicle. Test cages were equipped with a grid floor, two different levers and a pellet magazine located on the opposite wall. The complete protocol is illustrated in Fig. 7. The first two days of magazine training the animals obtained a pellet (Dustless Precision Pellets Rodent, Purified; Bioserv) each 10 "; the trial duration was 30'. After this habituation, the mice were initially trained to press the lever on a fixed ratio 1 (FR-1) reinforcement schedule whereby a single lever press elicits the delivery of a food pellet to the magazine. Only one lever is designated as 'active' (triggering delivery of food reward) and the allocation of right and left levers was counterbalanced between mice. After the food delivery, 8 s of inter-trial interval was added, during which levers were inactive. The inter-trial interval allows time for mice to consume the food pellet.

Following two successive sessions of obtaining 50 pellets, the schedule was increased to FR-2 in which two active lever presses triggered the delivery of the food pellet. Training on the FR-2 schedule lasted three days. Then, the schedule was increased to FR-3 and lasted three more days. Each FR training session lasted 1 h or when 100 pellets had been delivered (Tsutsui-Kimura et al. 2017). We analyzed the percent of inactive lever pressing (LP) [inactive LP/ (inactive and active LP) 100] as an index of associative learning.

The mice are then trained in the progressive ratio (PR) schedule of reinforcement (Richardson and Roberts 1996). The response ratio schedule during PR testing can be calculated according to the following formula:

$$= \left[5\, e^{(Rx0.2)} \right] - 5$$

Where R is equal to the number of food rewards already earned plus 1 (that is, the next reinforcement). Thus, the number of responses required to earn a food reward followed the order: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, and so on. The final ratio completed represented the break point. A PR session

lasted a maximum of 1h. Failure to press the lever in any 3' period resulted in termination of the session. PR schedule lasted three days.

The PR break point was defined as an index of instrumental motivation. The time spent to complete the PR (the mean time from the first active LP to achieving the required number of active LP) was defined as an index of instrumental motivation. Percent inactive LP was calculated and was defined as an index of associative learning.

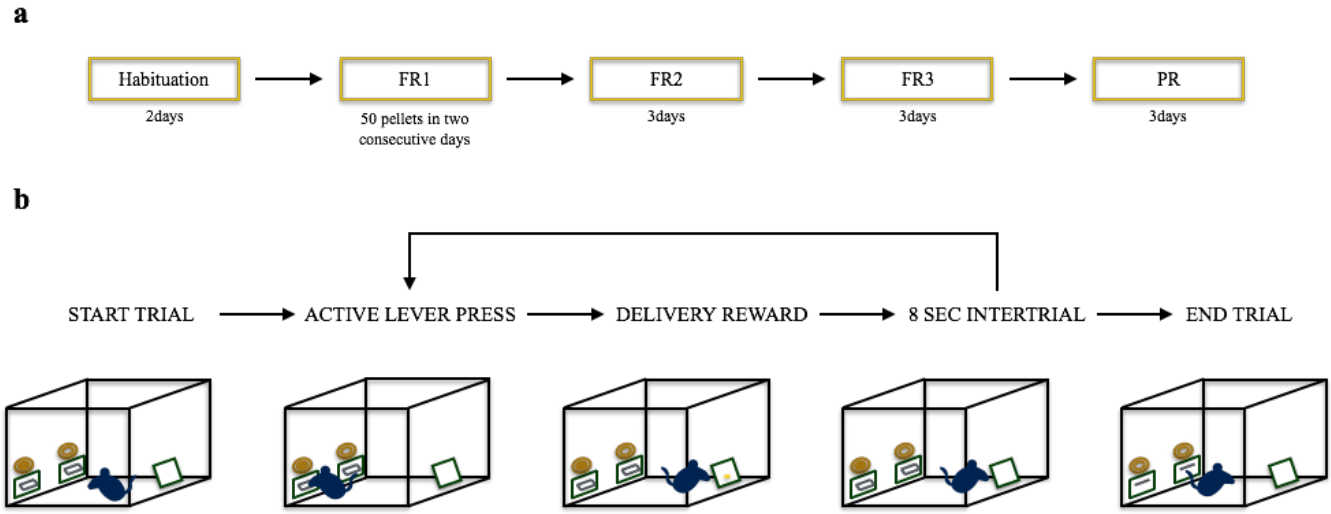

Fig. 7. *(**a-b**) Schematic illustration of the operant behavior protocol.*

Surgical procedure and PEN microinjection

Mice were anaesthetized using isoflurane and fixed by ear bars in a Kopf stereotaxis apparatus. The top of the mouse head was cleaned using sterile cotton swabs and betadine solution. A mid-sagittal incision was made to expose the skull and then cleaned with sterile saline followed by 30% hydrogen peroxide solution. A small hole through the skull (approximately 1mm) was bored with a drill, dura mater was removed using a needle and then the skull was cleaned and dried.

Stainless-steel guide cannulae (pedestal height 4mm, length 5.5 mm, 22 GA - Plastics One, Roanoke VA) were implanted unilaterally in the caudate-putamen (CPu) (coordinates from bregma: + 0.8 anteroposterior (AP); + 2.5 lateromedial (LM); –3.6 dorsoventral (DV)), or bilaterally (pedestal height , length 4.5 mm, 26 GA, center to the center 1.5 mm - Plastics One, Roanoke VA) above the NAc shell

(coordinates from bregma: + 1.3 anteroposterior (AP); ± 0.75 lateromedial (LM); –3.6 dorsoventral (DV)) (Fig. 8).

The guide cannula was held in place using dental cement, and occluded to prevent clogging using a 5.5 mm obturator. Mice were allowed to recover for 7–10 days before injections were performed. After recovery, 2 µL of PEN (Phoenix Pharmaceuticals, Inc.) was infused unilaterally by infusion pump at a rate of 0.5 µL/minute, using a 20 µL Hamilton syringe, 22 GA (Hamilton Company), attached by PE50 tubing, 0.58 internal diameter (Plastics One, Roanoke VA) to an unilateral infusion cannula (length 6 mm, 26 GA - Plastics One, Roanoke VA), or bilaterally using two 20 µL Hamilton to an infusion cannula (length 5 mm, 33 GA, center to center 1.5 mm - Plastics One, Roanoke VA). Immediately after the injection the animals were moved into the behavioral arena and tested in the open field as described above. We injected the animals with different doses of PEN as shown in Tab. 1.

At the beginning of the study, target coordinates and proper cannula placement were verified by slowly injecting 1 µL of methylene blue dye into the cannulas while the mice were under anesthesia. After waiting 5 min for the dye diffuse in the target area, the animals were killed. The brains were removed, sliced at the point of cannula entry, and target coordinates were verified. We repeated the same procedure after the experiments and we removed from the analysis the animals with an incorrected position of the cannula.

	PEN µg/uL
Unilateral - CPu	0.1 0.5 2.5 5
Bilateral - NAc	0.5 5

Tab. 1. *Doses of PEN injected unilaterally into CPu or bilaterally into NAc.*

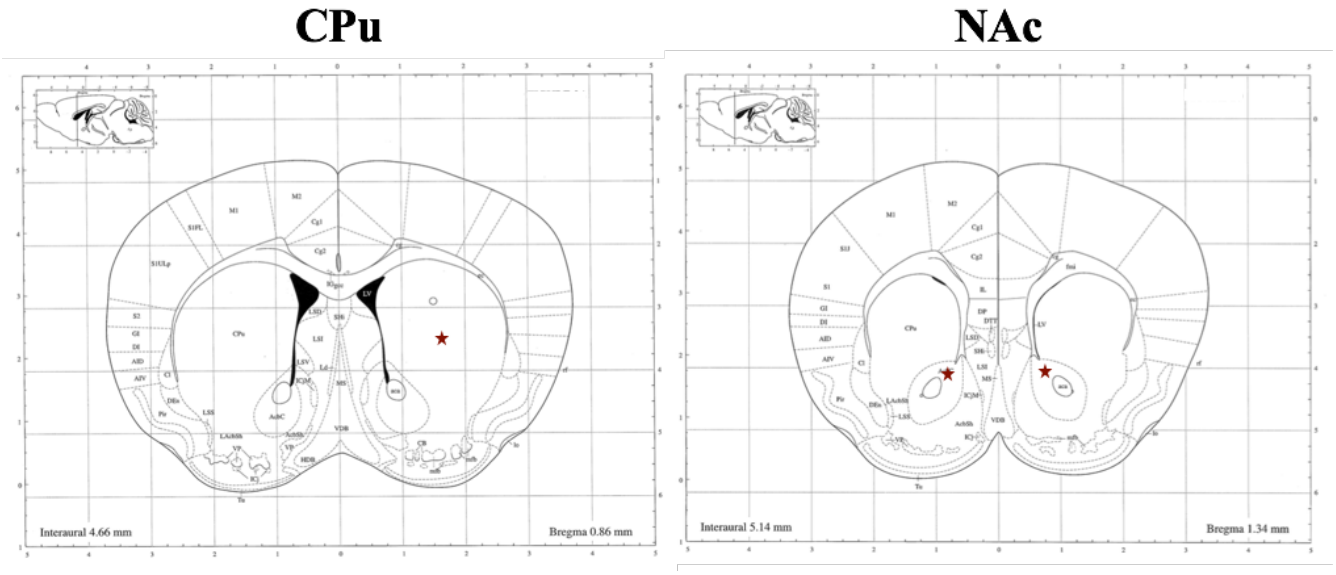

***Fig. 8**. Infusion cannula placements for CPu and NAc shell.*

Statistical analysis

Statistical analysis as performed by repeated measure (RP) analysis of variance (ANOVA). Post hoc analysis as performed by Bonferroni test.

To explore the changes of behavioral patterns over time, we divided the analyses into 5' time-bins. The differences at each time point between two groups or the summed data for these tests were analyzed using unpaired Student's two-tailed *t* test (Prism 6.0, GraphPad software). In all figures data are presented as mean ± standard error of the mean (SEM). $p < 0,05$ was considered as a threshold for significant difference.

RESULTS

Distribution of Lac-Z positive cells in GPR83 KO mice

The construct used to inactivate GPR83 by homologous recombination approach at the same time deleted exon 1 of the GPR83 gene and added a bacterial LacZ reporter gene that expresses the enzyme β-galactosidase. By revealing β-galactosidase positive cells using X-gal that gives a blue stain, we could therefore identify GPR83 positive cells in the GPR83 KO mice.

The distribution of X-gal positive cells tallies very well with the known distribution of GPR83 mRNA (see introduction). For instance, high level of staining was observed in limbic cortical regions, in periglomerular cells of the olfactory bulb and in large and medium neurons of the ventral striatum including the NAc and the olfactory tubercle. Few strongly positive cells were found randomly scattered in the dorsal striatum (Fig. 9). The size and distribution of these cells suggested their identification with cholinergic interneurons, the so-called tonically active neurons (TANs) (Pisani et al. 2001).

We wished to confirm this identification by double fluorescence experiments coupling fluorescent X-gal staining and immunofluorescent staining of ChAT, the biosynthetic enzyme of acetylcholine. We showed that ChAT positive cells also express X-gal, though, especially in the NAc, some ChAT negative cells also expressed X-gal (Fig. 10).

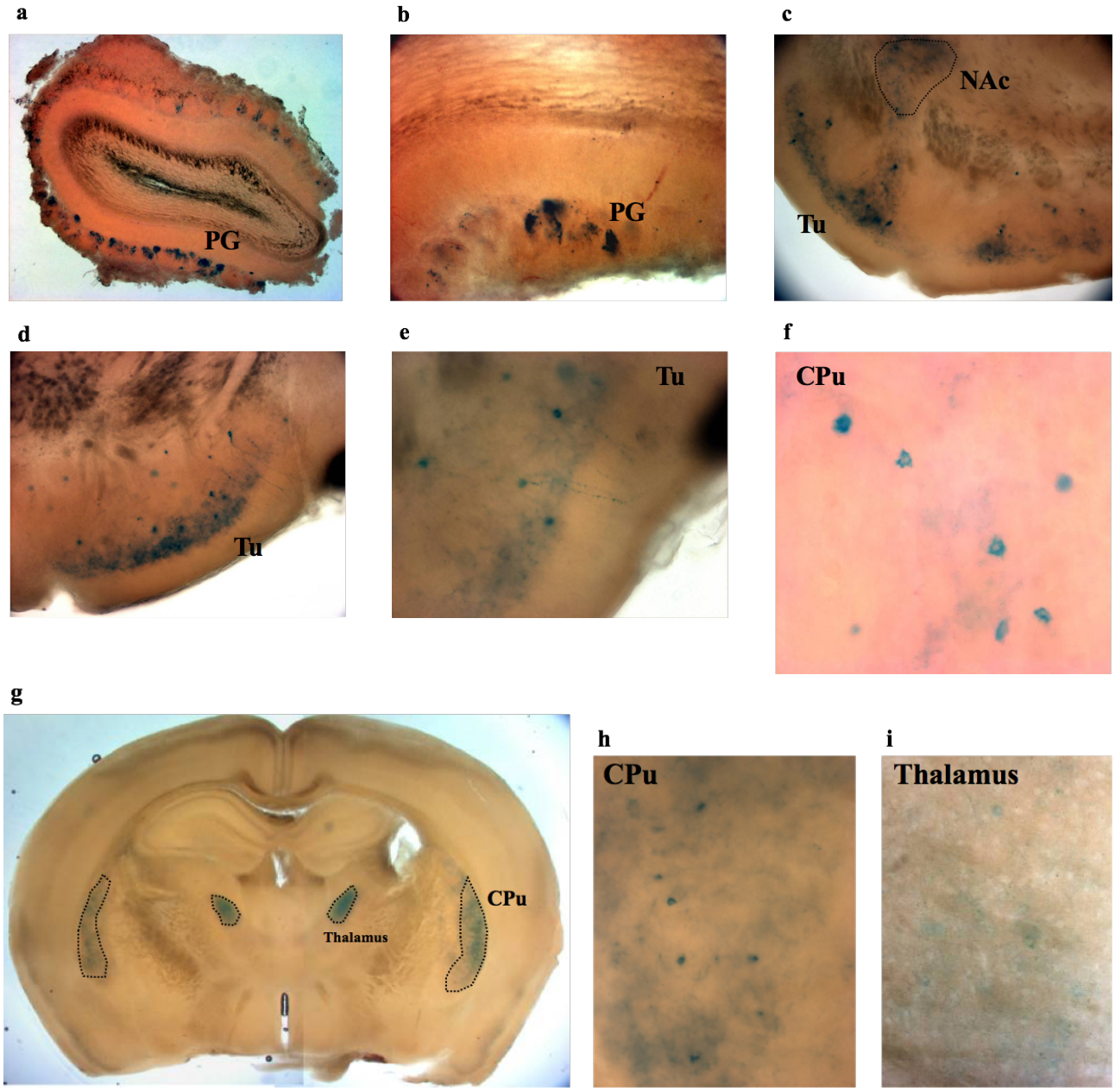

Fig. 9. *X-Gal staining of mouse brain slices reveal GPR83 expression.* ***(a-b)*** *Periglomerular cells (PG);* ***(c-e)*** *olfactory tubercle (Tu) and nucleus accumbens (NAc);* ***(f, h)*** *Caudate-putamen (CPu);* ***(g, i)*** *thalamus.*

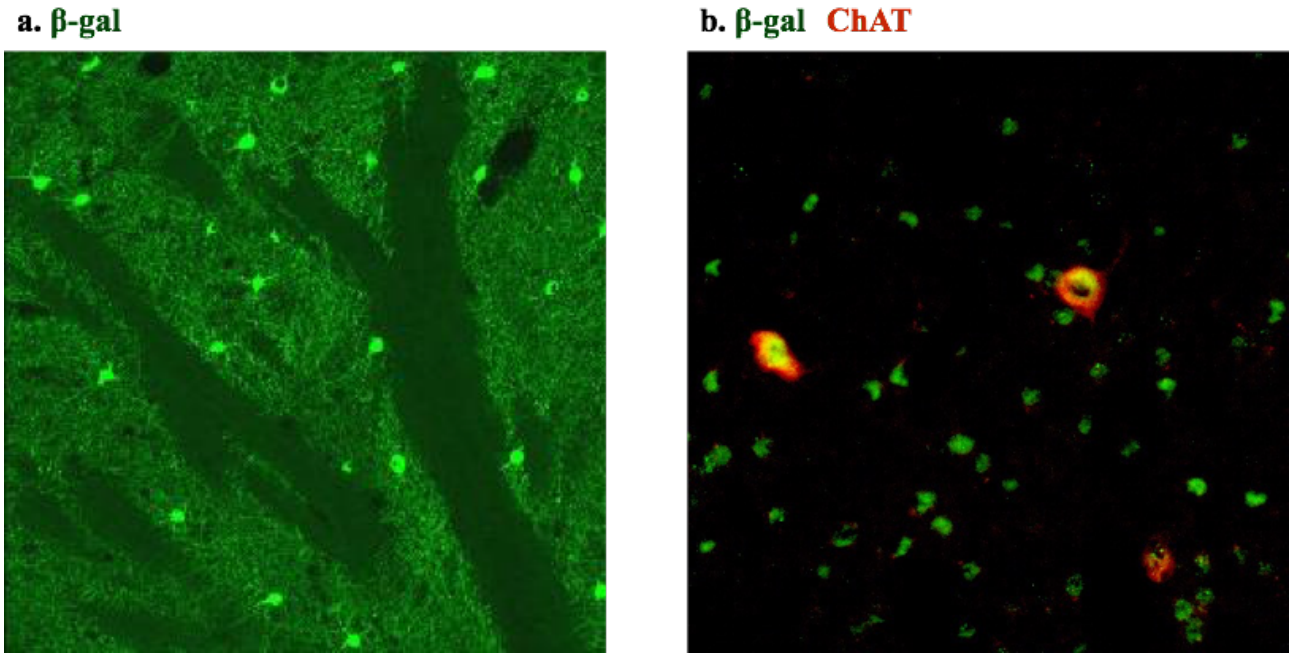

Fig.10. *Immunofluorescence localization of β -galactosidase (**a**) and co localization of β -galactosidase and ChAT positive cells in striatum (**b**)*

GPR83 KO mice have altered locomotor activity in light and dark condition.

Locomotor activity in a novel environment was measured in WT and GPR83 KO mice in light and dark condition using the open field test.

Fig.11 shows that locomotor activity was not altered in GPR83 KO mice compared to WT mice during the dark cycle, whereas during the light cycle the GPR83 KO mice showed a significant hyperlocomotion and did not show habituation to the novel environment during the time spent in the open field.

RP two-way ANOVA of the time course, revealed a significant effect of time in both conditions (dark: $F_{(5,110)} = 47.10$, $p < 0.0001$; light: $F_{(5,90)} = 17.37$, $p < 0.0001$), but only during the light cycle there was a genotype effect ($F_{(1,18)} = 5.941$, $p = 0.0254$). As shown in Fig 9d , the GPR83 KO mice presented a significantly higher total locomotor activity during the light condition as evaluated by the total distance traveled in 30' (Student's *t* test, $p = 0.0156$).

When a comparison of the locomotor activity was performed in the two cycle conditions for the same genotype, WT mice did not show any significant difference between the two conditions. Instead, GPR83 KO mice showed significantly higher locomotion in the light phase and a reduced adaptation to the cage (time ($F_{(5,85)} = 21.65$, $p < 0.0001$), light/dark effect ($F_{(1,17)} = 11.00$, $p = 0.0041$) and time x light/dark effect ($F_{(5,85)} = 2.988$, $p = 0.0156$). Finally, the GPR83 KO mice showed significantly increased

locomotion as evaluated as total distance traveled in light condition compared with dark condition (Student's *t* test p = 0.0041) (Fig 12).

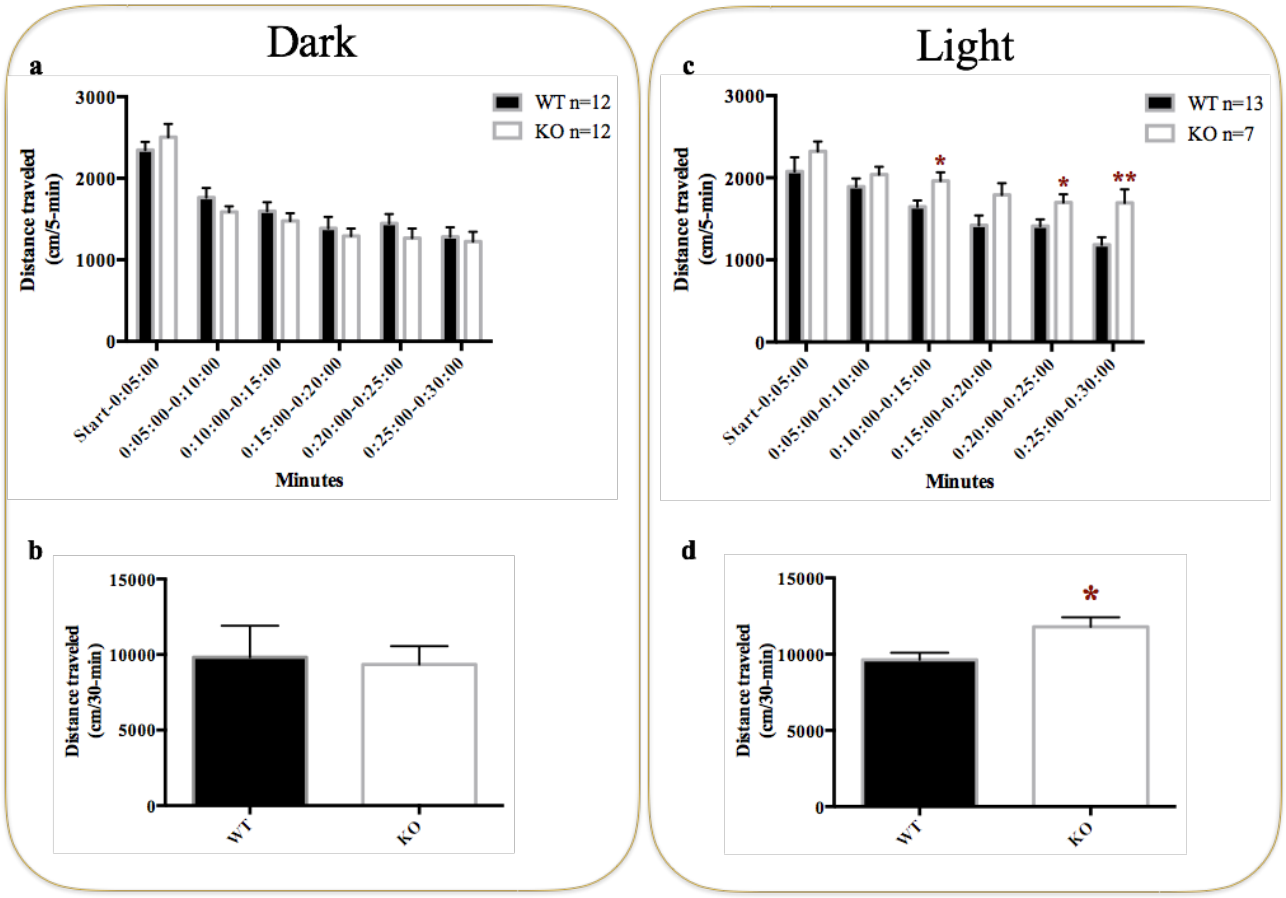

Fig. 11. WT and GPR83 KO mouse locomotor activity in an open field. ***(a)*** *Time course of the distance traveled in dark condition. Statistical analysis was performed by using RP ANOVA: time* $F_{(5,110)} = 47.10$, $p < 0.0001$; *genotype* $F_{(1,22)} = 0.4647$, $p = 0.5025$; *time x genotype* $F_{(5,110)} = 0.9697$, $p = 0.4396$. ***(b)*** *Total distance traveled in 30' in dark condition.* ***(c)*** *Time course of the distance traveled in light condition. Statistical analysis was performed by using RM ANOVA: time* $F_{(5,90)} = 17.37$, $p < 0.0001$; *genotype* $F_{(1,18)} = 5.941$, $p = 0.0254$; *time x genotype* $F_{(1,18)} = 5.941$, $p = 0.0254$. *A Student's t test revealed a significant difference between WT and KO mice after 15' (p= 0.0285), after 25' (p = 0.0475) and after 30' (0.0088).* ***(d)*** *Total distance traveled in 30' in light condition show a significant difference between groups. Statistical analysis according to Student's t test, p = 0.0156). Data are shown as mean ± SEM and the number of animals is shown in the figure.*

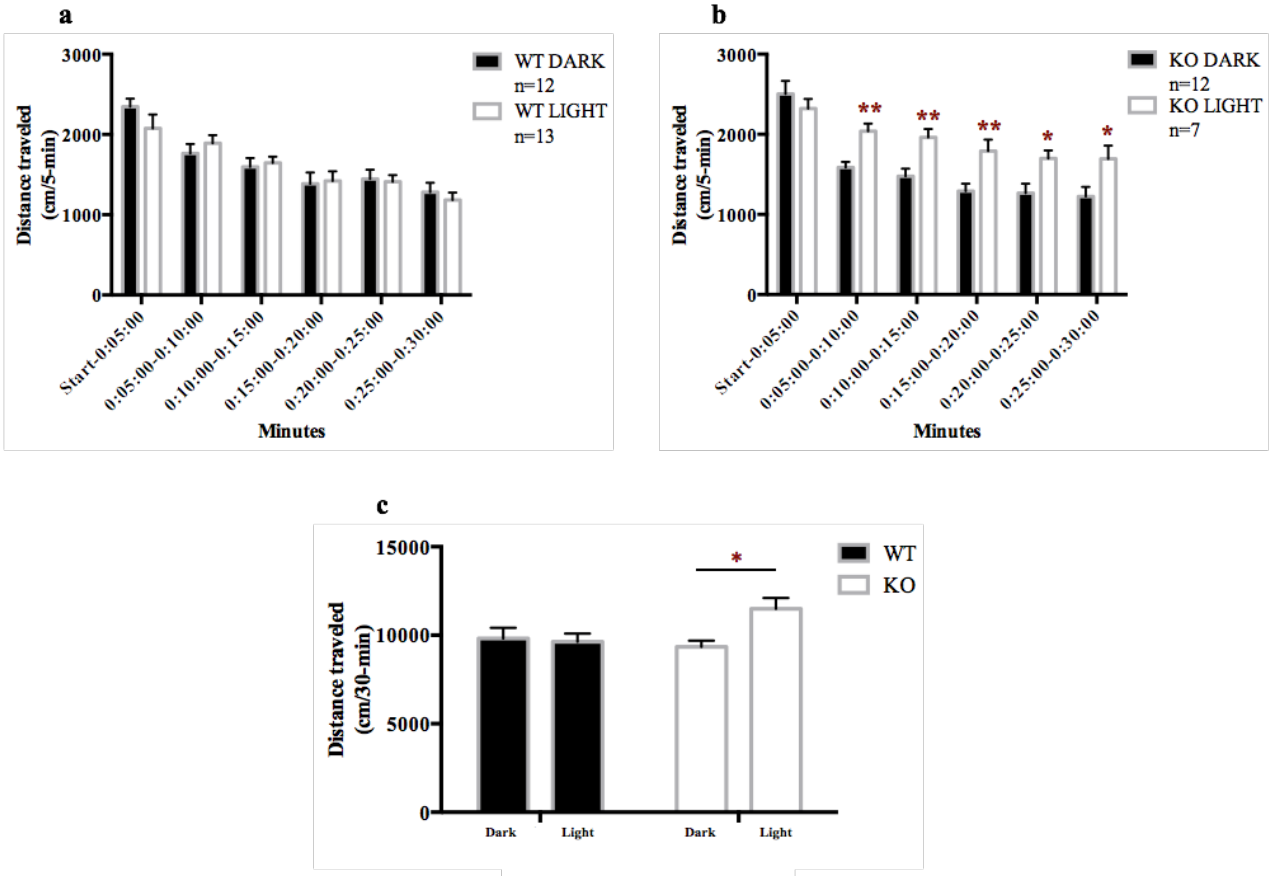

Fig. 12. Comparison of locomotor activity for the same genotype in light and dark. *(**a**) Time course of locomotor activity of WT mice in light and dark. Statistical analysis was carried out using a RP ANOVA: time $F_{(5,115)} = 39.27$, $p < 0.0001$; dark/light $F_{(1,23)} = 0.06300$, $p = 0.8040$; time x dark/light $F_{(5,115)} = 1.548$, $p = 0.1805$). (**b**) Time course of locomotor activity of GPR83 KO mice in light and dark. Statistical analysis was carried out using a RP ANOVA: time $F_{(5,85)} = 21.65$, $p < 0.0001$; dark/light $F_{(1,17)} = 11.00$, $p = 0.004$; time x dark/light $F_{(5,85)} = 2.988$, $p = 0.0156$. A Student's t test revealed a significant difference after 10' ($p= 0.0013$), 15' ($p= 0.0048$), 20' ($p= 0.0067$), 25' ($p= 0.0233$), 30' ($p= 0.0331$). (**c**) Total distance traveled by the GPR83 KO or WT mice during the light and dark cycle. Statistitcal analysis was performed using a Student's t test. GPR83 KO mice $p = 0.0041$. Data are shown as mean ± SEM and the number of animals is shown in the figure.*

GPR83 KO mice show increased anxiety-like behavior.

In the open field we analysed also the time spent in the internal zone as an index of anxiety-like behavior. GPR83 KO mice spent less time in the internal zone of the arena in comparison with WT mice only in dark condition (genotype $F_{(1,22)} = 7.053$ $p = 0.0144$, interaction time x genotype $F_{(5,110)} = 2.496$ $p = 0.0350$) (Fig. 13).

To confirm the anxiety-like behavior seen in the open field in dark condition, we performed the EPM during light cycle. In fact, this test is based on the fear of rodents for open space and altitude; in the absence of light the animal cannot distinguish the two different arms of the apparatus and for this reason the light was an unavoidable condition.

The anxiety-like behavior as not altered in the EPM, in fact there is no difference between GPR83 KO and WT in mice in term of time spent in the open arms, in the closed arms and in the central zone of the maze (Fig. 14).

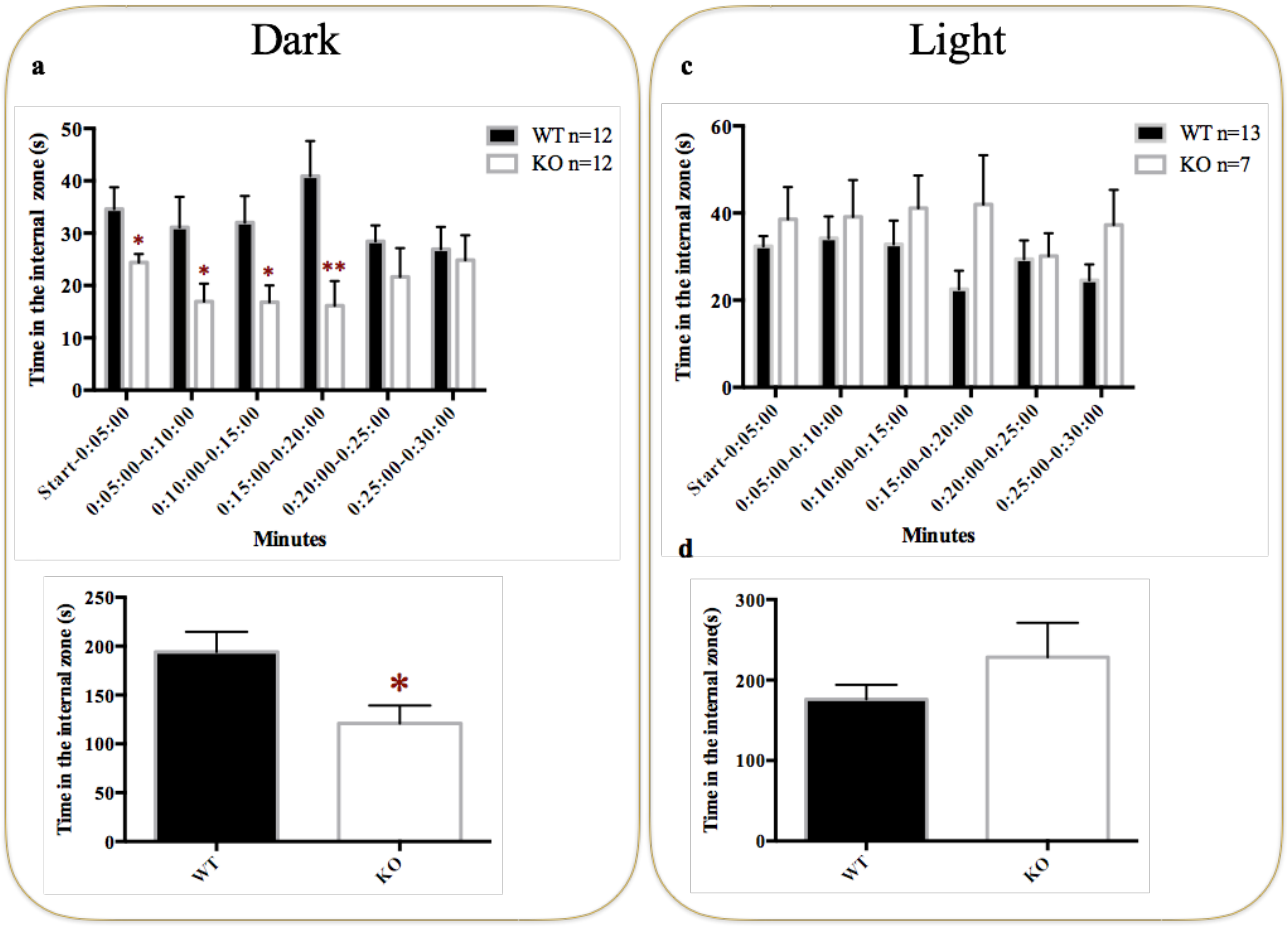

Fig. 13. Anxiety-like behaviour of GPR83 KO and WT mice in the open field. *(**a**) Time course of the time spent in the internal zone in dark condition. Statistical analysis was performed by using RP two-way ANOVA (time $F_{(5,110)}$ = 0.8404, p = 0.5239; genotype $F_{(1, 2)}$ = 7.053 p = 0.0144; time x genotype $F_{(5,110)}$ = 2.496 p = 0.0350). Student's t test revealed a significant difference between GPR83 KO and WT mice after 5' (p= 0.0322), 10' (p=0.0487), 15' (p=0.0188) and 20' (p=0.0062). (**b**) Total time spent in the internal zone in dark condition. Statistical analysis according to Student's t test, p= 0.0144. (**c**) Time course of the time spent in the internal zone in light condition. Statistical analysis was performed by using RP two-way ANOVA (time $F_{(5,90)}$ = 1.208, p = 0.3117; genotype $F_{(1,18)}$ = 1.766, p = 0.2005; time x genotype $F_{(5,90)}$ = 1.365, p = 0.2450). (**d**) Total time spent in the internal zone in light condition. No significant difference was shown. Data are shown as mean ± SEM and the number of animals is shown in the figure.*

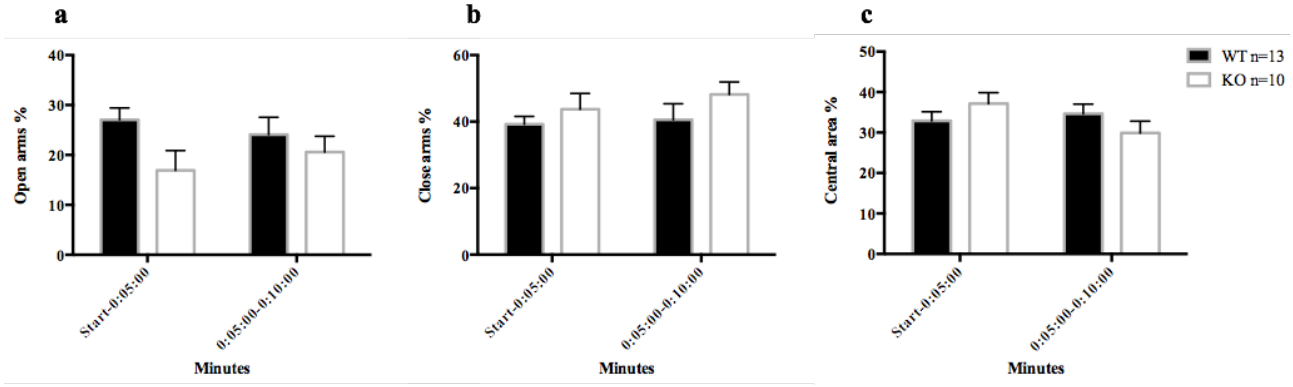

Fig. 14. Anxiety-like behavior of GPR83 KO and WT mice in the EPM. *(**a**) The animal showed a significant difference in the time spent in the open arms on the first 5'of test, and no difference in term of interaction, time and genotype. The statistical analysis was carried out by Student's t test, p= 0,013. No difference in the time spent in closed arms (**b**) and in the inside zone of the arena (**c**). Data are shown as mean ± SEM and the number of animals is shown in the figure.*

GPR83 KO mice have different sensitivity to cocaine.

No genotype effects and interaction between time and genotype were observed for baseline and post injection activities for both WT and GPR83 KO but only a time effect (baseline: $F_{(5, 110)} = 93.27$, $p < 0.0001$; post injection $F_{(11, 242)} = 19.30$, $p < 0.0001$). The Student's *t* test revealed a significant difference in locomotion after 15' post-injection (p= 0.0428) (Fig. 15).

To estimate a sensitization to cocaine we repeated the same protocol the day after. In WT mice a significative interaction between time and day, and a significant effect of time (interaction: $F_{(11, 242)} = 8.592$, $p < 0.0001$; time: $F_{(11, 242)} = 53.16$, $p < 0.0001$) was shown. Additionally the Student's *t* test revealed a significant increase of locomotion at the time of the injection in day 2 compared to day 1 (p= 0.0143). In GPR83 KO mice we found also an interaction time x day effect ($F_{(11, 242)} = 2.133$, $p = 0.0188$), a time effect ($F_{(11, 242)} = 31.64$, $p < 0.0001$), but not a day effect (Fig. 16).

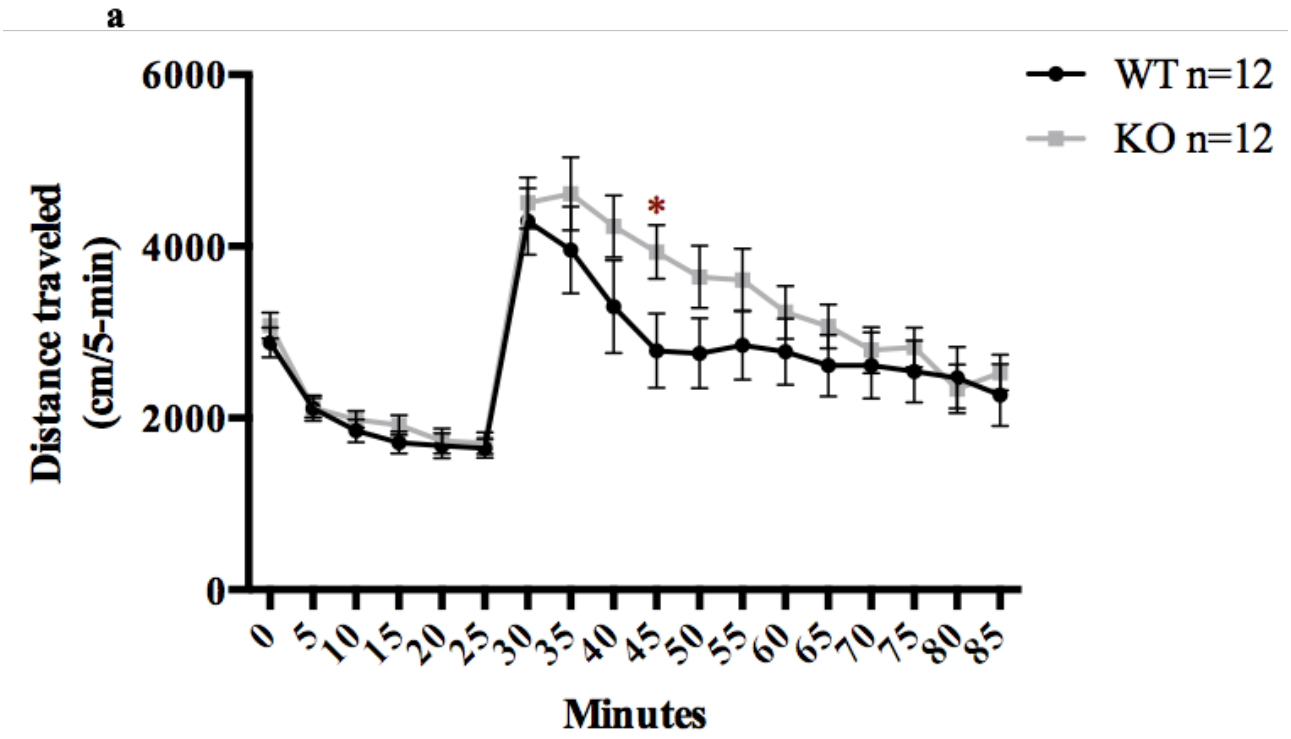

Fig. 15. Locomotor activity after acute injection 20 mg/Kg of cocaine. ***(a)*** *The cocaine was injected after 30' of baseline activity and the distance traveled before and after the injection was recorded. According to two-way RP ANOVA we found only a time difference in the baseline (time $F_{(5, 110)} = 93.27$, $p < 0.0001$; genotype $F_{(1, 22)} = 0.4397$, $p = 0.5141$; time x genotype $F_{(5, 110)} = 0.6340$, $p = 0.6742$), and after the injection (time $F_{(11, 242)} = 19.30$, $p < 0.0001$; genotype $F_{(1, 22)} = 1.431$, $p = 0.2443$; time x genotype $F_{(11, 242)} = 1.518$, $p = 0.1254$). At the minutes 45 the difference between WT and GPR83 KO mice was significant according to Student's t test (p = .,0428). Data are shown as mean ± SEM and the number of animals are show in the figure.*

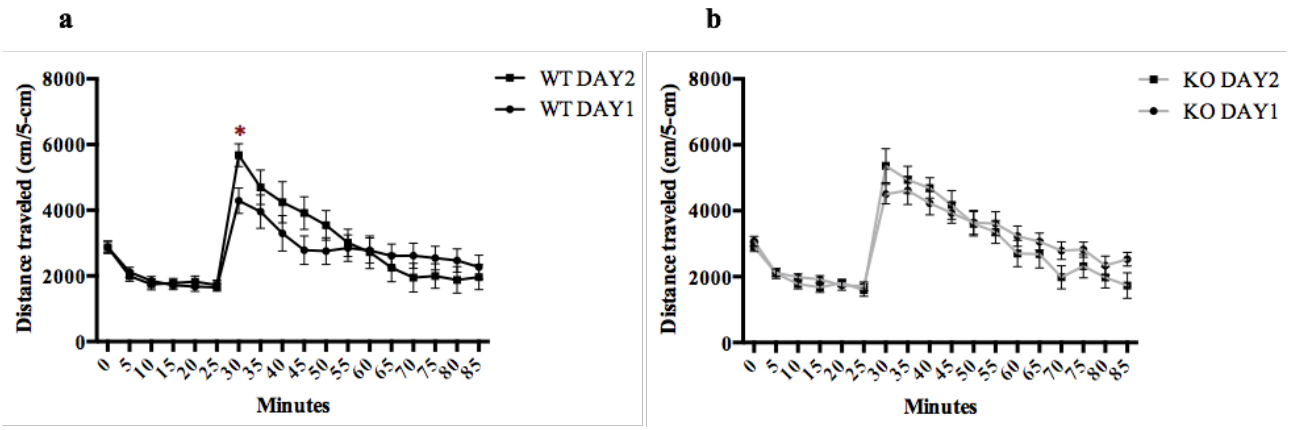

Fig. 16. Sensitization to 20 mg/Kg cocaine administration after 1 day. *(**a**) Distance traveled in control WT after a second treatment of cocaine. Statistical analysis was carried out by two-way RP ANOVA (time: $F_{(11, 242)} = 53.16$, $p < 0.0001$; day: $F_{(1, 22)} = 0.1510$, $p = 0.7013$; interaction: $F_{(11, 242)} = 8.592$, $p < 0.0001$). At the minute 30 the difference between WT on the day 1 compared with WT on the day2 of the injection of cocaine was significant according to Student's t test ($p = 0.0143$). (**b**) Distance traveled in GPR83 KO after a second treatment of cocaine. Statistical analysis was carried out by two-way RP ANOVA (time: $F_{(11, 242)} = 31.64$, $p < 0.0001$; day: $F_{(1, 22)} = 0.1705$; $p = 0.6836$; interaction: $F_{(11, 242)} = 2.133$, $p = 0.0188$). Data are shown as mean ± SEM and the number of animals are show in the figure.*

Altered food motivated behavior in GPR83 KO mice.

To investigate the involvement of GPR83 in motivation and reward, we performed a PR task.

We food restricted the mice starting from 6 days before the start of the learning protocol and until the end of the PR. We found a significant acceleration in the weight loss in GPR83 KO mice compared to WT mice (time $F_{(24,528)} = 55.73$, $p < 0.0001$; genotype $F_{(1,22)} = 2.240$; $p = 0.1487$; interaction: $F_{(24,528)} = 4.74$, $p < 0.0001$) (Fig. 17).

To complete the FR1 it was required to obtain 50 rewards in two consecutive days. For this reason, not all the animals completed FR1 after the same number of days. (Tab. 2). The Chi-Square test revealed a trend for a significant difference between the number of KO and WT mice that complete the task during FR1 sessions. ($p= 0.0704$).

Days to complete FR1	KO (n)	WT (n)
3	5	0
4	3	6
5	3	1
6	1	2

Tab. 2. *Number of mice that complete the FR1 after different days.*

We removed from the experiment the animals that did not obtain 50 pellets after 7 days of FR1 (WT n=2).

In the FR2 and FR3 we analysed the difference in learning in terms of number of inactive lever pressed, and in both of tasks there were no significant differences, however in the FR2 the KO mice seem to learn better than WT mice (Fig. 18).

The motivation of animals to obtain food was defined using a PR for three consecutive days. The GPR83 KO mice showed a significantly higher motivation to food as regards the break point, number of rewards obtained and time spent to complete the trial.

The RP two-way ANOVA showed for the break point an effect of time ($F_{(2,38)} = 20.31$, $p < 0.0001$), genotype ($F_{(1,19)} = 7.819$, $p = 0.0115$) and interaction $F_{(2,38)} = 3.774$, $p = 0.0320$). Bonferroni post-hoc test showed a significant effect between groups in the Day 1 ($p = 0.0009$). The difference in the total break points expressed as mean in the three days was significant (Student's *t* test $p = 0.0083$) (Fig. 19 a-b).

As regards the number of rewards obtained, we found a trend for a significant difference between GPR83 KO and WT mice (time $F_{(2,38)} = 20.04$ p, < 0.0001; genotype $F_{(1,19)} = 3.893$, $p = 0.0632$; interaction $F_{(2,38)} = 2.369$, $p = 0.1072$). Bonferroni post-hoc test showed a significant effect between groups in the Day 1 ($p = 0.0372$). The difference between the total number of rewards obtained expressed as mean for three days was significant (Student's *t* test $p = 0.0051$) (Fig. 19 c-d)

Finally, the time spent to complete the task showed a significant difference between the two groups (time $F_{(2, 38)} = 0.6630$, $p = 0.5212$; genotype $F_{(1,19)} = 6.589$, $p = 0.0189$; interaction $F_{(2, 38)} = 0.7227$, $p = 0.4920$). Bonferroni post-hoc test showed a significant effect between groups in the Day 1 ($p= 0.0258$). The difference in the mean of the time in three days was significant (Student's *t* test $p = 0.0189$) (Fig. 19 e-f).

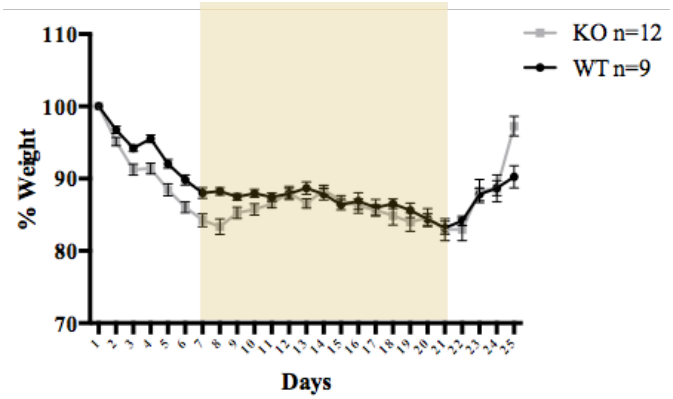

Fig. 17. *Graphic representation of the % of weight loss in WT and GPR83 KO mice during the operant behavior protocol. Statistical analysis was carried out by RP two-way ANOVA (time $F_{(24,528)} = 55.73$, $p < 0.0001$; genotype $F_{(1,22)} = 2.240$; $p = 0.1487$; time x genotype: $F_{(24,528)} = 4.74$, $p < 0.0001$). Data are shown as mean ± SEM and the number of animals is shown in the figure.*

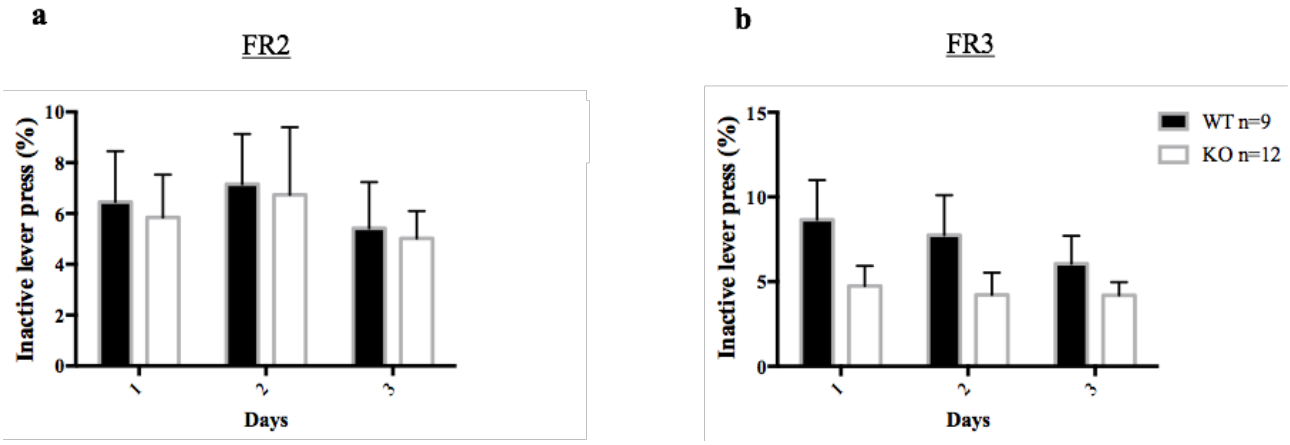

Fig. 18. Percent of inactive lever press during the FR2 and FR3. *Difference in learning in term of number of inactive lever pressed in FR2 (**a**) and in FR3 (**b**). According to RP two-way ANOVA there was no significant difference between GPR83 KO and WT mice. Data are shown as mean ± SEM and the number of animals is shown in the figure.*

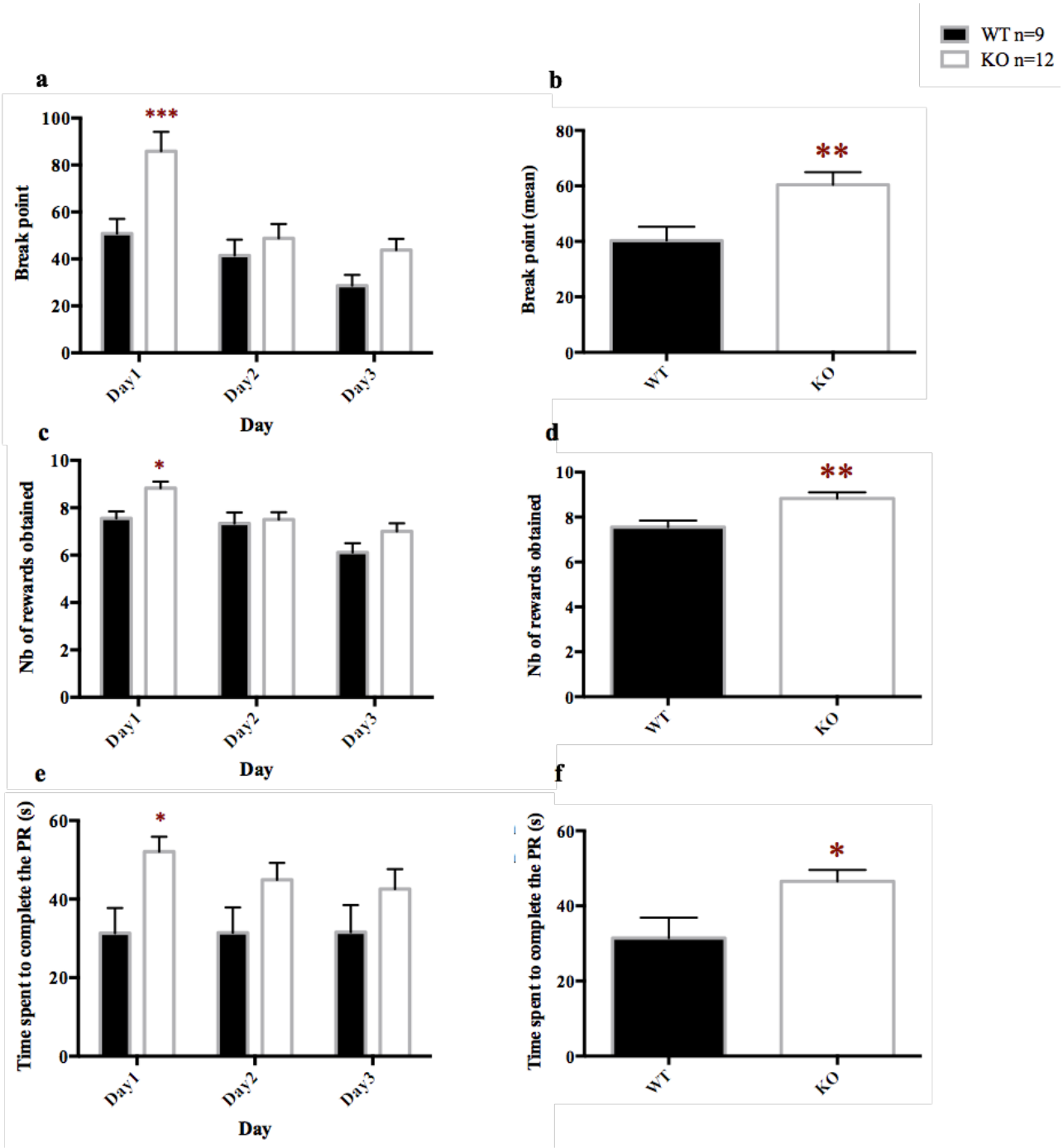

Fig. 19. Motivated food behavior in GPR83 KO and WT mice. ***(a)*** *Break point in three consecutive days. The RP two-way ANOVA showed a time effect ($F_{(2,38)} = 20.31$, $p < 0.0001$), genotype ($F_{(1,19)} = 7.819$, $p = 0.0115$) and time x genotype interaction $F_{(2,38)} = 3.774$, $p = 0.0320$). Post-hoc analysis was performed by Bonferroni test (KO vs. WT in the Day1, $p = 0.0009$).* ***(b)*** *Total break points expressed as mean in three days (Student's t test $p = 0.0083$).* ***(c)*** *Number of rewards obtained in three consecutive days. The RP two-way ANOVA showed a time effect ($F_{(2,38)} = 20.04$ p, < 0.0001), a trend for a significant effect for genotype ($F_{(1,19)} = 3.893$, $p = 0.0632$) and no time x genotype effect ($F_{(2,38)} = 2.369$, $p = 0.1072$). Post-hoc analysis was performed by Bonferroni test (KO vs. WT in the Day1, $p = 0.0372$).* ***(d)*** *Total numbers of rewards obtained expressed as mean for three days (Student's t test $p = 0.0051$).* ***(e)*** *Time spent to complete the task. The RP two-way ANOVA showed a genotype effect ($F_{(1,19)} = 6.589$, $p = 0.0189$), but no time ($F_{(2, 38)} = 0.6630$, $p = 0.5212$) and interaction effect ($F_{(2, 38)} = 0.7227$, $p = 0.4920$). Post-hoc analysis was performed by Bonferroni test (KO vs. WT in the Day1, $p = 0.0258$).* ***(f)*** *Total time spent to complete the PR expressed as mean in three days (Student's t test $p = 0.0189$). Data are shown as mean ± SEM and the number of animals is shown in the figure.*

Effects of acute PEN microinjection in CPu and NAc in the open field test.

Previous in vitro studies showed high affinity of PEN for the GPR83 in hypothalamic and hippocampal membranes, which are brain areas that display a high expression of the receptor (Pisani et al. 2001). The receptor appears highly expressed also in the striatum (Pesini et al. 1998), thus we investigated the behavioral effects of PEN microinjected into the CPu and NAc.

The purpose of the first in vivo experiment was to test the effect of the injection of PEN into the dorsal striatum in a novel environment.

The injection of PEN directly into the CPu did not show an effect on the distance traveled for all the doses that we tested (Fig.20).

Instead, we found some significant effect on the anxiety like-behavior. When we injected 0.5 µg/µL PEN, during the first 5' post-injection the mice spent less time in the internal zone of the arena. The RP two-way ANOVA revealed a time effect ($F_{(5,45)} = 2.526$, $p = 0.0424$) and a treatment effect ($F_{(1,9)} = 6.389$, $p = 0.0324$), but no interaction ($F_{(5, 45)} = 1.489$, $p = 0.2124$) (Fig. 22). On the contrary, a higher dose (2.5 µg/µL) reverted the effect, with an increase of the time in the internal zone, implying a decrease of anxiety-like behavior (treatment: $F_{(1,9)} = 5.529$, $p = 0.0432$; time: $F_{(5, 45)} = 1.230$, $p= 0.3107$; interaction: $F_{(5, 45)} = 0.6918$, $p= 0.6323$) (Fig. 23).

The lowest (0.1 µg/µL) and the highest (5 µg/µL) dose did not show any difference between peptide treatment and saline treatment (Fig. 21, 24).

After these experiments, that show an effect of PEN in the anxiety-like behavior, we decided to repeat the experiment injecting the peptide into the NAc, but in this case bilaterally to have a more pronounced effect and using only two doses.

Also, in this case there was no difference in the locomotor activity for both of the doses injected (Fig. 25).

In the first 5' after the injection of 0.5 µg/µL of PEN, mice showed an increase in the time spent in the internal zone, implying a decrease of the anxiety-like behavior (Student's *t* test, $p = 0.0226$) (Fig. 26); this effect was not found after the injection of 1.5 µg/µL (Fig. 27).

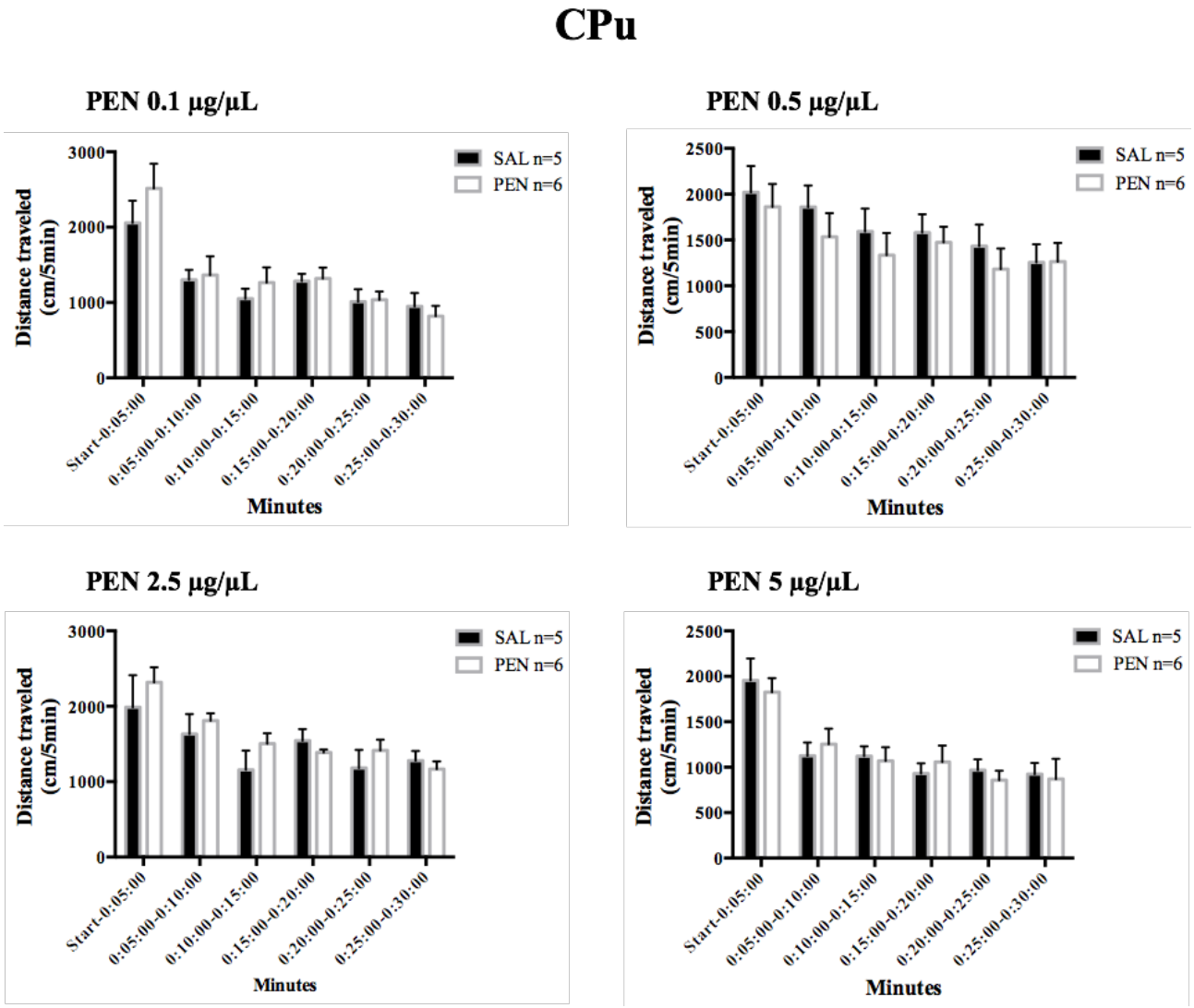

Fig. 20. Acute locomotor effects of PEN microinjection into the CPu. *0.1, 0.5, 1.5 and 2.5 µg/µL PEN injected into CPu have no effect on the locomotor activity. Data are shown as mean ± SEM and the number of animals is shown in the figure.*

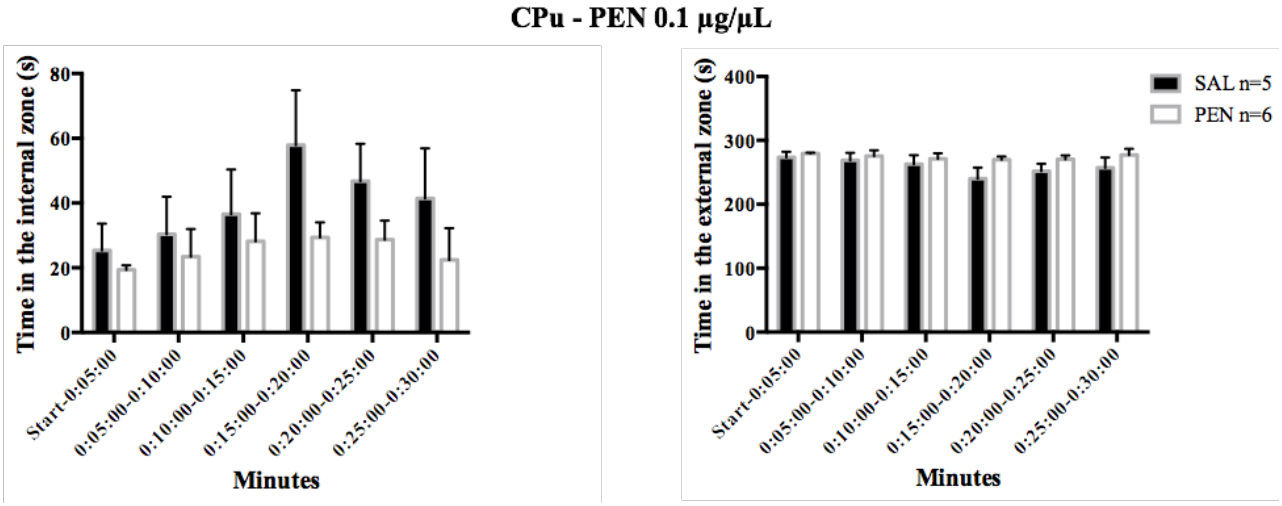

Fig. 21. Acute effects of 0.1 µg/µL PEN unilateral microinjection into the CPu in open field . *0.1 µg/µL PEN has no behavioral effect in the open field test. Data are shown as mean ± SEM and the number of animals is shown in the figure.*

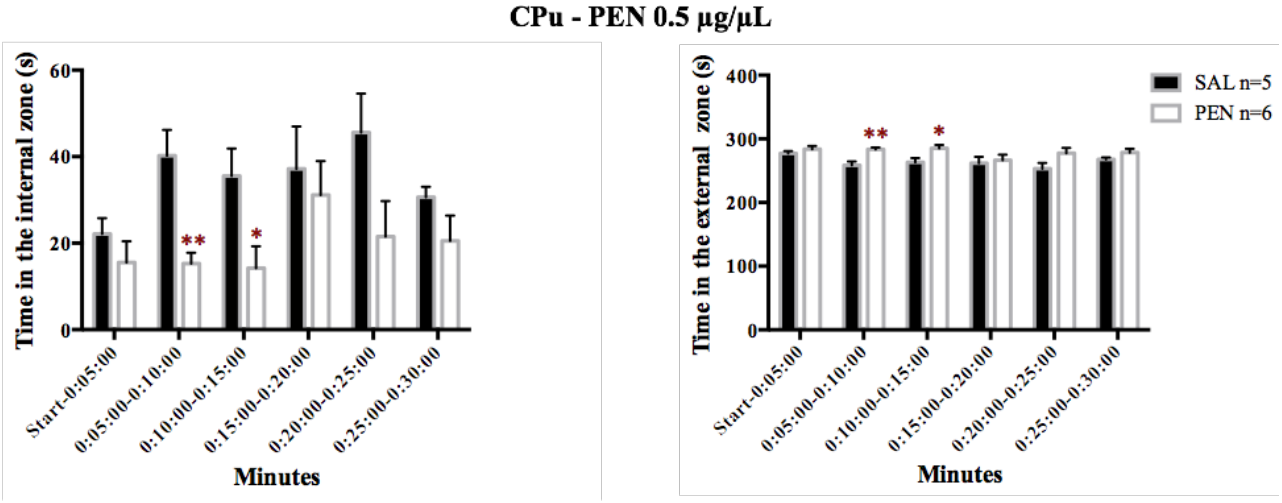

Fig. 22. Acute effects of 0.5 µg/µL PEN unilateral microinjection into the CPu in open field . *0.5 µg/µL PEN decreases the time spent in the internal zone (RP two-way ANOVA: time $F_{(5,45)} = 2.526$, p= 0.0424; treatment $F_{(1,9)} = 6.389$, p= 0.0324; time x treatment $F_{(5,45)} = 1.489$, p= 0.2124), and increases the time in the external zone (RP two-way ANOVA: time $F_{(5,45)} = 2.567$, p= 0.0398; treatment $F_{(1,9)} = 6.004$, p= 0.0367; time x treatment $F_{(1,9)} = 6.004$, p= 0.0367). A Student's t test revealed a significant difference in the internal zone during the 5-10' interval (p=0.0025) and the 10-15' interval (p= 0.0264), and in the external zone during the 5-10' interval (p= 0.0028) and the 5-10' interval (p= 0.0263). Data are shown as mean ± SEM and the number of animals is shown in the figure.*

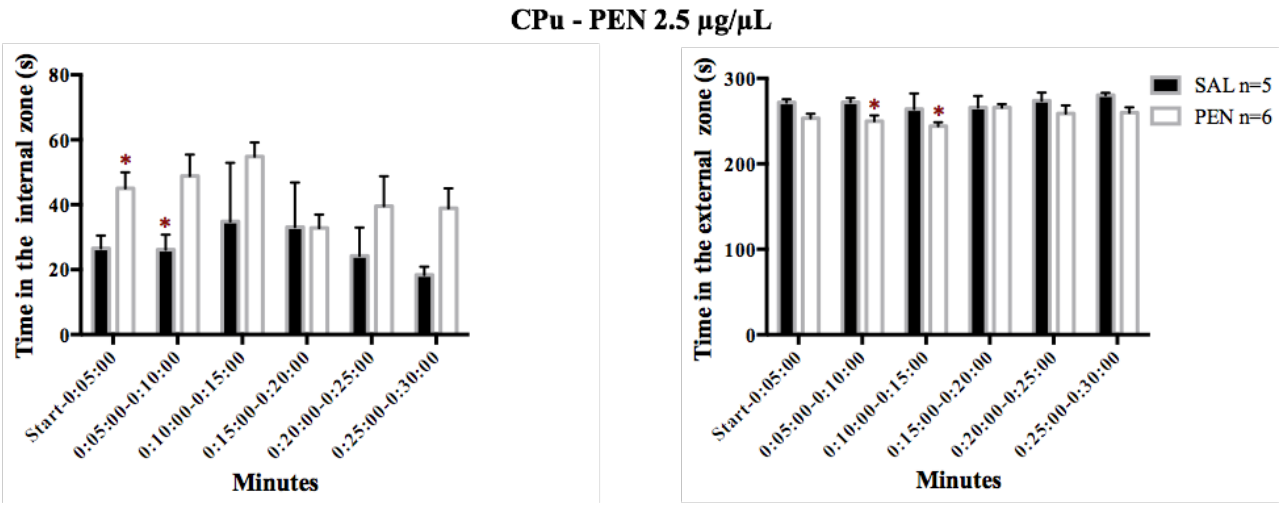

Fig. 23. Acute effects of 2.5 µg/µL PEN unilateral microinjection into the CPu in open field *2.5 µg/µL PEN causes an increase of the time spent in the internal zone (RP two-way ANOVA: time $F_{(5,45)} = 1.230$, p= 0.3107; treatment $F_{(1,9)} = 5.529$, p= 0.0432; time x treatment $F_{(5,45)} = 0.6918$, p= 0.6323) and a decrease in the time in the external zone (RP two-way ANOVA: time $F_{(5\ 45)} = 1.149$, p= 0.3491; treatment $F_{(1,9)} = 5.246$, p= 0.0477; time x treatment $F_{(1,9)} = 5.246$, p= 0.0477). A Student's t test revealed a significant difference in the internal zone in the 0-5' interval (p= 0.0199) and 5-10' interval (p= 0.0240), and in the external zone in the 0-5' interval (p=0.0207) and 5-10' interval (p= 0.0290). Data are shown as mean ± SEM and the number of animals is shown in the figure.*

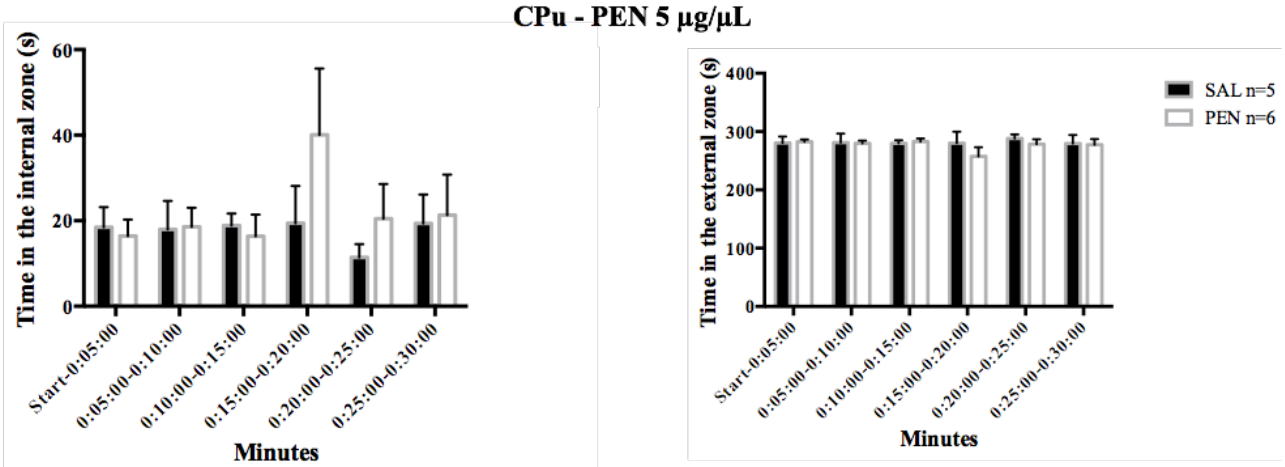

Fig. 24. Acute effects of 5 µg/µL PEN unilateral microinjection into the CPu in open field. *5 µg/µL PEN has no behavioral effect in the open field. Data are shown as mean ± SEM and the number of animals is shown in the figure.*

NAc

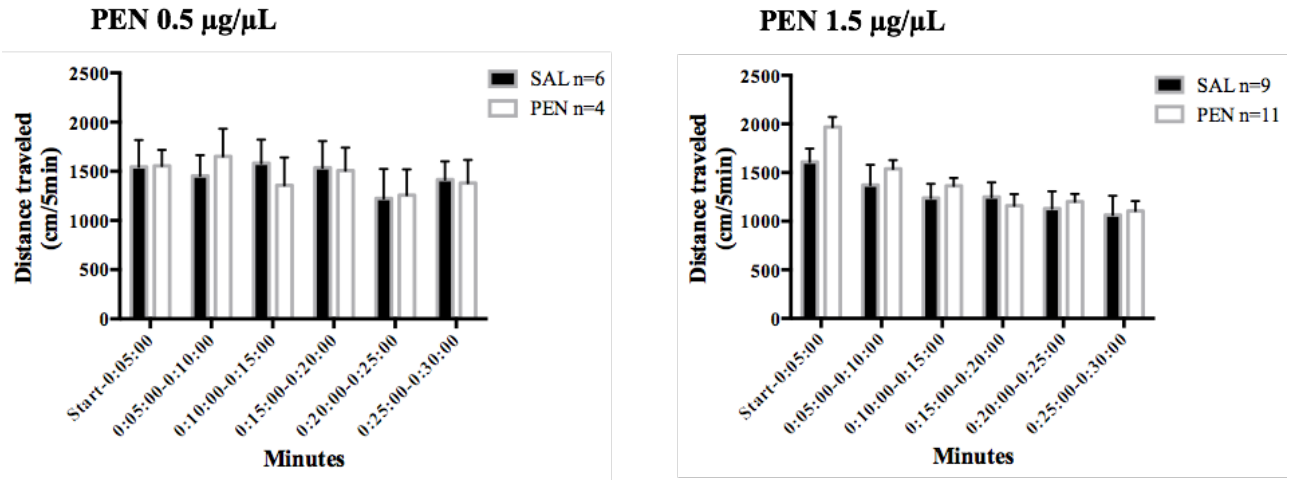

Fig. 25. Acute locomotor effects of PEN microinjection into the NAc. *0.5, and 1.5 µg/µL PEN into NAc have no effect on the locomotor activity. Data are shown as mean ± SEM and the number of animals is shown in the figure.*

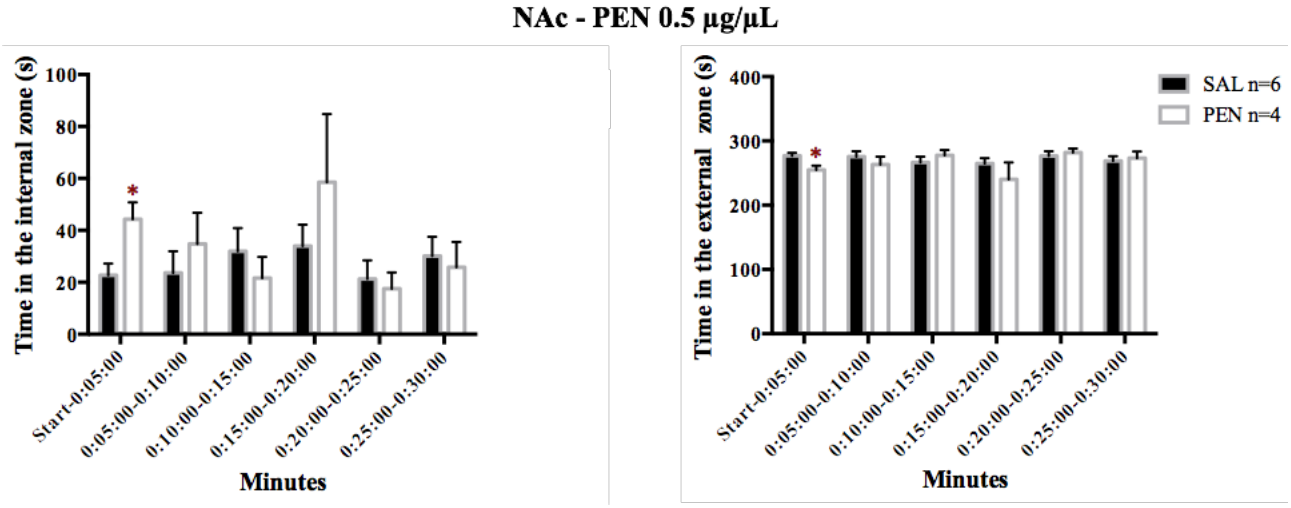

Fig. 26. Acute effects of 0.5 µg/µL PEN bilateral microinjection into the NAc in an open field. *0.5 µg/µL PEN cause an increase of the time spent in the internal zone immediately after the injection (Student's t test p= 0.0226), and a decrease of the time spent in the external zone (Student's t test p= 0.0237). Data are shown as mean ± SEM and the number of animals is shown in the figure.*

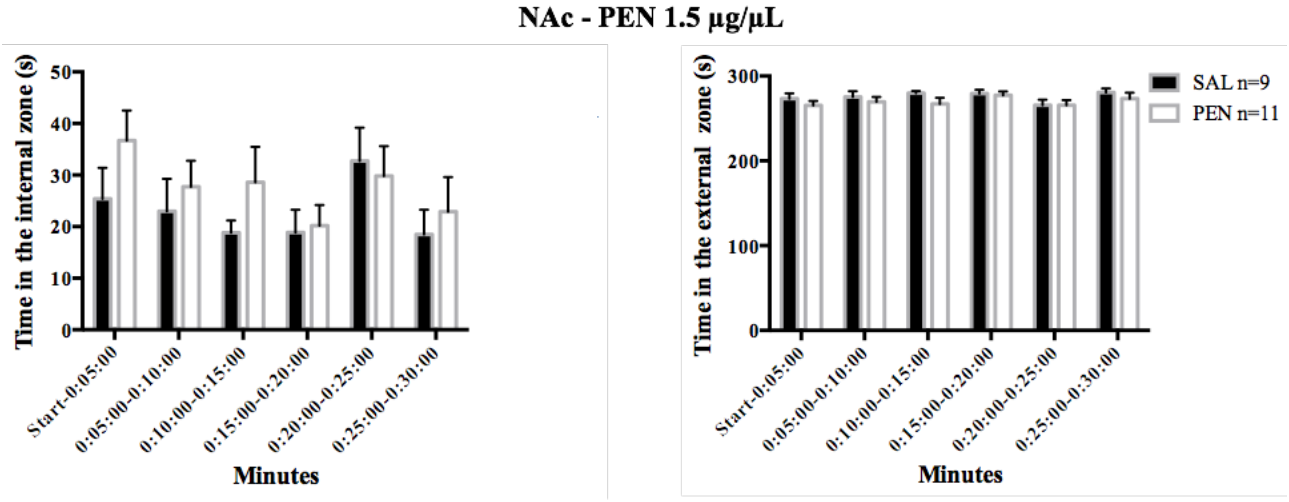

Fig. 27. Acute effects of 1.5 µg/µL PEN bilateral microinjection into the NAc in an open field *1.5 µg/µL PEN has no behavioral effect in the open field test. Data are shown as mean ± SEM and the number of animals is shown in the figure.*

DISCUSSION

We started to analyze the role of GPR83 in locomotor activity in a novel environment using GPR83 KO mice and WT mice in their active (dark) and inactive phase (light). During the dark phase we did not find any significant difference in terms of locomotor activity, whereas during the light phase the KO mice showed an hyperlocomotion compared with WT mice. The light could be considered as stress factor in a novel environment, suggesting a higher sensitivity of KO mice to stressful conditions.

Stress has been defined by various physiological changes that include activation of the HPA axis (Dallman et al. 1995). Another widely adopted definition of stress is any alteration in psychological homeostatic processes (Burchfield 1979). A further neurobiological mechanism demonstrated in acute stress in rodents is the increased levels of neurotransmitters in mesolimbic pathway, for instance the increase in dopamine and acetylcholine neuron activity and extracellular levels (Sorg and Steketee 1992) (Abercrombie et al. 1989) (Imperato et al. 1991) (Imperato et al. 1992).

GPR83 was initially defined as a stress-responsive gene because it is significantly down-regulated in principal brain areas linked to stress (ventral and dorsal striatum, hippocampus and different hypothalamic nuclei) following dexamethasone administration (Adams et al. 2003); in addition, Vollmer and coworkers found that GPR83 has a role in sensitivity to stress since GPR83 KO mice were insensitive to the behavioral effects of restraint stress test, though they did not show any significant difference in the effects of this test on HPA axis (Vollmer et al. 2013).

The open field test is thought to evaluate two components of mouse behavior, locomotion and anxiety both induced by the novel environment represented by the cage where the mouse is put.

Total locomotion in a novel environment is an index of spontaneous exploratory behavior in rodents, and exploration is naturally a dark phase behavior for mice. In the dark, GPR83 KO mice did not show any significant difference from WT mice suggesting that when the novel environment is modestly stressing, GPR83 transmission is not relevant. Instead, KO mice showed a significant hyperlocomotion in the light phase. Overall, these results indicate that hyperlocomotion in GPR83 KO mice is not due to an alteration in the mechanisms of locomotion but rather on the impact that stress and/or novelty exert on locomotion control. As a consequence, GPR83 transmission can be hypothesized to be physiologically involved in stress and/or anxiety circuits rather than in locomotion circuits.

In WT mice the permanence of the animal in the open field arena causes a progressive reduction in locomotion that is due to the progressive reduction of the novelty and the consequent exploratory activity.

On the contrary, increased locomotion in KO mice is more evident at late times of the test, suggesting that the animal maintains a higher sensitivity to the novel environment and the associated stressful situation. At cellular/molecular level a possible mechanism that could underlie the observed behavioral alterations in GPR83 KO mice is either an intrinsic hyperdopaminergic state or an increased reactivity to stress stimuli.

In the open field test, reduced time in the central part of the arena is considered an index of anxiety. Rodents are spontaneously disposed to remain close to the walls of the cage to exploit the protection of a side from where no danger can arrive. Accordingly, anxious states increase the time past close to the peripheral walls while anxiolytic drugs increase the time spent in the less protected central zone. The analysis of anxiety-like behavior in a novel environment did not show any significant difference between the genotypes in the light phase, but only in the dark phase, as showed by the markedly decreased time spent by the GPR83 KO mice in the internal zone of the open field arena. This evidence clearly implies GPR83 transmission in the physiological counter-regulation of anxiety states and tallies well with the evidence discussed above of an increased sensitivity to stressful stimuli in GPR83 KO mice.

GPR83 expression is predominant in brain regions regulating motor activity such as the CPu and NAc. We noted a difference in locomotor activity inside an open field in a situation of novelty under light condition. This suggests that GPR83 is physiologically recruited to suppress the stress response elicited by novelty. Moreover, the presence of GPR83 in limbic areas such as the amygdala, hippocampus and prefrontal cortex suggested its role in the orchestration of emotional outcomes related to anxiety and fear. Accordingly, GPR83 deficient mice showed anxiety-like behavior in the open field during the dark phase. Overall, known regional expression pattern of GPR83 tallies well with the behavioral alterations we detected in GPR83 KO mice, though a finer behavioral characterization and the use of region-specific KO mice will be necessary to better define the involvement of GPR83 transmission in specific circuits.

Then, we investigated the role of GPR83 in the response to addictive drugs, namely the psychostimulant cocaine.

The acute administration of cocaine after habituation to a novel environment induces hyperlocomotion that typically exceeds novelty-induced increased locomotion. Acute cocaine effects were different between GPR83 KO and WT mice; in fact, the cocaine-elicited locomotor activity in KO was significantly higher compared to WT mice, in the period following the peak cocaine effect though not at

peak level (5 minutes post-injection) or in the late plateau (more than one hour post-injection). This pattern of change suggests that GPR83 transmission influences the activity of specific subcircuits mediating cocaine-elicited hyperlocomotion.

Cocaine is a psychostimulant that blocks dopamine transporter (DAT), and to a lesser extent noradrenaline transporter (NET) and serotonin transporter (SERT), thus increasing dopamine extracellular levels. The higher concentration of dopamine in the synaptic space determines the increased locomotion in animals acutely treated with cocaine (Rocha 2003). On the one hand, these results imply a role of GPR83 in the response to cocaine effects. On the other hand, as in the case of the open field test, the analysis of cocaine hyperlocomotion points to a physiological regulation of dopamine-related striatal mechanisms by GPR83 transmission.

Repeated exposure to cocaine results in a progressive and long-lasting enhancement of the locomotor response to the drug, a phenomenon called locomotor sensitization. The enhanced locomotor activity following daily cocaine treatments has been associated with an increase in extracellular dopamine levels in the mesolimbic dopamine system. Sensitized responses can be observed after several weeks, months, or up to at least a year of drug-free period (Lodge and Grace, 2011). In rodents, sensitization has been shown to correlate with enhanced predisposition to self-administer psychostimulants (Schenk and Partridge 2000) (Vezina et al. 2002) and reinstatement of extinguished self-administration (De Vries et al. 1998) (Suto et al. 2004).

Interestingly, during the second day of cocaine injection, GPR83 KO mice did not show increased cocaine-elicited locomotion compared to WT mice. Our results show an absence of sensitization in KO mice, suggesting a possible involvement of GPR83 transmission in the processes leading to drug addiction.

In agreement with the evidence of high levels of GPR83 expression in the hypothalamus, a recent study discovered an influence of GPR83 in feeding and metabolism (Müller et al. 2013). Hypothalamus has a crucial role in energy balance and food intake, however is also associated to neural networks that integrate other factors involved in appetite, such as sensory factors, emotional processing, decision making and learning. These processes are important for the normal homeostatic mechanisms that drive the motivation to food and are determinants of when to eat or not. Ingestion of food provides a form of reward, particularly if the food is rich in simple sugars or fat, and eating can be a source of comfort in depression or stressful states (Kelley et al. 2005).

The amygdala is more generally involved in assigning emotional valence, including reward (Baxter and Murray 2002). In particular the basolateral amygdala, another region where GPR83 is highly expressed, has been the subject of numerous behavioral studies examining how this structure might play a role in reward processes (Hatfield et al. 1996)(Balleine, Killcross, and Dickinson 2003). GPR83 is expressed at high levels in several regions, that constitute key stations of the brain reward circuit, such as hypothalamus, amygdala, and ventral striatum,. Additionally it was already demonstrated an association of GPR83 with hedonic and reward-related behavior (Vollmer et al. 2013).

These considerations led us to hypothesize an involvement of the receptor in motivation for food. Operant conditioning tasks provide an effective means to evaluate changes in the motivational properties of food. The experimental procedures that we performed permit to train mice to lever press for food rewards and stably respond on a PR schedule of reinforcement. PR schedule are used to evaluate the reinforcing efficacy of a self-administered drug or food by increasing the response requirements for each successive reinforcement and determining the break point at which the animal will no longer respond (Arnold and Roberts 1997).

To investigate the involvement of GPR83 in motivation and reward we performed a PR task. In the training phase (FR schedule) we required that the animals press the correct lever to obtained a food pellet, being the percent of inactive lever press considered as an index of learning. The results showed notsignificant difference between GPR83 KO and WT mice in the FR1, FR2 and FR3. Instead, as shown from the data on break point, number of rewards obtained and the time to complete the task, the motivation to get food tested by PR schedule was higher in GPR83 KO compared to WT mice. These results imply GPR83 transmission in the physiological regulation of circuits controlling food reward.
Mice with chronically elevated dopamine levels showed a high motivation to food but no deficit in the acquisition of Pavlovian learning (Wise 2004) (Cagniard et al. 2006). Again this suggests the possibility that GRP83 KO mice have increased activity of dopamine neurons in the mesolimbic system.

The behavioral evidence pointing to a hyperdopaminergic transmission in striatum of GPR83 KO mice and the histological evidence for strong expression of GPR83 in ChAT positive cells of the striatum suggest a possible functional explanation at circuit level. ChAT positive TANs control striatal dopamine transmission (Exley and Cragg 2008). Experiments in reconstituted systems indicate that GPR83 activates PLC and induces an increase of Ca^{2+} in target cells (Gomes et al. 2016). GPR83 transmission

into ChAT neurons would therefore stimulate these neurons while inactivation of GPR83 would depress ChAT activity thus disinhibiting dopamine release (Rizzi and Tan 2017).

The experiments discussed above on GPR83 KO mice showing the behavioral alterations induced by the suppression of the endogenous activity of this receptor, clearly establish a physiological involvement of GPR83 in a number of brain functions including control of anxiety, stress response and food reward. In a further series of experiments we wanted to test the behavioral effects of GPR83 activation using the brain region-specific administration of GPR83 agonists.

Acute microinjection of several proSAAS-derived peptides was performed from different Authors to assess their effect on feeding, showing an increase in food intake. These results confirm the orexigenic role proposed for the proSAAS peptides (Wardman et al. 2011)(Ye et al. 2017).

To test the behavioral effects of the GPR83-specific ligand PEN, we injected the peptide into the CPu or the NAc. In general, we found behavioral effects in parameters related to stress and anxiety at the test of the open field.

The injection of 0.5 μg/μL PEN into the CPu had an effect on anxiety parameter, whereas a dose 5 times higher (2.5 μg/μL PEN) showed an opposite effect. The lowest (0.1 μg/μL) and highest (5 μg/μL) doses injected into the CPu did not show any significant effect.

Considering the involvement of GPR83 in anxiety-like behavior showed by the experiments with KO mice and the high expression of GPR83 in NAc, we decided to test the peptide also in this region. Our data showed a reduction of anxiety behavior in a novel environment after bilateral injection of 0.5 μg/μL PEN into the NAc, whereas 2.5 μg/μL had no significant effect.

The expression of GPR83 in the NAc is much higher compared to CPu; for this reason it is possible that a lower dose of the peptide is necessary to activate neurons in the ventral striatum compared to the dorsal striatum.

Ye and colleagues (2017) showed an effect of intracerebral injection of PEN into the NAc of rats to assess the role of proSAAS peptides in locomotor activity. They found that 10 μg/0.5 μL PEN increases locomotor activity in rats. In our study, we did not find any effect on locomotion injecting PEN either into the CPu or the NAc. This difference may due to species-specific expression patterns of GPR83. In fact, mice and rats have different patterns of GPR83 expression in ventral striatum. In mice there is a strong labeling, while in rats the expression is restricted to scattered cells (Pesini et al. 1998) (Sah et al. 2005) (Brézillon et al. 2001b). Microinjection into NAc may therefore activate GPR83 in different cell populations in these two rodent species and therefore elicit different effects on locomotor activity.

Overall, the central effects of PEN confirm a role of the GPR83 in neural systems involved in anxiety and stress-related behavior, but not directly in locomotion. Moreover, the restricted window of effective doses and the opposite effects of different doses in certain behaviors suggest that the receptor is expressed by different neuronal populations in the striatal circuits, with opposite functional roles. Therefore, a finer analysis of these circuits will be required using cell-specific approaches such as coupling inactivation of GPR83 expression within specific populations with shRNAs and in vivo region-specific injections.

CONCLUSION

The present study provides the first evidence of a physiological involvement of the GPR83 in the modulation of behavioral effects related to stress response in a novel environment, cocaine-elicited locomotion and motivation for food. Additionally, the central effect of the specific GPR83 ligand PEN suggest an involvement in anxiety-related behaviors.

Future experiments are required to define which specific neuronal populations express GPR83 and what are their involvements in the behaviors that are regulated by GPR83. Specific attention should be devoted to the influence of GPR83 expressing neurons on dopaminergic transmission whose implication has been repeatedly suggested by present behavioral evidence.

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ACKNOWLEDGEMENTS

It is not easy to thank in a few words all the people who made a contribution during my PhD years. However I will try to do my best to extend my acknowledgements to all those who have helped me on a scientific and emotional level.

I cannot begin to express my thanks to my supervisor Prof. Michele Zoli, for his unwavering support, guidance and insight throughout these years and this project.

I am also grateful to Prof. Alban de Kerchove D'Exaerde, who gave me an opportunity to join his team at the Université Libre de Bruxelles. Thank you for the warm welcome, the precious support and for helping me start and finish this work.

I would also like to extend my deepest gratitude to Patrick Laurent. This thesis would not have been possible without your advices, your encouragement and the motivation you gave me during my experience in Brussels. Especially for the good coffee every morning in your office.

Many thanks to all the neuroscience group of Brussels for the support and help during my experience in their laboratory of Neurophysiology. In particular to Katerina, Adria, Elisa, Elena, Ramiro, Lars, Pierre, for being not only my coworkers but also friends. I have very fond memories of my time there. Many thanks in addition to all the people who helped me finish the experiment in time.

Particularly helpful to me during this PhD were my Italian coworkers. To Antonietta for her teachings and her laboratory experience during these years; to Giuseppina and Eleonora who have always been there to help me. But most of all for making these three years fun and enjoyable even during the working hours.

Moreover, I would like to extend my thanks to the people I met during these years at the University of Modena: Chiara, Martina, Arianna, Carmela, Anna, Federica, Ilaria, Laura, Marcello, Michele and Simone. I am glad to have shared this experience with all of you.

Some special words of gratitude go to my friends who have always been a major source of support when things were a bit discouraging: Sara, Piero, Valeria, Giacomo, Luca, Andrea F., Andrea A., Marina, Michela, Manuel and Giulia. Thanks guys for always being there for me.

Finally, last but not the least, also to my parents, who believed in me and wanted the best for me. And in particular to my brother and best friend Benedetto, who supported me in every possible way.