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Master thesis

The interplay of autophagy and the κ- opioid receptor in the nervous system

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Abstract

The κ- opioid system, composed of the κ- opioid receptor (κ-OR) and its endogenous ligand dynorphin, is widely expressed in the CNS. Apart from pain perception and analgesia, the κ-opioid system is implicated in the manifestation and treatment of several diseases, such as anxiety and depression disorders, drug addiction and epilepsy. Moreover, activation of the κ-OR directly regulates neurotransmission and synaptic plasticity in key brain regions, such as the cortex, the hippocampus, the ventral striatum and several midbrain structures, however the molecular mechanisms that serve these functions are not well characterized. Recent observations from Dr. Georgoussi lab indicate that activation of the κ-OR by the specific agonist U50,488H induces autophagy in the neuroblastoma Neuro2A cell line (unpublished data). Autophagy is a dynamic and tightly regulated multistep procedure, responsible for the degradation of malfunctioning or superfluous proteins, macromolecules and organelles in order to maintain cellular homeostasis and quality control. The aim of this master thesis was to examine the interplay between κ-OR activation and autophagy in the nervous system, under physiological conditions. Our results demonstrate that activation of κ-OR can induce autophagy in a dose and time dependent manner *in vitro* in primary neuronal cultures and upregulate *de novo* autophagosomal biogenesis. Moreover, our preliminary data support that activation of κ-OR *in vivo* differentially regulates autophagy in the mouse cortex, hippocampus and striatum. These findings will be the starting point of my PhD study, where we intent to further investigate the role of κ-OR induced autophagy *in vivo* and access its involvement in physiology and behavior.

Key words:
Autophagy, LC3, nervous system, kappa opioid receptor, U50,488H, *Mus musculus*

A. Introduction

A.1. Opioid receptors

Opioid receptors belong to the superfamily of G protein coupled receptors (GPCRs) and are widely expressed in the central and peripheral nervous system, exerting their analgesic actions. There are three types of opioid receptors, mu (μ-OR), delta (δ-OR) and kappa (κ-OR), which share 50-70% homology between their genes (*Stein, 2016).* For each type there are additional subtypes, arising from receptor oligomerization, post-transcriptional and post-translational modifications *(Law et al, 2013).* Alternative classifications include in the opioid receptor family a fourth receptor, the nociceptin-orphanin opioid receptor (NOP), which exhibits high structural similarity but lacks of common pharmacological properties and thus is not considered a classical opioid receptor *(Terenius & Johansson, 2010)*.

Structurally, opioid receptors consist of 7 transmembrane alpha helices that are aligned around a central ligand-binding pocket and are connected with three extracellular and three intracellular loops. The transmembrane regions are conserved between the three types of opioid receptors, whereas the extracellular and intracellular loops show more extensive variation, conferring ligand selectivity and unique trafficking properties to the receptors *(Cox 2012).* The endogenous ligands of opioid receptors derive from three independent genes that encode the precursor proteins proopiomelanocortin, proenkephalin and prodynorphin. Proteolytic cleavage of the precursor proteins leads in the production of the major opioid peptides β-endorphin; Leu- and Met-enkephalin; and different dynorphins including dynorphin A, dynorphin B, big dynorphin, a/b-neo-endorphin, respectively. These peptides exhibit different affinity and selectivity for the μ-OR (β-endorphin, Met-enkephalin), δ-OR (Leu-enkephalins, β-endorphin), and κ-OR (dynorphin) *(Mansour et al., 1995)*. Furthermore,

opioid receptors bind exogenously administered natural and synthetic opiates, such as morphine, heroin, oxycodone and salvinorin A, that mimic the functions of endogenous ligands *(Trescot et al, 2008)*.

Ligand binding results in conformational changes of the receptor, allowing intracellular coupling of heterotrimeric Gi/o proteins. At the Gα subunit, GTP replaces GDP and dissociation of the trimeric G protein complex into Gα and Gβγ subunits follows. The Gα subunit inhibits adenylyl cyclase and activates the MAPKs, whereas the Gβγ heterodimer directly blocks Na^{+} and Ca^{2+} ion channels in the membrane, and activates G protein–coupled inwardly rectifying K+ (GIRK) channels, resulting in decreased action potential generation and presynaptic inhibition of neurotransmitter release *(Standifer & Pasternak, 1997)*. Moreover, the intracellular domain of the opioid receptors serves as a platform for the interaction with numerous proteins, such as RGS proteins, β-arrestins and GRKs, which tightly regulate the receptor signaling and intracellular trafficking *(Georgoussi et al, 2012)*.

A.2. The κ- opioid system

The κ-opioid system is involved in a broad spectrum of physiological responses, such as pain perception and analgesia, water diuresis, lipid metabolism and inflammation. *(Simonin et al 1995, Brooks et al, 1993, Czyzyk et al, 2010, Chuang et al, 1995, Finley et al, 2008)*. Similarly with μ-ORs and δ-ORs, the κ-ORs are distributed not only in the CNS and PNS, but also in other peripheral systems such as the enteric nervous system and the immune system.

A.2.1. Functional Anatomy

In the mammalian brain, the κ-ORs and its endogenous ligand dynorphin are expressed in multiple locations, which do not necessarily coincide. The discrepancies in the literature arise from either differences in the sensitivity of the employed technical approaches, such as mRNA *in situ* hybridization, ligand binding autoradiography, immunohistochemistry or inter-species and gender disparities *(Racz et al, 1998, Merrer et al, 2009)*. Nevertheless, most publications commonly accept the fact that the κ- opioid system is expressed in the cortex, dorsal and ventral striatum, hippocampus, amygdala, thalamus, as well as monoaminergic midbrain structures such as the VTA and PAG *(Benarroch et al, 2012, Crowley & Kash, 2015).*

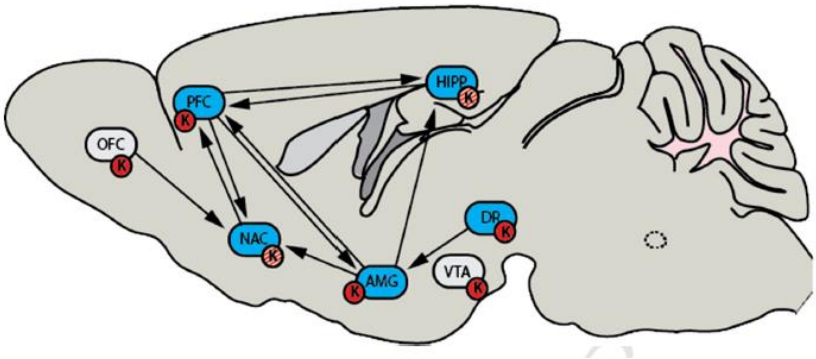

Figure 1: The κ-opioid system is widely expressed in the mouse brain. Key regions involve the cortex and numerous subcortical regions, such as the hippocampus, the nucleus accumbens, the amygdala, the ventral tegmental area and the dorsal raphe nuclei. Adapted from *Crowley & Kash, 2015*.

In the hippocampus, κ-OR immunoreactivity appears to be more dense in the dentate gyrus, and particularly in the granule cells axons, which constitute the mossy fiber pathway connecting the dentate gyrus to CA3, and the terminals of perforant path, which provide input from the entorhinal cortex to the dentate gyrus. Dynorphins are also abundantly localized in the granule cells, mostly in the mossy fibers, but also in dendrites and granule

cells somata *(Drake et al, 2007)*. As far as the remaining hippocampal areas are concerned, although literature appears to be controversial, findings support the expression of the κ-opioid system beyond the dentate gyrus *(Halasy et al, 2000)*.

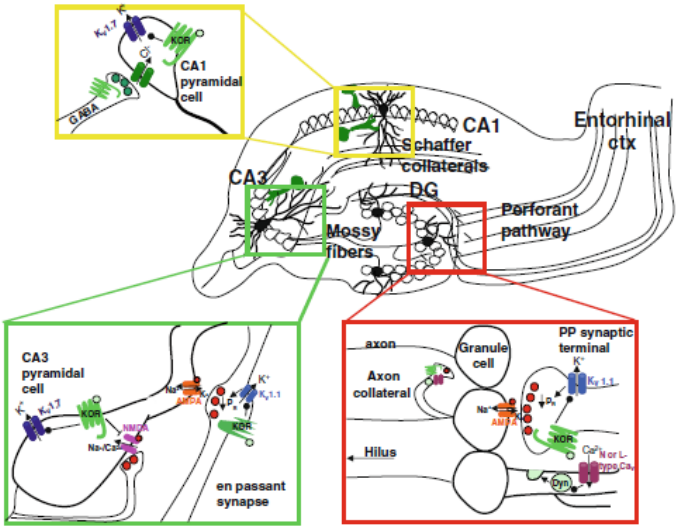

Figure 2: In the hippocampus the κ-OR is localized mainly in the granule cells of the dentate gyrus, but also in the CA1 and CA3 areas. Adapted from Lemos & Chavkin 2010.

A.2.2. κ-OR regulates physiology and behavior

Due to its widespread localization, the κ-opioid system is a master regulator of a plethora of physiological responses. It is of great importance to notice that even though the distributions of the μ-, δ- and κ- opioid systems largely overlap, activation of κ-OR has numerous distinct and in some cases opposing effects compared to μ-OR and δ-OR (as described in Table 1); for instance, activation κ-OR leads in a dysphoric state whereas μ-OR activation produces euphoria *(Lemos & Chavkin 2010)*. These effects can be either short term or long term and are served by alterations of normal neurotransmission and synaptic plasticity.

Comparison of actions of μ and κ opioid receptors

Parameter	μ Receptor	κ Receptor	Reference
Feeding	Stimulation	Stimulation	(Cooper et al. 1985; Gosnell et al. 1987; Jackson and Cooper 1985; Morley et al. 1985)
Drinking	Decrease	Increase	(Hartig and Opitz 1983; Locke et al. 1982)
Locomotion	Increase	Decrease	(Iwamoto 1981)
Place conditioning	Preference	Aversion	(Mucha and Herz 1985)
Submissive behavior	Decrease	Increase	(Benton 1985; Benton et al. 1985)
Seizures	Convulsant	Anticonvulsant	(Lee et al. 1989; Tortella et al. 1986, 1987)
Urinary output	Antidiuretic	Diuretic	(Smith et al. 2008)
Body temperature	Hyperthermia	Hypothermia	(Chen et al. 2005; Handler et al. 1992)
Dopamine release	Increase	Decrease	(Di Chiara and Imperato 1988)
Serotonin release in dorsal raphe	Increase	Decrease	(Tao and Auerbach 2002, 2005)

Table 1: Opposing action of μ- and κ- opioid receptors. Adapted from Wee & Koob, 2010.

As mentioned above, the signaling cascade downstream activation of the κ-OR results in presynaptic inhibition of neurotransmitter release, postsynaptic hyperpolarization and decrease in neuronal excitability. It is well established that activation of κ-OR by dynorphin or exogenous agonists, such as U69,593 and U50,488H, inhibit LTP and facilitate LTD in *in vitro* and *in vivo* electrophysiological recordings in the hippocampus *(Wagner et al, 1993)*, amygdala *(Huge et al, 2009)* and striatum *(Atwood et al, 2014)*. Interestingly, κ-OR exerts its neuro-modulatory functions not only on glutamatergic excitatory neurons, but also on inhibitory interneurons and DAergic neurons in the NAc, VTA, BNST and amygdala, regulating excitation – inhibition balance and neurotransmitter release *(Graziane et al, 2013, Tejeda et al, 2017, Margolis et al, 2003, Crowley et al, 2016, Przybysz et al, 2017, Gilpin et al, 2014)*.

The hippocampal synaptic plasticity constitutes another example of κ-OR/μ-OR opposing actions. In the dentate gyrus, endogenous enkephalins acting on μ-OR and δ-OR facilitate LTP, either by hyperpolarizing GABAergic interneurons *(Madison and Nicoll, 1988)*, or by blocking GABAergic input and thus inducing disinhibitory effects *(Neumaier et al., 1988)*.

On the contrary, endogenous dynorphins have been extensively shown to block LTP at three synapses: the perforant pathway to molecular layer, the mossy fiber pathway to CA3 pyramidal cell layer and recurrent collateral contact of mossy fiber pathways to dentate granule cells *(Drake et al, 2007).*

A.2.2.1. Stress, anxiety and depression

These neuroplastic alterations on key forebrain and midbrain regions constitute the molecular mechanism that κ-opioid system exploits to regulate a broad spectrum of behavioral responses beyond nociception and analgesia. To begin with, it is well documented that κ-OR / dynorphin mediate anxiety and stress related behaviors. Stress has been shown to increase endogenous dynorphin levels and up regulate κ-OR signalling in the NAc and CA3 region of the hippocampus *(Shirayama et al, 2004)*, while administration of κ-OR antagonists have been shown to exert consistent anxiolytic effects in different animal models (reviewed in *Hang et al, 2015).* Furthermore, ablation of κ-OR from brain dopamine neurons produced anxiolytic effects, confirming that the regulation of dopaminergic neurotransmission by κ-ORs is critical for expression of stress and anxiety *(Van't Veer et al, 2013).*

Several preclinical and clinical data highlight the role of κ-OR antagonists as potent antidepressants; currently there are two separate phase 3 and phase 2 clinical trials evaluating the κ-OR antagonists ALKS 5461 and CERC-501 as treatment for major depressive disorder and treatment resistant depression respectively *(clinical trials.gov).* This is in line with evidence supporting that chronic administration of κ-OR agonists decrease BDNF mRNA and protein levels in the cortex and hippocampus, while acute treatment with the κ-OR antagonist nor-BNI increase BDNF expression in the hippocampus and the amygdala *(Droga et al, 2015, Zhang et al, 2007)* .

A.2.2.2. Addiction and Reinstatement of drug abuse

The dysphoric and negative reinforcing properties of κ-OR agonists raise the possibility of potential therapeutic value to treat addiction *(Shippenberg, 2009).* Indeed, a wealth of studies demonstrates that κ-OR agonists functionally attenuate cocaine-induced place preference *(Shippenberg et al, 1996),* cocaine self-administration *(Schenk et al, 1999),* as well as ethanol reward *(Longrip et al, 2009).* These effects are mediated by the inhibition of dopamine release on the mesolimbic reward pathway *(Wang et al 2010).*On the other hand, under stress conditions, κ-agonists can potentiate the rewarding effect of psychostimulants and enhance condition place preference (CPP). Likewise, κ-antagonism attenuates stress-induced potentiation of cocaine reinforcement by blocking the actions of endogenous dynorphins that are released during stress *(Mague et al, 2003, McLaughlin et al, 2003).* Therefore, is has been suggested that antagonists of κ-ORs may represent powerful therapeutic tools for protecting individuals from relapse to drug abuse. In a follow up study by the Chavkin group, the significance of temporal correlation between the κ-OR activation and cocaine administration has been examined. κ-OR activation by the selective agonist U50,488H 60 minutes prior to cocaine potentiated the cocaine-CPP, whereas administration of U50,488H 15 minutes before cocaine suppressed the cocaine-CPP *(McLaughlin et al, 2006).* Additional studies must be conducted to further delineate how the κ-opioid system interacts with the reward and stress systems on the network level to enhance or inhibit drug abuse.

A.2.2.3. Epilepsy

Ample preclinical and clinical evidence supports the implication of hippocampal κ- opioid system in temporal lobe epilepsy (TLE) and drug induced seizures. In the clinical population, pro-dynorphin is upregulated in the dentate gyrus of patients with TLE *(Houser et al, 1990,*

Pirker et al., 2009) and polymorphisms in the promotor of the pro-dynorphin gene are associated with vulnerability to develop TLE *(Stongmann et al, 2002, Gambardella et al, 2003).* In animal models of epilepsy, hippocampal dynorphin reduces pilocarpine-induced seizure activity *(Bausch and Chavkin, 1997)* and pro-dynorphin knockout mice show decreased seizure threshold and faster seizure onset, a phenotype that is rescued after administration of the κ-OR agonist U50,488H *(Loacker et al, 2007).* Moreover, exogenously administered κ-OR agonists have been well documented to protect against maximal electroshock-, pilocarpine- and bicuculline- induced convulsions *(Bausch et al., 1998, Yajima et al., 2000, Manocha et al., 2003).*

A.3. Autophagy

Autophagy is a major catabolic process, responsible for the degradation of superfluous or damaged intracellular constituents. Unlike the Ubiquitin-Proteasome System (UPS) that only degrades specific ubiquitinated proteins, autophagy can target several types of macromolecules and organelles, such as proteins, lipids, ribosomes, nuclei, ER and mitochondria, to safeguard cellular homeostasis and produce new building blocks. Apart from its housekeeping role under physiological conditions, autophagy is upregulated in response to nutrient deprivation, in order to recycle cytosolic components and compensate for the negative energy equilibrium.

There are three types of autophagy: microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy. In microautophagy, invaginations of the lysosomal membrane directly engulf small molecules, while in CMA the chaperone HSC70 and its co-chaperones deliver substrate proteins with a KFERQ amino acid motif to the lysosomal protein LAMP2A, which

in turn translocates them across the membrane into the lysosome for degradation. On the contrary, macroautophagy, hereafter referred to simply as autophagy, is a multistep complex mechanism that entails the formation of a new organelle, the autophagosome, which allows the delivery of the autophagic cargo to the lysosome.

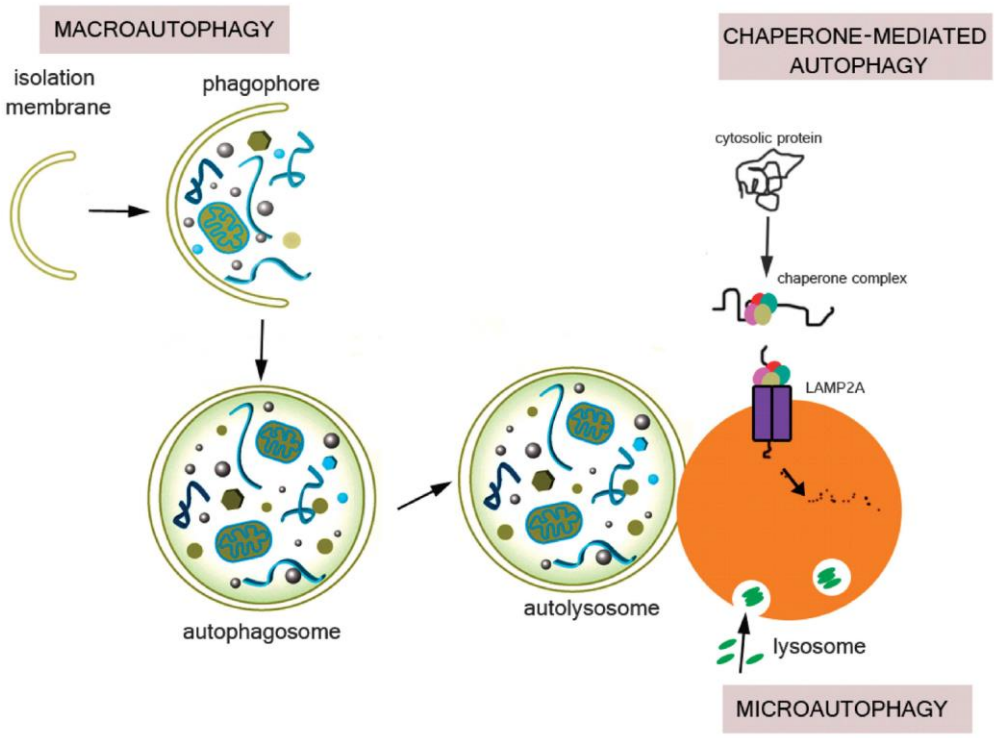

Figure 3: There are three mechanistically distinct types of autophagy. In chaperone-mediated autophagy substrates with the KFERQ motif are recognized by HSC70 and delivered to the lysosome in a LAMP2A dependent manner. In microautophagy substates are directly engulfed in the lysosome. On the contrary, macroautophagy is a multistep process, characterized by the formation of the autophagosome, a unique double membrane structure that contains the autophagic cargo and fuses with the lysosome to degrade its constituents. Adapted from Nikoletopoulou et al., 2015.

Although once characterized as a bulk process, it is now well appreciated that autophagy is highly selective process *(Stolz, et al, 2014).* In the heart of this selectivity lies the LC3 protein, a dedicated marker of the autophagosomal structure. The lipidated form LC3-II is

incorporated both in the inner and outer membranes of the growing phagophore and can directly interact with autophagic protein substrates through the LIR (LC3 interacting region) motif. Moreover, there are several autophagy receptors or adaptor proteins, the role of which is to interact with both target proteins and LC3 and lead to substrate into the autophagosome. Characteristic examples of adaptor proteins are p62/sequestosome 1 (SQSTM1), optineurin, NDP52, NBR1 and Alfy.

A.3.1. The molecular mechanism of autophagy

Initiation

In mammalian cells, key players in autophagic mechanisms are the autophagy-related (ATG) proteins. Autophagy is initiated with the self-assembly of the pre-autophagosomal structure (PAS) to form a cup shaped membranous formation known as the phagophore or isolation membrane. Key molecules of this step are the PAS components, namely the Ulk1, FIP200, ATG13 and ATG101, which comprise the Ulk1 complex. The activated Ulk1 complex recruits the (Ptdlns)3- kinase complex (including Ambra1, Beclin 1, Atg14(L)/barkor, Vps15 and Vps34) at the phagophore assembly site.

Vesicle Nucleation and Elongation

Afterwards, a specific ER structure is formed, named the omegasome, that serves as a membranous platform for the formation of the phagophore and the initiation of autophagosome biogenesis. Key modulators of vesicle nucleation are DFCP1 και WIPI proteins. Subsequently, the phagophore elongates with the involvement of two ubiquitin-like conjugated complexes. The first complex results in the covalent conjugation of ATG12 to ATG5, aided by ATG7 and ATG10, and the non-covalent conjugation of ATG12-ATG5 with

ATG16-like 1(ATG16L1). The second complex facilitates the maturation of LC3 (ATG8) into LC3-I by the ATG4 protease and then the conjugation of LC3-I to phosphatidylethanolamine (PE) by ATG7 and ATG3 to form the PE-LC3 / LC3-II protein. The LC3-II is integrated into the autophagosomal membrane and is involved in maturation of autophagosomes and cargo recognition as mentioned above *(Nikoletopoulou et al, 2015)*. When the elongation is completed, the two ends of the phagophore fuse together to create the double membrane mature autophagosome that encapsulates the autophagic cargo.

Fusion with lysosome and degradation

When the autophagosome reaches complete formation, proteins involved in autophagosome formation are released and recycled. Upon maturation, complete autophagosomes fuse with endosomes or lysosomes to produce amphisomes or autolysosomes. Inside the autolysosomes, the autophagic cargo finally is degraded lysosomal hydrolases.

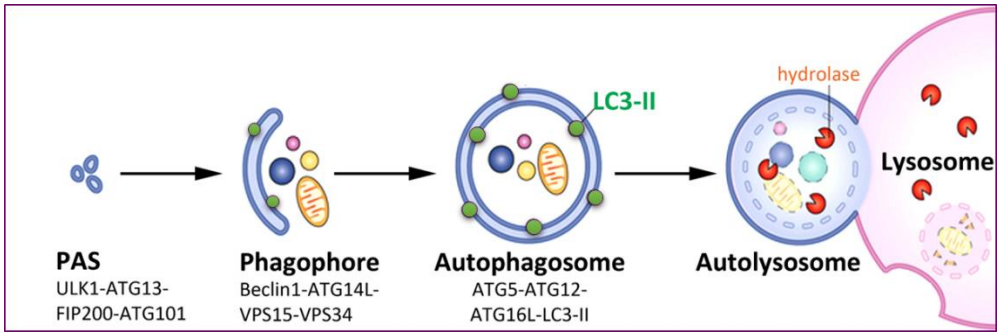

Figure 4: Each step of the autophagic process is characterized by the expression of specific ATG and other proteins. The *de novo* formation of autophagosomes begins with the assembly of the pre autophagosomal structure (PAS) that further nucleates and expands to form the phagophore or isolation membrane, which sequestrates cellular constituents targeted for degradation. Elongation of the phagophore leads in the formation of the autophagosome, which further fuses with a lysosome to form the autolysosome and enable degradation of the autophagic cargo. Adapted from Kaur & Debnath, 2015.

A.3.2. Regulation of autophagy

The classical regulation of autophagy is governed by the serine/threonine protein kinase mTOR, which negatively regulates this process. The mTOR pathway apart from autophagy also regulates other vital cellular functions such as translation and cell growth. Three distinct signalling pathways converge on mTORC1 activation to inhibit autophagy; the Rag/mTORC1 pathway that senses availability of amino acids and nutrient sufficiency, the PI3KC1a/Akt/TSC/mTORC1 pathway that senses growth factors sufficiency and the AMPK/TSC/mTORC1 pathway that integrates intracellular stress signals and energy levels *(Sarkar, 2013).* The active mTORC1 can in turn phosphorylate the Ulk1 and ATG13 proteins, resulting in the inhibition of their kinase activity and subsequent prevention of PAS complex assembly and autophagosomal biogenesis *(Jung et al, 2010).*

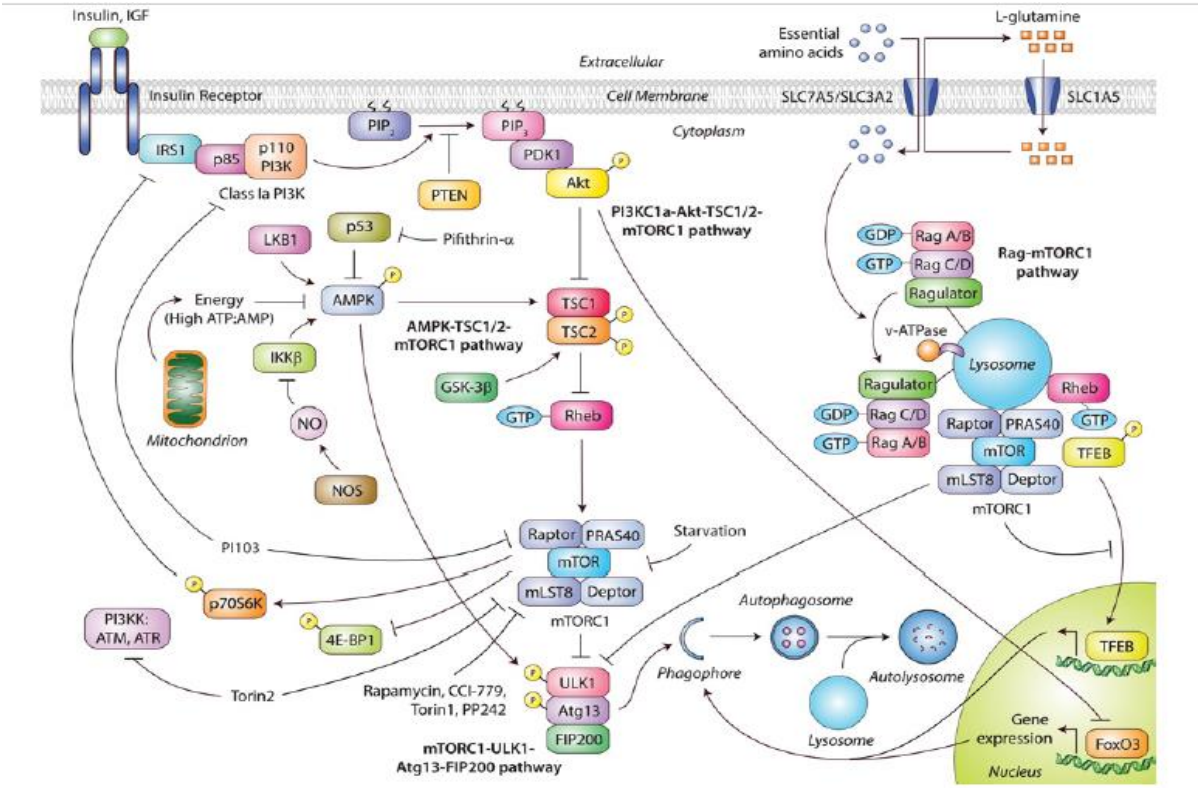

Figure 5: Regulation of autophagy by mTOR-dependent pathways. Adapted from Sarkar, 2013.

Moreover, there are mTORC1-independent signaling pathways that regulate autophagy in different steps. Firstly, G proteins-mediated activation of PLC increases IP3 and DAG intracellular levels resulting in release of Ca^{+2} from the ER and inhibition of autophagosomes synthesis. Additionally, modulation of ion channels conductance may lead to increased intracellular Ca^{+2} and subsequent calpain activation to block autophagy in later steps. Finally, the JNK1/Beclin-1/PI3KC3 pathway can positively regulate autophagy, since the active JNK phosphorylates both Beclin-1 and Bcl-2, resulting in the dissociation of the autophagy-inhibitory complex Beclin-1–Bcl-2, thus promoting the formation of the autophagy-stimulatory complex Beclin-1–hVps34 *(Sarkar, 2013).*

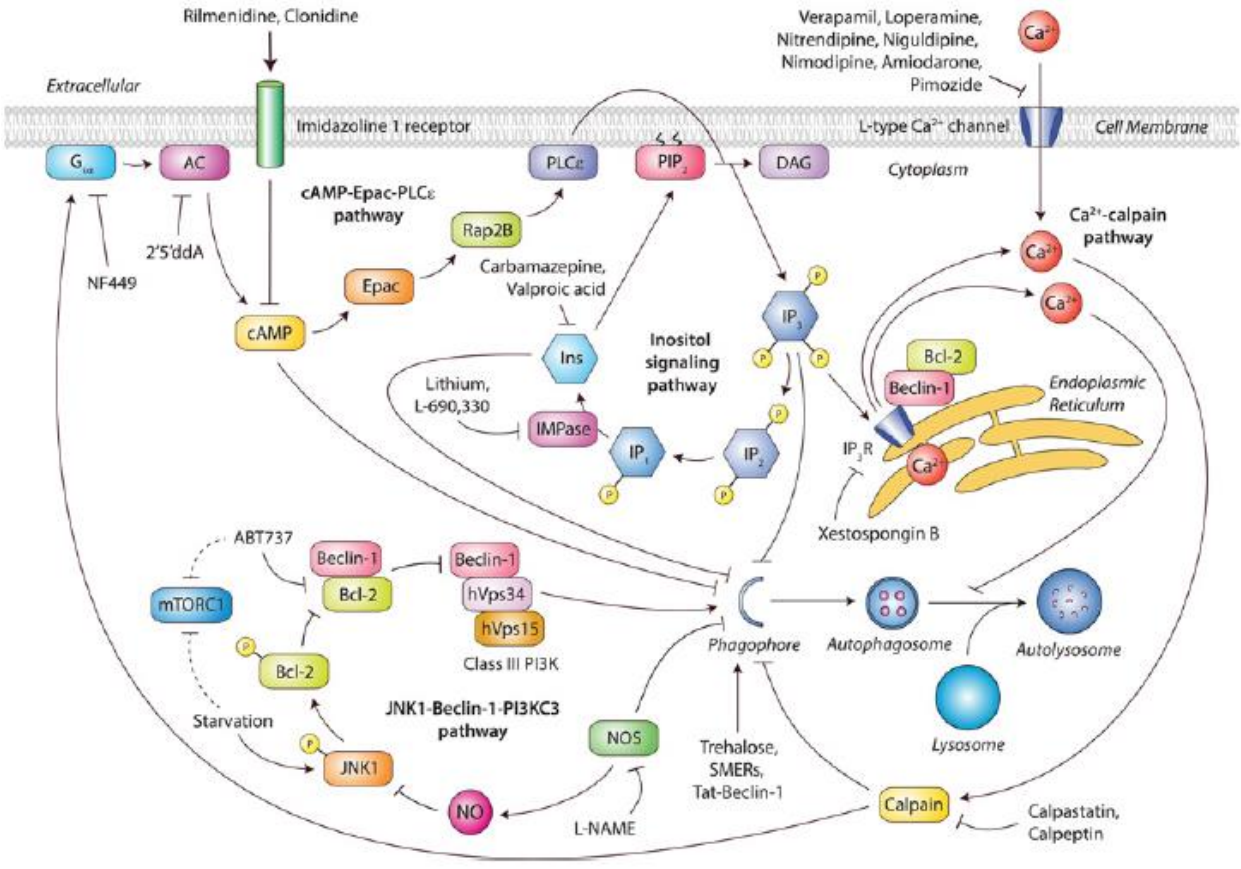

Figure 6: Regulation of autophagy by mTOR-independent pathways. Adapted from Sarkar, 2013

A.3.2.1. Distinct features of neuronal autophagy regulation

Among all possible ways to induce autophagy, starvation represents the most widely studied paradigm. *In vitro*, nutrient deprivation of neuronal cell cultures, similarly to other cell types, leads to suppression of TOR signalling and thus induction of autophagy *(Young & La Spada, 2009)*. However, the situation *in vivo* appears to be more complex, as the effects of starvation are not uniform in different brain areas. Our recent work demonstrated that fasting upregulates autophagy in the hypothalamus, but suppresses autophagy in the cortex and hippocampus. Interestingly, in pre-adult mice, fasting resulted in an increase of autophagy in all examined brain areas, suggesting that autophagy *in vivo* is both regionally and temporally differentially regulated *(Nikoletopoulou et al, 2017).*

The same study highlighted the role of BDNF, the major growth factor of the CNS, on the regulation of neuronal autophagy. BDNF application suppresses the autophagic flux *in vitro* through activation of PI3K/Akt pathway, resulting in transcriptional downregulation of several autophagic genes. As anticipated, *in vivo* ablation of BDNF in the neural lineage was found to increase baseline autophagy in the mouse cortex and hippocampus regulated *(Nikoletopoulou et al, 2017).*

A.3.3. Autophagy in the CNS

Autophagy is a well-orchestrated and tightly regulated process that controls a plethora of crucial physiological functions. The examination of tissue specific autophagy knockout animals has revealed the critical impact of autophagy implication in metabolism *(Kim & Lee,. 2014)*, immunity, inflammation *(Levine et al, 2011)*, differentiation and development *(Mizushima & Levine, 2010)*. Furthermore, it is now well appreciated that autophagy is

involved in the manifestation of severe pathologies such as cancer and numerous infectious, neurodevelopmental and neurodegenerative diseases, such as autism spectrum disorders, lysosomal storage disorders, Parkinson's, Alzheimer's and Huntington's diseases *(Rubinsztein et al, 2012, Tang et al 2014, Nah et al, 2015).*

Focusing on the CNS, it is increasingly appreciated that basal autophagy, referring to autophagy that is constitutively active under physiological conditions, is indispensable for neuronal maintenance and quality control. Even though autophagosomes accumulation is very low in neurons under stress free conditions, recent data support that this is due to the highly efficient neuronal autophagic machinery, which constantly degrades the autophagic cargo, rather than the absence of basal autophagy. As indicated by experiments using pharmacological manipulations to block autophagosome-lysosome fusion, neurons are capable of possessing a rapid autophagic flux, which does not allow autophagosomes to accumulate in high levels *(Nikoletopoulou et al, 2015*). Mice with ablation of autophagy in the neural lineage exhibit late-onset neurodegeneration causing a significant reduction in the number of pyramidal neurons in the cerebral cortex and of Purkinje cells in the cerebellar cortex *(Hara et al., 2006; Komatsu et al., 2006)*. However, it is difficult to infer which of these effects can be attributed to autophagy deficiency in neurons rather than glial cells.

Recent evidence emphasizes the role of neuronal autophagy on synaptic pruning, a developmental procedure that takes place during adolescence to eliminate excess dendritic spines in the cortex and employs LTD-like mechanisms *(Tang et al, 2014).* Additionally, in line with pre-existing evidence, our recent work revealed that autophagy directly regulates synaptic plasticity, as on the one hand suppression of autophagy is necessary for BDNF induced enhancement of LTP and on the other hand autophagy dynamically participates in

synapse remodeling, by directly degrading key synaptic proteins *(Nikoletopoulou et al, 2017).* Furthermore, unpublished work from our lab suggests that both NMDAR- and mGluR- dependent LTD induce a rapid and sustained increase in neuronal autophagy, by increasing the levels of PAS components, and that autophagy is required for the induction of LTD *(Kallergi et al., submitted).* Interestingly, the role of autophagy on the pre-synaptic site appears to be more clear, as it may regulate neurotransmitter release, trafficking of synaptic vesicles, as well as homeostasis of mitochondria *(reviewed in Nikoletopoulou & Tavernarakis, 2018).*

A.4. Opioid receptors and autophagy: an unexplored relationship

A.4.1. μ-OR mediated autophagy

Given the malleable nature of autophagy, it is stands to reason to wonder if opioids and opioid receptor mediated signaling exploit the autophagic machinery to induce some of their beneficial and adverse effects. Although morphine has a high abusive potential, its extraordinary analgesic and euphoric effects render it a commonly administered and highly abused drug. Thus, the majority of researchers choose morphine as the prototypical opioid to address their scientific questions. Treatment with morphine has been shown to upregulate numerous autophagic markers, such as LC3-II, ATG proteins, members of the Ulk1 complex and Beclin1, in several *in vitro* systems, such as the human neuroblastoma SH-SY5Y cell line *(Zhao et al, 2010),* the rat pheochromocytoma PC12, the rat glioma C6 *(Feng et al, 2013)* and primary cultures such as human primary astrocytes *(Cao et al, 2016),* human microglia *(El-Hage et al, 2015),* human pulmonary microvascular endothelial cells

(HPMECs) *(Dalvi et al, 2016),* and mouse and rat primary hippocampal *(Cai et al, 2016),* cortical *(Feng et al, 2013)* and microglial *(Pan et al, 2017)* cultures. Furthermore, *in vivo* acute or chronic morphine administration has been documented to induce autophagy in the rat *(Zhao et al, 2010)* and mouse *(Feng et al, 2015, Cai et al, 2016, Pan et al, 2017)* hippocampus.

However, the signalling pathway leading from μ-OR activation to induction of autophagy, as well as the physiological impact of the autophagic upregulation are poorly understood.

Feng et al, suggested that morphine induces mitochondrial defects that subsequently are substrate to autophagic degradation, resulting in mtDNA copy number reduction and that the increased autophagy in primary neurons negatively regulates neurite outgrowth and dendritic arborization, assessed by Tuj1 staining of neurons.

Pan et al supported that morphine upregulates autophagy in the hippocampus *in vivo,* as a protective, pro-survival response towards morphine induced neuronal death and subsequent spatial memory deficit. Additionally, they propose that autophagy mediates the morphine-induced reduction of inflammatory cytokines in hippocampal microglia.

Cai et al provided evidence that chronic morphine administration alters synaptic plasticity in the hippocampus, resulting in a reduction of spines as well as of excitatory synapse densities and enhancement of inhibitory synapse densities, via the upregulation of ROS production and ER stress that ultimately induce autophagy. Interestingly, they proved that pharmacological and genetic manipulations that block autophagy rescue the morphine- induced deficits of synaptic plasticity, recognizing autophagy as a critical regulator of morphine-mediated synaptic alterations.

A.4.2. κ-OR mediated autophagy

Currently, there are no published data addressing the question whether activation of κ-OR by a specific agonist can induce autophagy. Nonetheless, two independent publications state that methamphetamine induces autophagy in a κ-OR dependent manner, as indicated by upregulation of LC3-II and Beclin1 levels, in human umbilical vein endothelial cells (HUVECs), SVGA astrocytes and human fetal astrocytes *(Ma et al, 2014, Cao et al, 2016)*. Additionally, both papers attribute methamphetamine-induced autophagy with a pro-survival role, as pharmacological inhibition of autophagy upon methamphetamine treatment further increases methamphetamine evoked apoptotic cell death.

In agreement with the aforementioned results, unpublished data from Dr. Georgoussi lab indicate that κ-OR activation by the specific κ-OR agonist U50,488H induces autophagy in a dose dependent manner, in the mouse neuroblastoma cell line Neuro2A stably expressing the human κ-OR. The signalling pathway leading from κ-OR activation to autophagic upregulation is currently under investigation.

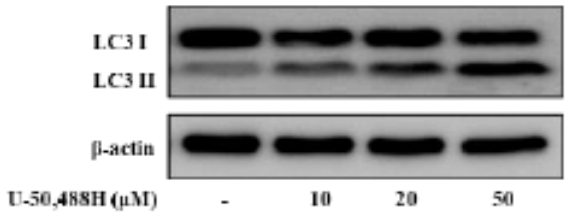

Figure 7: Activation of the κ-opioid receptor (κ-OR) by t-he specific agonist U50,488H for 6 hours can dose dependently upregulate LC3-II expression in Neuro2A cells stably expressing the κ-OR.

A.4.3. κ-OR directly interacts with autophagic proteins

Although κ-OR does not contain a LIR motif (LC3-interacting region motif), and thus is unlikely to be a direct substrate of autophagic degradation *(Wild et al, 2014)*, it was

intriguing to find that it can directly interact with GABARAPL1 (GEC1) *(Chen et al, 2006)*. GABARAPL1 along with GABARAP-1, GABARAP-2, GATE-16 and MAP1LC3 and yeast protein Atg8 comprise the GABARAP family, all members of which are involved in protein and vesicle intracellular transport, as well as the formation and elongation of autophagosomes *(Chakrama et al, 2010)*. Despite the early belief that GABARAP proteins serve redundant functions in the autophagic mechanism, nowadays it is well established that the MAP1LC3 proteins are involved in the elongation of the double-membrane phagophore, while GABARAP-1, -2 and -L1 are required for the late maturation of the autophagosomes *(Guittaut et al, 2014)*. The exact outcome of κ-OR and GABARAPL1 interaction is not yet adequately defined, but evidence support that in neuronal cell lines overexpressing both proteins, GABARAPL1 enhances κ-OR total and surface expression and is implicated in κ-OR transport from ER/Golgi to plasma membranes *(Chen et al, 2011, Le Grand et al, 2011)*. More studies must be done to elucidate a possible role of κ-OR GABARAPL1 interaction regarding autophagy.

B. Aim of the study

The aforementioned studies indicate the breadth of functions that are served by autophagy in the nervous system, as well as the key modulatory role of the endogenous opioid system in neural physiology and pathology. Sparse evidence suggests the implication of autophagy in μ-OR mediated neural functions, such as morphine- induced memory impairment and neuronal apoptosis. Additionally, it has been proven that opioid administration may directly induce autophagy in vitro via the μ- and κ- opioid receptor. However, the interaction of autophagy and the κ-OR in neurons remains elusive.

The aim of this study is to directly examine the interplay between κ-OR activation and autophagy, under physiological conditions. Firstly, we tested whether activation of κ-OR by a specific agonist can induce autophagy *in vitro* in neuronal primary cultures. Secondly, we assessed whether activation of κ-OR *in vivo* may directly induce autophagy in the mouse brain and further characterized the specific brain regions that κ-OR induced autophagy occurs.

C. Results

C.1. U50,488H can dose-dependently induce autophagy *in vitro.*

We firstly sought to confirm that κ-OR activation may induce autophagy in primary neuronal cultures. To this end, we isolated and culture cortical neurons from B6 embryos at E15.5-16.5 for 10 days *in vitro* (DIV10) and treated them with 10-50μM U50,488H, a specific κ-OR agonist, for 6 hours. Additionally, to ensure that the observed effects resulted from κ-OR activation, we pre-treated neurons with a saturating concentration (50μM) of the general opioid receptor antagonist naloxone for 45 minutes. Cell lysates were analysed with Western blot and the levels of LC3-II were assessed. As mentioned above, LC3-II, which is the lipidated form of the LC3 protein, is highly associated with autophagosomes and consist a dedicated marker of autophagy *(Klionsky et al, 2016).* Our results indicate that U50,488H can dose dependently increase the LC3-II levels, by activating the κ-OR, even at the low concentration of 10μM (Figure 8 A, B).

Moreover, we prepared primary cortical cultures of Thy1-EYFP/Cre neurons, treated them with 10μM U50,488H for 6 hours and analyzed immunocytochemically against LC3 levels. In these cells, the Thy1 promoter drives EYFP expression in excitatory projection neurons,

enabling their labeling. In accordance with the biochemical data, κ-OR activation specifically upregulated LC3 levels, as indicated by increased overall intensity and puncta formation (Figure 8C).

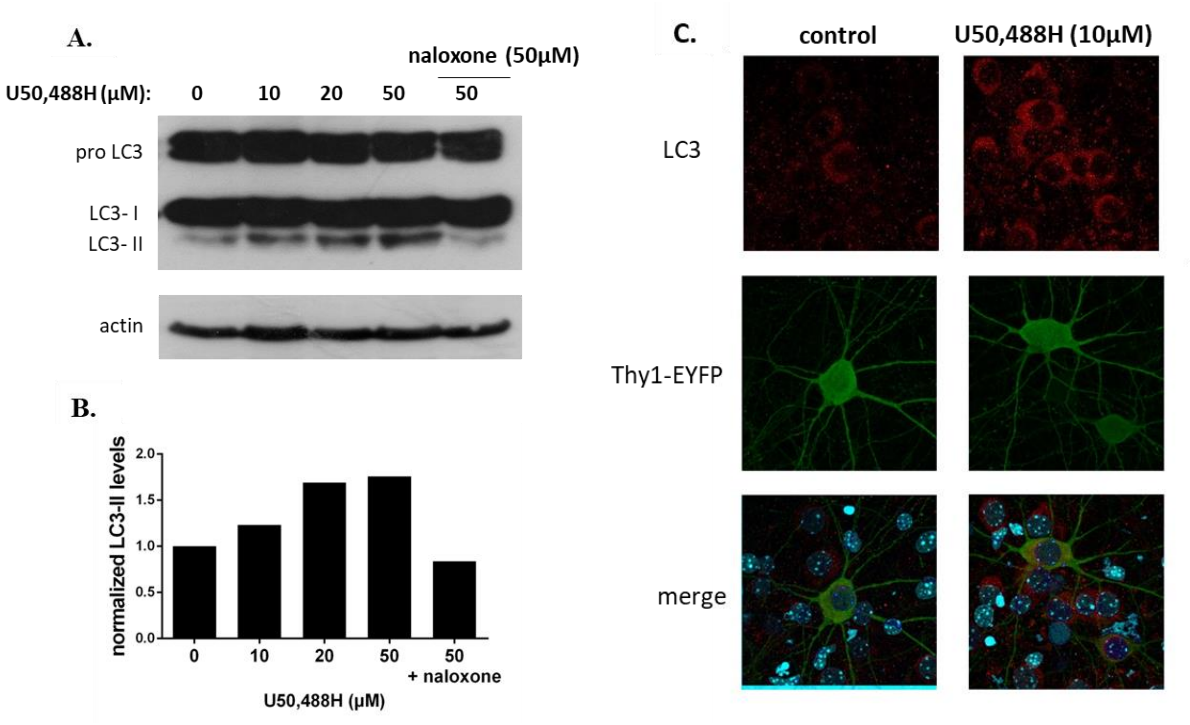

Figure 8: Activation of the κ-OR by the specific agonist U50,488H can dose dependently upregulate LC3-II levels *in vitro*, an effect that is abrogated when neurons are pretreated with the antagonist naloxone.

A. 50μg of protein lysates were analysed by SDS-PAGE electrophoresis on 15% gel and blotted with antibodies against LC3 and actin.

B. Quantification of LC3-II levels normalized to actin.The relative ratio of LC3-II/actin of control sample was set to equal 1. (N=1)

C. Representative confocal images of Thy1-EYFP/Cre neurons treated with 10μM U50,488H for 6hours.The presented image is indicative of three independent experiments.

C.2. The effect of κ-OR activation is not local at synapses.

Recent work from our lab demonstrated that acute activation of the NMDA and mGlu receptors leads in induction of autophagy locally at synapses *(Kallergi et al, submitted).* We wondered whether this is also the case with κ-OR induced autophagy. To address this question, we biochemically isolated functional synapses from the mouse forebrain using centrifugations and a discontinuous sucrose gradient, as described in methods section. The synaptosomal preparation was treated with 20 or 50 μM U50,488H for 1 or 2 hours, as due to methodological limitations treatment for longer periods of time cannot be performed. Western blot analysis of LC3-II and ATG13 protein levels revealed no significant differences among all conditions, suggesting that activation of the κ-OR probably does not induce autophagy locally at synapses (Figure 9). However, there is a possibility that the time of κ-OR activation was not sufficient to evoke a response of the autophagic machinery.

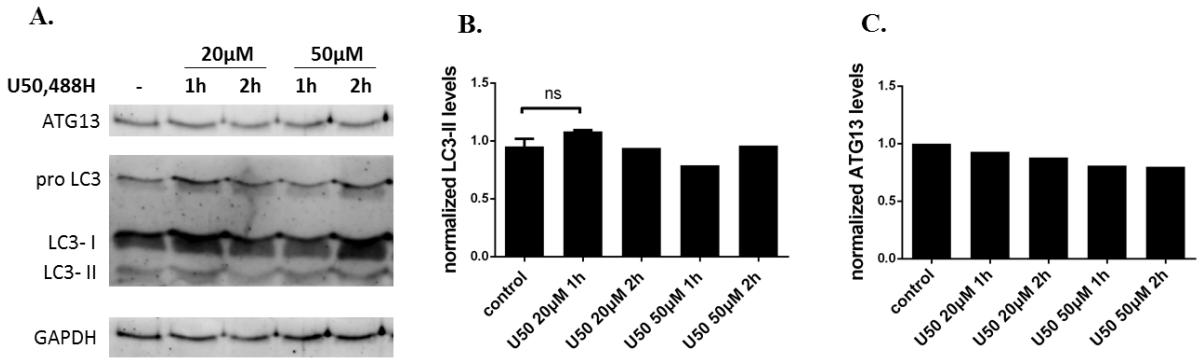

Figure 9: Activation of κ-OR locally at synapses does not upregulate autophagy.

A. 50μg of synaptosomes were analysed by SDS-PAGE electrophoresis on 15% gel and blotted with antibodies against LC3, ATG13 and GAPDH.

B. Quantification of LC3-II levels normalized to GAPDH upon treatment with U50,488H as indicated. The relative ratio of LC3-II/GAPDH of control sample was set to equal 1. Bars represent mean values ± SEM. Statistical analysis was performed using Student's t test to compare control

condition with U50,488H 20μM 1hour (N=2). For the remaining conditions the experiment was performed once, therefore statistical analysis cannot be implemented.

C. Quantification of ATG13 levels normalized to GAPDH upon treatment with U50,488H as indicated. The relative ratio of ATG13/GAPDH of control sample was set to equal 1. No differences were detected among all exprerimental conditions.

C.3. Sustained activation of κ-OR is required for induction of autophagy *in vitro.*

As κ-OR activation failed to induce autophagy locally at synapses, we turned back to intact neurons to evaluate the time course of κ-OR induced autophagy. To do so, we treated primary cortical neurons with 10μM U50,488H and assessed LC3-II levels at different time points of treatment, namely 1, 6 and 24 hours. High protein levels of LC3-II reflect an increase in the number of autophagosomes; however this accumulation of autophagosomes may arise from either increased autophagy that requires more autophagosomes, or by a blockade at the late steps of autophagy, when the autophagic cargo is degraded. To confirm that κ-OR activation upregulates autophagy, we treated neurons with U50,488H in the presence of 10nM BafilomycinA1, a specific inhibitor of autophagosome – lysosome fusion. The inhibition of autophagosome – lysosome fusion enables us to assess the number of autophagosomes that would be degraded under physiological conditions, thus upregulation of LC3-II levels upon Bafilomycin A1 treatment is an indisputable marker of increased autophagic flux. Western blot analysis of neuronal lysates showed that activation of the κ-OR for 1 hour is not sufficient to induce autophagy, whereas after 6 and 24 hours of κ-OR activation LC3-II levels are significantly increased (Figure 10 A-B). In the presence of Bafilomycin A1, LC3-II levels were further increased upon 6 and 24 hours of κ-OR activation, compared to control sample treated with Bafilomycin A1 (Figure 10 C-E).

Additional experiments will be performed to gain confidence of on our results and ensure statistical significance.

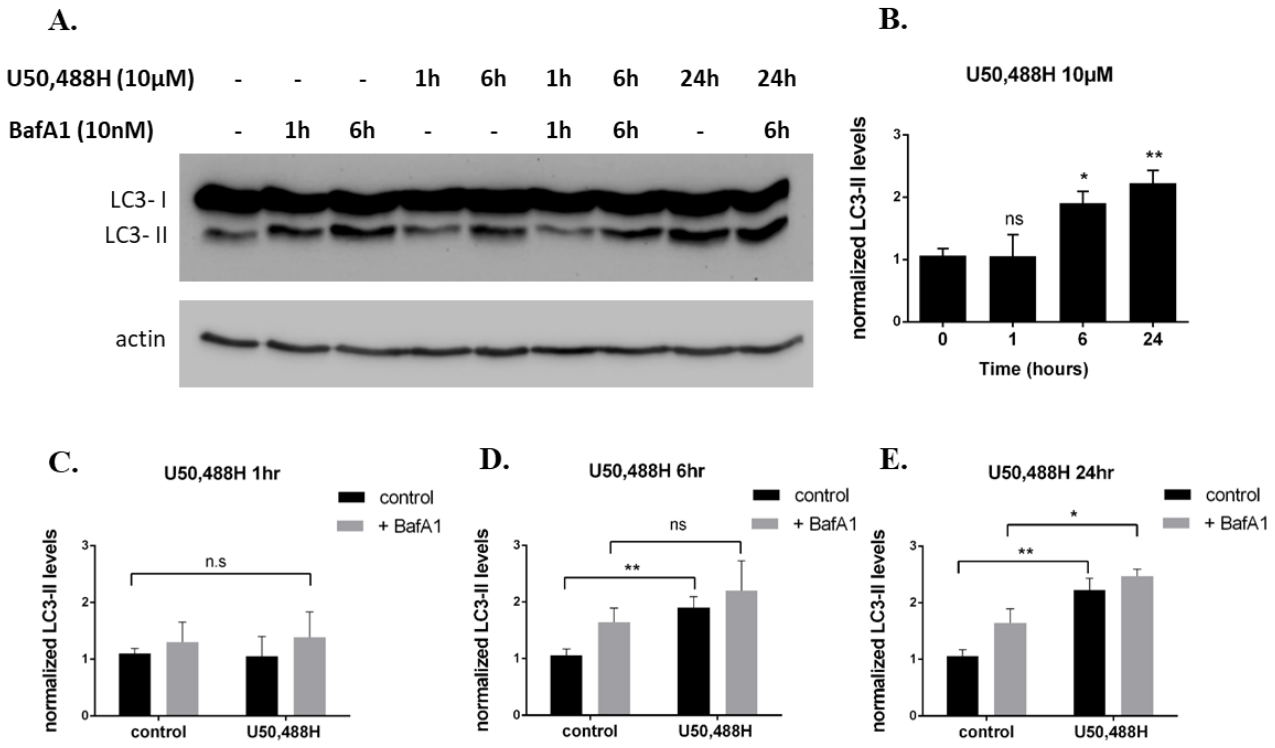

Figure 10: Sustained activation of the κ-OR by the specific agonist U50,488H is required to upregulate LC3-II levels and increase the autophagic flux *in vitro.*(N=2)

A. 50μg of protein lysates were analysed by SDS-PAGE electrophoresis on 15% gel and blotted with antibodies against LC3 and actin.

B. Quantification of LC3-II levels normalized to actin upon 1, 6 and 24 hours of 10μM U50,488H treatment. The relative ratio of LC3-II/actin of control sample was set to equal 1. Bars represent mean values ± SEM. Statistical analysis was performed by one-way ANOVA. * compared to control

C. Quantification of LC3-II levels normalized to actin upon 1h treatment with 10μM U50,488H in the presence or absence of 10nM BafilomycinA1. The relative ratio of LC3-II/actin of control sample was set to equal 1. Bars represent mean values ± SEM. Statistical analysis was performed by two-way ANOVA. Not significant differences were detected.

D. Quantification of LC3-II levels normalized to actin upon 6h treatment with 10μM U50,488H in the presence or absence of 10nM BafilomycinA1. The relative ratio of LC3-II/actin of control sample was set to equal 1. Bars represent mean values ± SEM. Statistical analysis was performed by two-way

ANOVA. Upon 6 hours treatment, U50,488H significantly increases LC3-II levels compared to control.

E. Quantification of LC3-II levels normalized to actin upon 24h treatment with 10μM U50,488H in the presence or absence of 10nM BafilomycinA1. The relative ratio of LC3-II/actin of control sample was set to equal 1. Bars represent mean values ± SEM. Statistical analysis was performed by two-way ANOVA. Upon 24 hours treatment, U50,488H significantly increases LC3-II levels compared to control.

C.4. κ-OR activation induces autophagosomal biogenesis *in vitro*.

Our next step was to evaluate whether activation of the κ-OR may affect the early events of the autophagic process, namely the *de novo* formation of the phagophore and the biogenesis of autophagosomes. For this reason, we treated cortical neurons with 10μM U50,488H for 1,6 and 24 hours and analyzed the protein levels of the PAS components FIP200 and ULK1. These proteins are the key modulators of the self-assembly of the phagophore during the initiation of autophagy. Our analysis revealed that upon at least 6 hours of treatment the levels of all PAS components are upregulated, suggesting that the κ-OR signaling leads in activation of the autophagic machinery at the early steps (Figure 11).

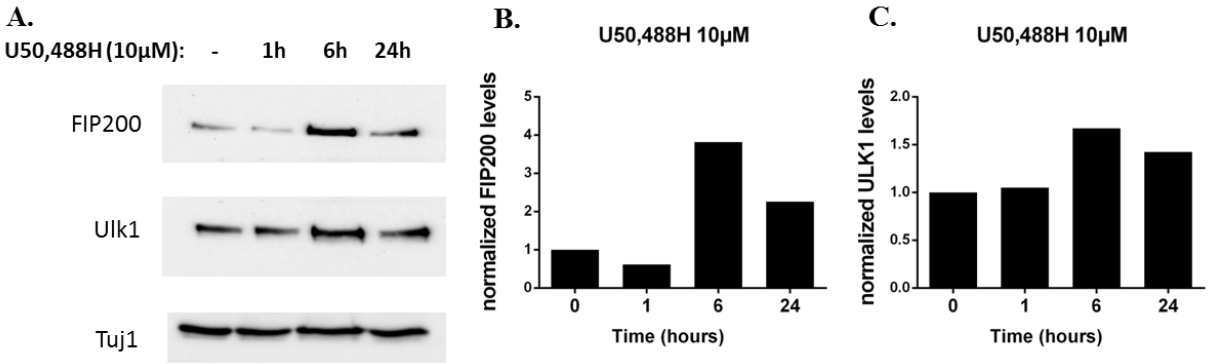

Figure 11: κ-OR activation upregulates the protein levels of the PAS components FIP200 and ULK1, indicating an increase of the autophagosomal biogenesis (N=1).

A50μg of protein lysates were analysed by SDS-PAGE electrophoresis on 7.5% gel and blotted with antibodies against FIP200, ULK1 and β3 tubulin / Tuj1.

B. Quantification of FIP200 levels normalized to Tuj1 upon 1, 6 and 24 hours of 10μM U50,488H treatment. The relative ratio of FIP200/Tuj1 of control sample was set to equal 1.
C. Quantification of ULK1 levels normalized to Tuj1 upon 1, 6 and 24 hours of 10μM U50,488H treatment. The relative ratio of ULK1/Tuj1 of control sample was set to equal 1.
More experiments will be done to define statistical significance.

C.5. Is κ-OR induced autophagy relevant in the mouse brain?

To further investigate whether activation of the κ-OR may induce autophagy in the mouse brain, we firstly attempted an *ex vivo* approach. Using a vibratome we prepared 100μm thick coronal brain slices from fresh tissue, and treated them with 10μM U50,488H in oxygenated aCSF for 5 hours. Subsequently, we isolated the hippocampus and performed immunohistochemical analysis of the LC3 protein levels with confocal microscopy. We co-stained our slices with MAP2 or GFP and Hoechst to visualize the hippocampal neuronal architecture and nuclei respectively. Our results, obtained from both B6 and Thy1-EYFP/Cre animals, indicated a reduction of LC3 expression in the hippocampal CA1 area, as well as in the dentate gyrus (DG), but no change in the hippocampal CA3 area (Figure 12 A, B). Similarly, we prepared and treated with U50,488H 400μm thick brain slices, from which we isolated the cortex, hippocampus and striatum, produced protein lysates that were analyzed biochemically with western blot. In accordance with the immunohistochemical data, a reduction of LC3-II species was observed in the hippocampus, while the in the cortex we did not observe any changes and in the striatum we detected an increase (Figure 12 C, D). Unfortunately, due to the increased technical difficulty of our experimental procedure, until today each experiment has been conducted only once and statistical significance cannot be determined.

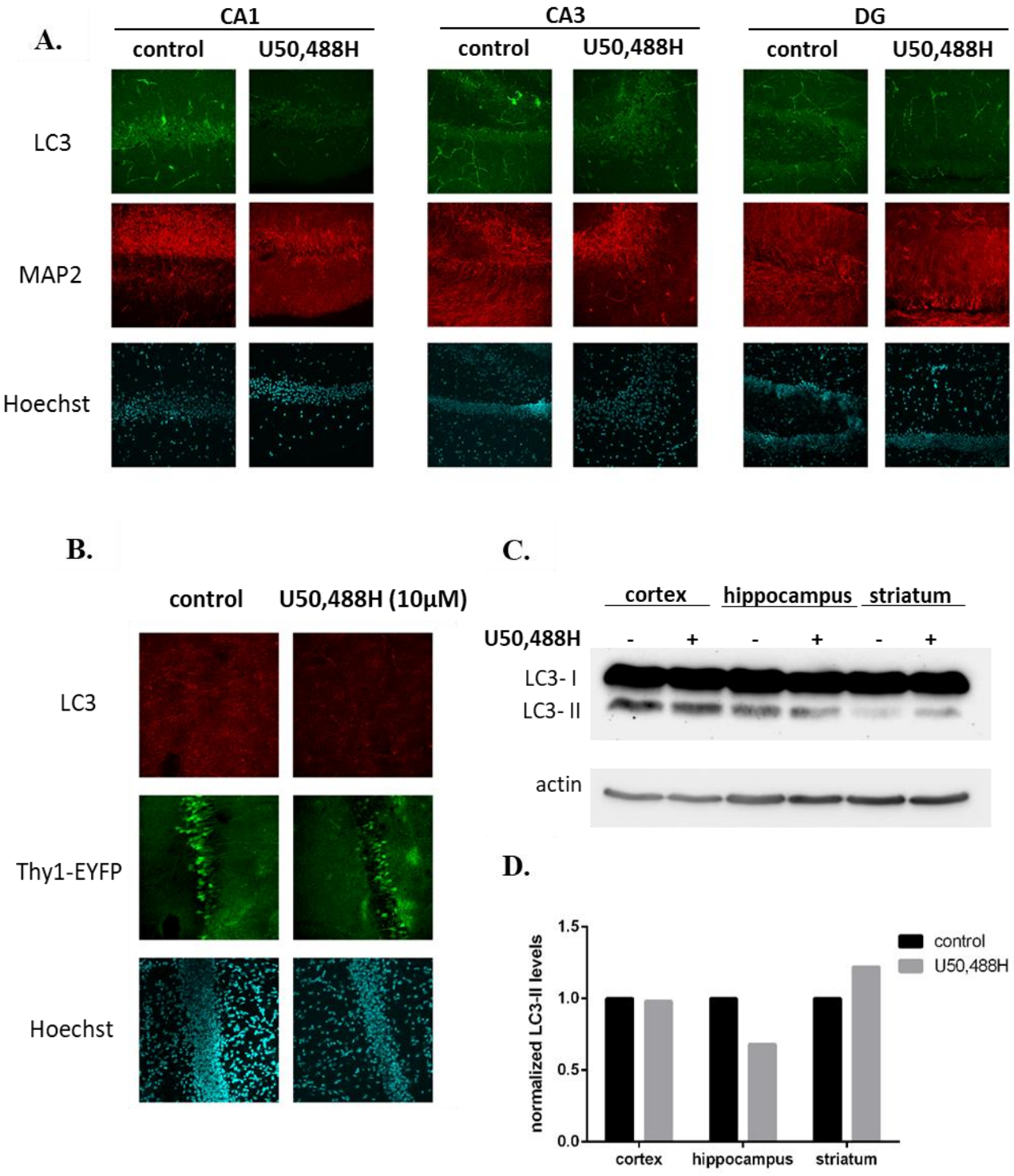

Figure 12: κ-OR activation regulates autophagy in the mouse brain.

A. Representative confocal images of the hippocampal CA1, CA3 and DG areas of B6 mice. Upon treatment with 10μM U50,488H, LC3 levels are downregulated in the CA1 and DG areas of the

hippocampus. To visualize the hippocampal architecture we co-stained with MAP2, a neuronal dendritic marker, and Hoechst. (N=1)
B. Representative confocal images of the hippocampal CA1 area of Thy1-EYFP/Cre mice. Upon treatment with 10μM U50,488H, LC3 levels are downregulated in the CA1 area of the hippocampus. To visualize the excitatory projection neurons we co-stained with GFP and Hoechst. (N=1)
C. 100μg of protein lysates were analysed by SDS-PAGE electrophoresis on 15% gel and blotted with antibodies against LC3 and actin. Upon treatment with 10μM U50,488H, LC3-II levels are downregulated in the hippocampus, upregulated in the striatum, and not affected in the cortex (N=1)
D. Quantification of LC3-II levels normalized to actin upon U50,488H treatment. The relative ratio of LC3-II/actin of control sample for each brain region was set to equal 1.

Our preliminary *ex vivo* data, indicating a reduction of autophagy upon κ-OR activation in the hippocampus, appeared to be contradictory to our *in vitro* evidence. To ensure that our results were not a byproduct of technical issues, we decided to employ an *in vivo* approach and administer U50,488H in an intact organism. As described in figure 13A, a B6 mouse received daily intraperitoneal (i.p.) injections with 5mg/kg U50,488H for seven days, while a littermate control received saline injections of the approximately same volume. We chose this dosing protocol based on the bibligraphy, as 5mg/kg is an efficient dose, that does not produce tolerance when administered chronically *(Droga et al, 2015).* At the eight day, mice were sacrificied, and the cortex, hippocampus and striatum were isolated. Protein lysates were produced and LC3 levels were analyzed with western blot. Our *in vivo* results suggest that κ-OR activation by U50,488H suppresses autophagy in the hippocampus and striatum, whereas promotes autophagy in the cortex (Figure 13B,C). Additional experiments are planned, in order to validate our results and determine statistical significance.

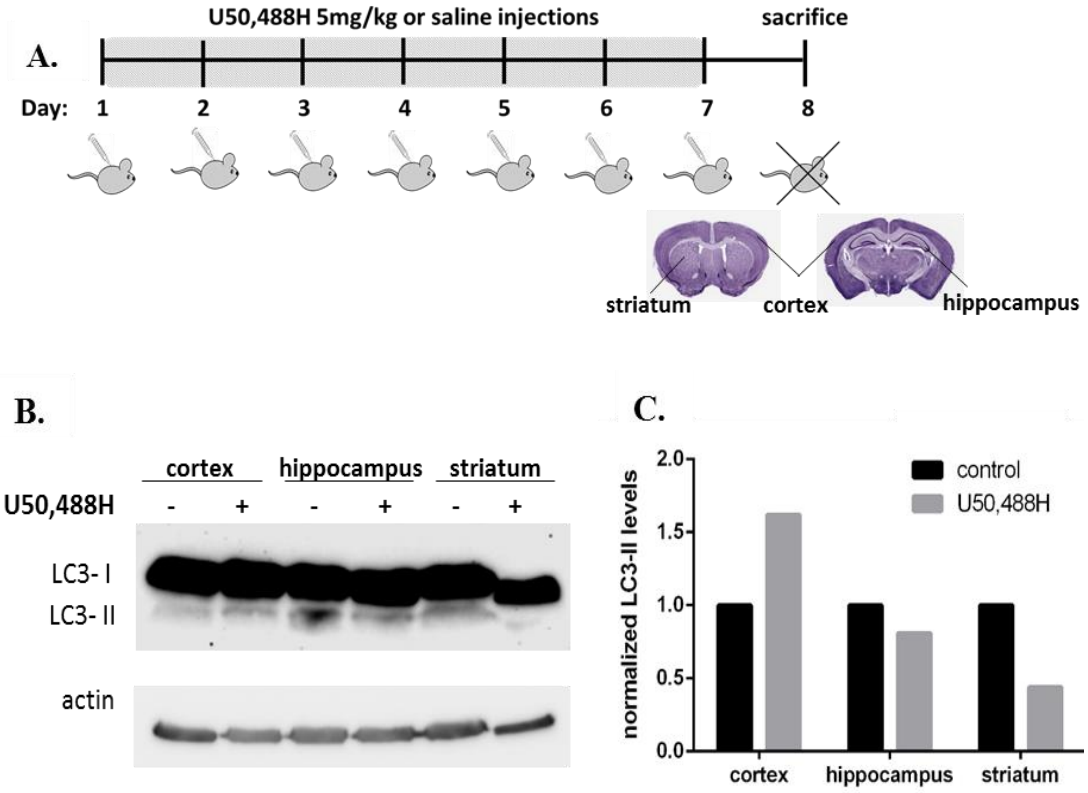

Figure 13: Chronic activation of the κ-OR regulates autophagy in the mouse brain *in vivo.*

A. Experimental procedure. From day 1-7, mice were submitted to systemic administration of 5mg/kg U50,488H or saline i.p. On day 8, mice were sacrificed and different brain areas were isolated.

B. 100µg of protein lysates were analysed by SDS-PAGE electrophoresis on 15% gel and blotted with antibodies against LC3 and actin. Upon chronic U50,488H administration, LC3-II levels are upregulated in the cortex, and downregulated in the hippocampus and striatum. (N=1)

C. Quantification of LC3-II levels normalized to actin upon U50,488H treatment. The relative ratio of LC3-II/actin of control sample for each brain region was set to equal 1.

D. Discussion and future plans

Our results point towards an interaction of the κ-opioid system and autophagy in neurons under physiological conditions. We proved that *in vitro* activation of κ-OR by U50,488H administration in primary cortical neurons upregulates autophagy in a dose and time

dependent manner. Parallel work from Dr. Georgoussi's lab focuses on deciphering the signaling pathways and potential transcriptional events leading from κ-OR activation to induction of autophagy in the neuroblastoma cell line Neuro2A. We plan to confirm their findings in cortical neurons, as the unique physiology of neuronal cells raises the possibility that different mechanisms may exist.

Our main focus is to verify our *in vivo* results, as the surprising finding that in the hippocampus autophagy is suppressed upon κ-OR activation is contradictory to our *in vitro* results. A possible explanation for the observed discrepancy is that hippocampal and cortical neurons may exhibit intrinsic differences in their response to U50,488H. To test this hypothesis, we will establish primary hippocampal cultures and examine the effect of κ-OR activation on induction of autophagy. Alternatively, the strange effect of κ-OR activation in the hippocampus may arise from cell specificity differences; for example in the hippocampus the κ-OR may be expressed in distinct neuronal populations and the effects produced by U50,488H administration may arise from a network effect. To explore this idea, our approach will be to use the lhx6-EYFP/Cre mice, in which the lhx6+ interneurons are marked, and examine whether U50,488H administration differentially affects autophagy in the excitatory and inhibitory neuronal populations. In parallel, we will use an antibody against κ-OR to evaluate the site of its expression both in neuronal cultures and in brain slices.

In addition, we would like to investigate if autophagy participates in κ-OR mediated synaptic plasticity and behavior. Ample evidence supports that activation of the κ-opioid system diminishes LTP and facilitates LTD in the rodent brain. Our recent work established the interplay between autophagy and synaptic plasticity (*Nikoletopoulou et al, 2017, Kallergi et al, submitted).* Therefore, our intention is to examine whether autophagy is necessary for κ-

OR mediated synaptic plasticity. To this end, we will take advantage of our *Nestin-cre;Atg5$^{f/f}$* and *Thy1-cre;Atg5$^{f/f}$* animals that exhibit autophagy deficiency in the neural lineage and excitatory neurons respectively, and proceed with electrophysiological field recordings upon chronic U50,488H administration. Furthermore, we plan to examine how well-established κ-OR mediated behaviors are affected in these animals, upon U50,488H administration. Numerous studies have characterized the implication of κ- opioid system in stress, anxiety, depression, spatial memory and epilepsy *(Bausch et al 1998, Daumas et al, 2007, Smith et al, 2012, Droga et al, 2015).* To study these behaviors we will perform a series of behavioral tests in WT and KO mice upon U50,488H administration, such as elevated plus maze, open field and light dark box for anxiety, forced swim test for depression, Morris water maze for spatial memory and pilocarpine induced seizures for epilepsy.

Finally, it is should be mentioned that a recent study demonstrated that κ-OR activation by U50,488H downregulates BDNF expression in primary neuronal cultures *(Droga et al 2015*), while κ-OR antagonists have been shown to upregulate BDNF expression *(Zhang et al, 2007).* In view of our recent publication, which established the role of BDNF as a negative regulator of autophagy, it is possible that κ-OR induced autophagy relies on alterations of BDNF levels.

Our effort to characterize how autophagy interacts with the κ- opioid system to regulate physiology and pathology will shed light on long standing questions regarding the endogenous opioid system and the molecular mechanisms that mediate its effects. A detailed understanding of how distinct systems and signaling pathways are inter-regulated in the brain holds the key to develop promising therapeutic interventions with minimized side-effects.

E. Methods

E.1. Mice

Mice were housed at a 12 hours light/dark cycle with ad libitum access for food and water. All experiments were performed on C57BL/6, and Thy1-EYFP/Cre [Tg(Thy1-cre/ERT2,-EYFP)HGfng/PyngJ] mice obtained from Jackson laboratory. The tamoxifen inducible Thy1-EYFP/Cre mice were used in the present study without crossing with floxed mice, as they constitutively express EYFP protein under the Thy1 promoter, in order to specifically mark the excitatory projection neurons *in vivo* or *in vitro.* All experiments were performed in accordance with the guidelines of FORTH ethics committee.

E.2. Primary cortical cultures

Pregnant mice were sacrificed on embryonic day E15.5-E16.5 and embryos were kept in PBS on ice. Cortex and hippocampus were extracted, minced with forceps and treated with 0.5% trypsin for 30min at 37^{o}C to dissociate. After centrifugation (5min at 1000rpm), cells were plated on 12-well plates containing 15mm glass coverslips or 6-well plates, coated overnight with poly-D-lysine, at an initial density of 1 or 3 million cells/well correspondingly. Neurons were cultured in neurobasal medium supplemented with B-27 (2%), L-glutamine (300μM), β-mercaptoethanol (25μM) ,penicillin (5μg/ml) and streptomycin (12.5μg/ml), which was half-changed every 3-4days. After 10 or 21 days *in vitro*, neurons were treated with U50,488H (Tocris, 10-50μM), naloxone (Tocris, 50μM), Bafilomycin A1(Sigma-Aldrich 10μM) for 6hr, unless indicated otherwise.

E.3. *Ex vivo* treatments

Male B6 or Thy1-cre mice at approximately p60 were sacrificed by cervical dislocation. The brain was removed immediately, glued onto the stage of a vibratome (Leica) and placed in ice cold, oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 3.5 KCl, 26 NaHCO3, 1 MgCl2 and 10 glucose (pH = 7.4, 315 mOsm/l). 100 or 400 mm thick brain slices (for immunohistochemistry or western blot) were taken and transferred to a submerged chamber with oxygenated aCSF containing (mM): 125 NaCl, 3.5 KCl, 26 NaHCO3, 2 CaCl2, 1 MgCl2 and 10 glucose (pH = 7.4, 315mOsm/l) in room temperature (namely control aCSF). The slices were incubated for 6 hr in control aCSF, or aCSF containing U50,488H 10μM. After treatments, brain slices were processed as described below under Western blot or Immunostaining paragraphs.

E.4. Western blot

Cells were lysed with sonication in RIPA buffer (500 mM Tris-HCl pH 7.2, 1MNaCl, EDTA, Triton 100-X, Na-deoxycholate,10% SDS), supplemented with protease inhibitors (Roche) and placed for at least 1hr on ice, followed by 30 min centrifugation at 13,000 rpm. For tissues additional sonication was used to achieve homogenization. Samples were separated on a 7.5% or 15% polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore). After blocking for 1 hr at room temperature in 5% skim milk, membranes were incubated in the primary antibodies overnight at 4^{o}C. The primary antibodies used were LC3 (Santa Cruz), p62 (Progen), actin (Santa Cruz), b-III tubulin (Santa Cruz), ULK1 (Cell Signaling), FIP200 (Cell Signaling) and ATG13 (Sigma Aldrich). After three 10 min washes in PBS-T (100 mM Na2HPO4, 100mM NaH2PO4, 0.5N NaCl, 0.1% Tween-20), membranes

were incubated for 1 hr at room temperature in corresponding secondary horseradish peroxidase-conjugated antibodies (Abcam). Blots were developed by chemiluminescence using the Chemidoc (Biorad)

E.5. Synaptosomal preparation

For the isolation of synaptosomes, brains were isolated from C57BL/6 mice of both sexes at postnatal days between 20 and 25. The brains were rinsed and homogenized in solution A (0.32M Sucrose, 1mM $NaHCO_3$, 1mM $MgCl_2$, 0.5mM $CaCl_2 \cdot H_2O$, 10mM Na pyrophosphate, nanopure water, protease inhibitors) using a glass homogenizer. Following dilution to 10% w/v in solution A, and centrifugation (10min at 710g, 4^oC), the pellet was resuspended in solution A supernatant using the homogenizer. After a centrifugation (10min at 1400g, 4^oC) for the removal of nuclei, the supernatant was collected and centrifuged (10min at 13.800g, 4^oC). The pellet was resuspended in solution B (0.32M sucrose, 1mM $NaHCO_3$) using the homogenizer and 8ml of the resulting sample were layered on a discontinuous sucrose gradient (10ml-layers of 1.2M, 1.0M and 0.85M sucrose). Following centrifugation (2hrs at 82.500g, 4^oC), synaptosomes were isolated between the 1.2M and 1M sucrose layers.

E.6. Immunostaining and confocal imaging

Cultured neurons were rinsed in PBS and fixed for 15 min in 4% paraformaldehyde (PFA) in PBS. Following fixation, cells were rinsed in PBS and incubated for 1 hour in blocking solution containing 10% fetal bovine serum and 0.2% Triton-X in PBS. Neurons were then incubated in blocking solution containing primary antibody overnight at 4^oC. The following primary antibodies were used: LC3 (1:1000, mouse monoclonal, Santa Cruz), MAP2

(1:2000, guinea pig, SYSY), GFP (1:1000, homemade), p62 (1:2000, guinea pig, Progen), diluted in blocking solution. Neurons were rinsed in PBS and incubated with the following secondary antibodies (Abcam) for 1 hr at room temperature: anti-rabbit Alexa 488, anti-mouse Alexa 488 or 594, and anti-guinea pig Alexa 647. The nuclear dye Hoechst was used (1:5000) to stain nuclei. Neurons were rinsed in PBS and mounted onto slides. Confocal images of fluorescently labeled proteins were captured using the LSM 710 NL multi-photon microscope (Zeiss), using the 63x objective.

Isolated hippocampal slices were rinsed in PBS and fixed for 20 min in 4% paraformaldehyde (PFA) in PBS. Following fixation slices were incubated in permeabilization solution (0.5% Triton X-100 in PSB) overnight at 4^{o}C and subsequently in blocking solution (20% BSA in PBS) for at least 5 hours at room temperature. The above mentioned primary antibodies were used, but were diluted in 5% BSA in PBS and incubated for 48hr at 4^{o}C. Slices were washed with 5% BSA in PBS and incubated with the above mentioned secondary antibodies for at least 5hr at room temperature. Slices were rinsed in PBS and mounted onto slides. Confocal images of fluorescently labeled proteins were captured using the LSM 710 NL multi-photon microscope (Zeiss), using the 40x objective.

E.7. Statistical analysis

Statistical analyses were performed with the GraphPad Prism software, and the data are presented as mean ± SEM. For statistical significance of the differences between the means of two groups we used two-tailed Student's t tests, while for more groups we used ANOVA. Statistical significance was established at p-value $\leq$0.05.

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