1. General information about the project

1.1. Project title: **Dissecting the role of neuronal activity and Gs- and Gi-linked GPCR signaling in guiding the *in vivo* trafficking of autophagic vesicles and the cargo disposal in noradrenergic neurons of the *Locus coeruleus***

1.2. Research area: Molecular and Cellular Neurobiology

1.3. Project leader Dr. rer. nat. Anna Karpova, DOB: 20/10/1972, Russian

Leibniz Institute for Neurobiology, RG Neuroplasticity Brenneckestr. 6, 39118 Magdeburg, Germany akarpova@lin-magdeburg.de | +49 (0)391 6263-94371; The employment of the project leader at the LIN is contractually fixed for the duration of the proposed funding period

1.4 Legal issues

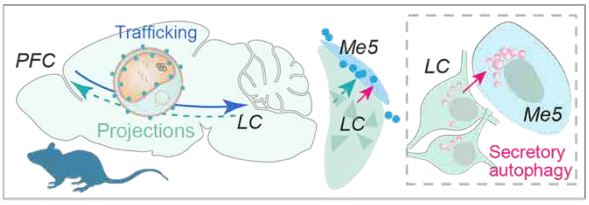
This project includes

1.	research on human subjects or human material.	no
2.	clinical trials.	no
3.	experiments involving vertebrates.	yes
4.	experiments involving recombinant DNA.	yes
5.	research involving human embryonic stem cells.	no

2. Project proposal

2.1 Summary

Maintenance of proteostasis depends on efficient endo-lysosomal and autophagy systems and is particularly important for survival of long-range projecting norepinephrinergic neurons of the *locus coeruleus* (*LC-NE*). The extensive use of volume transmission suggests presynaptic regulation, involving G-protein coupled receptors (GPCRs) to control autophagic vesicle (AVs) trafficking in *LC-NE* axons for timely cargo disposal. How *LC-NE* neurons navigate AVs through their projections, and the role of sleep-/ wakefulness-related activity in this process, remain unknown.



In the proposed project context-dependent AVs trafficking in *LC-NE* will be assessed by combining *in vivo* 2P-imaging with monitoring norepinephrine release or with chemo- and optogenetic manipulations. This will be complemented by analyzing fiber-mediated photoconversion in behaving animals and by cell biological/ biochemical assays.

A particular focus will be on the role of β2- and α2-adrenergic receptors for retrograde trafficking of AVs in *LC-NE* axons projecting to M1-PFC, characterizing the downstream molecular mechanisms controlling their logistical complexity (e.g. pausing, velocity), such as differential regulation of cAMP/PKA and spinophilin/PP1gamma.

Intrigued by unexpected observations, further research will elaborate on the disposal of autophagic cargo through the interplay between *LC-NE* neurons and adjacent mesencephalic trigeminal nucleus (*Me5*) neurons via secretory autophagy mechanisms involving Rab27a and Sec22b downstream of somatic GPCRs.

2.2 State-of-the-art

The highly polarized organization of neurons is essential for communication within the brain via chemical synapses. The axonal compartment contains a few terminal synapses as well as numerous *en passant* presynaptic boutons, which are specialized for the release of neurotransmitters and neuromodulators (Liu & Tsien, 1995; Südhof, 2021). The high rate of membrane turnover at the presynapse, combined with substantial metabolic demands, presents a distinct challenge for maintaining neuronal proteostasis – a process critical for neuronal survival. The main machinery for neuronal protein synthesis and degradation is localized within the neuronal soma, while synaptic junctions are often situated at considerable distances from the cell body. Consequently, **precise regulation of transport mechanisms** is essential to ensure the efficient delivery of newly synthesized proteins, the removal of misfolded proteins and damaged organelles, and the execution of somatic disposal processes, all of which are necessary to maintain proper neuronal function.
Neurons sequester non-functional cytosolic material - including oxidized metabolites, misfolded macromolecules, and damaged organelles - into double-membrane organelles known as autophagosomes and recent studies have highlighted the significance of autophagy in neuronal

function (Hara et al., 2006; Sulzer et al., 2008; Hernandez et al., 2012; Soukup et al., 2016; Murdoch et al., 2016; Okerlund et al., 2017; Zucca et al., 2018; Glatigny et al., 2019; Hill et al., 2019; Kuijpers et al., 2021; Bademosi et al., 2023; Kallergi et al., 2023; reviewed in Stavoe & Holzbaur, 2019; Lieberman & Sulzer, 2020; Fleming & Rubinsztein, 2020; Andres-Alonso et al., 2021). The catabolic function of autophagosomes largely depends on their fusion with lysosomes, which are mainly present within the cell soma. To reach lysosomes, autophagosomes utilize molecular motors for transport (Maday and Holzbaur, 2016; Kulkarni and Maday, 2018). Both dynein (Jahreiss et al., 2008; Batlevi et al., 2010; Kimura et al., 2008; Cai & Ganesan, 2022) and kinesins (Maday et al., 2012) participate in the transport of AVs along microtubules (reviewed in Kast & Dominguez, 2017), and both co-fractionate with axonal autophagosomes (Maday et al., 2012). Additionally, AVs navigate through the axons using motor-adaptor-mediated strategies (Cheng et al. 2015, Jordens et al. 2001, Wijdeven et al. 2016) and association with scaffolding proteins (Fu et al. 2014, Wong & Holzbaur 2014b), to enable fast and slow AV motility, stationary pauses, prolonged stops with varying dwell times, and fusion with other organelles (Cason et al., 2021; Cason & Holzbaur, 2023). Intriguingly, axonal autophagosomes can fuse with late endosomes to form hybrid organelles known as amphisomes, which are then transported to the neuronal soma via a shared adaptor-motor mechanism acquired upon fusion (Cheng et al., 2015; Kononenko et al., 2017; Andres-Alonso et al., 2019; reviewed in Andres-Alonso et al., 2021; reviewed in Grochowska et al., 2022). Intriguingly, amphisome possess both degradative and signaling capacities (Andres-Alonso et al., 2019; reviewed in Andres-Alonso et al., 2021; Kuijpers et al., 2021). The involvement of various dynein adaptors adds further complexity to the logistics of AV trafficking (Cheng et al., 2015; Andres-Alonso et al., 2019; Cai and Ganesan, 2022; reviewed in Nambiar & Manjithaya, 2024). Stationary pauses at synaptic boutons have attracted particular interest and were recently shown to depend on two molecular mechanisms operating downstream of cAMP and PKA activation (Fig. 1.; Andres-Alonso et al., 2019): (a) modulating the interaction of amphisomes with the dynein motor complex, a process facilitated by the motor adaptor protein Snapin (Cai et al., 2010; Xie et al., 2015; Andres-Alonso et al., 2019); and (b) regulating motor processivity by modulating the GTPase-activating protein (GAP) activity of RapGAP SIPA1L family members, which have been identified in association with the outer membrane leaflet of autophagosomes (Andres-Alonso et al., 2019; Goldsmith et al., 2022).

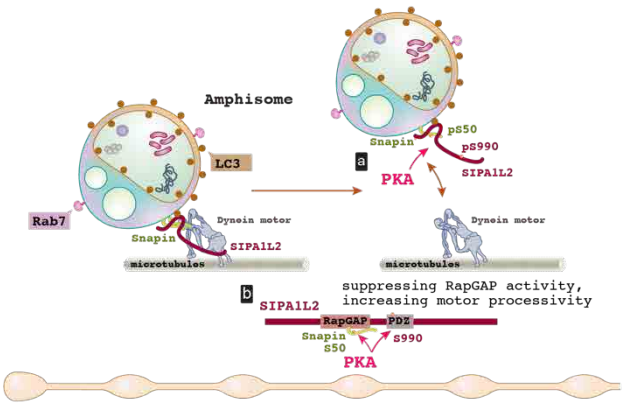


Fig.: 1: *Molecular mechanism of amphisome immobilization/pausing at synaptic boutons.*
(a) a LC3B/Rab7-positive hybrid organelle, amphisome, retrogradely traffics along the axon and association of LC3B promotes RapGAP activity of SIPA1L2 ensuring its fast velocity. The PKA-dependent phosphorylation of the dynein adaptor Snapin, which results in the dissociation of SIPA1L2/Snapin complex from the Dynein intermediate chain (DIC), leads to the immobilization of amphisomes at synaptic boutons. (b) SIPA1L2 is a substrate for PKA, and the SIPA1L2 S990D mutant exhibited a downregulation of its RapGAP activity favoring ERK signaling involved in motor processivity (Andres-Alonso et al., 2019).

After PKA-dependent pausing at synaptic boutons, LC3B-positive amphisomes resume their retrograde movement. Pharmacological inhibition of the PKA activity significantly reduces their dwell time (Andres-Alonso et al., 2019), suggesting that the reassembly of LC3B-positive vesicles with the motor complex for retrograde transport is dependent on the **dephosphorylation** of the PKA substrate, the motor-adaptor protein Snapin (Chheda et al., 2001; Di Giovanni & Sheng, 2015; Andres-Alonso et al., 2019), to allow for recoupling with DIC. Phosphatases from various families are abundant at synapses, where they regulate synaptic transmission and plasticity, and their phosphatase activity is tightly controlled (Foley et al., 2023; Woolfrey & Dell'Acqua, 2015). However, the specific phosphatase responsible for Snapin dephosphorylation and the promotion of Snapin-DIC recoupling remains to be identified. Intriguingly, the role of the protein phosphatase calcineurin and calcineurin-dependent dephosphorylation of the BDNF scaffold huntingtin in "setting the endosome moving in a retrograde direction" has been shown (Scaramuzzino et al., 2022), suggesting that the dephosphorylation of transport-related proteins might indeed **represent a general mechanism** involved in the mobilization of various organelles.

Spinophilin, also known as Neurabin-2/PP1R9B, is a crucial modulator of protein phosphatase 1 (PP1) enzymatic activity (Allen et al., 1997; Feng et al., 2000; Baucum et al., 2012). Spinophilin influences the phosphorylation state of various proteins by targeting them (Terry-Lorenzo et al., 2002; Grossman et al., 2004; Ragusa et al., 2010) and sequestering PP1 away from its synaptic substrates, a process known as spinophilin-dependent sequestration of PP1 (Salek et al., 2019). Thus, the modulation of the spinophilin-PP1 interaction is involved in both the regulation of PP1 binding to its synaptic substrates and the modulation of PP1 phosphatase activity (Salek et al., 2019). Originally described as highly enriched at the post synapse (Allen et al., 1997; Morishita et al., 2001; Peti et al., 2013; Matsuura et al., 2022), spinophilin is also present at the presynaptic sites (Muhammad et al., 2015) and interacts with multiple presynaptic proteins (Baucum et al., 2012; Watkins et al., 2018). Electron microscopy studies have further revealed that spinophilin associates with the vesicular compartment (Allen et al., 1997; Feng et al., 2000; Sarrouilhe et al., 2006). Intriguingly, endogenous spinophilin has been found in complex with the autophagy marker LC3B in hippocampal lysates (Karoussiotis et al., 2022) and directly interacts with the SIPA1L family protein member SIPA1L1 (Matsuura et al., 2022). Additionally, brain-derived autophagosome profiling detected spinophilin on the cytoplasmic leaflet of the autophagosome, where SIPA1L family proteins, including SIPA1L1 and SIPA1L2, are also present (Goldsmith et al., 2022). Altogether, these findings suggest that spinophilin, associates with the amphisome, likely regulates local PP1 activity to mediate the dephosphorylation of PKA substrates and potentially promotes Snapin-DIC recoupling, facilitating the trafficking of autophagic cargos for disposal. Importantly, the modulation of the trafficking velocity of LC3-containing vesicles along the axon, as well as their temporally intermediate pausing, can significantly influence the time window available for the delivery of autophagic cargo to the cell soma for disposal (Fig. 2). Surprisingly, little is known about the regulation of these processes *in vivo* in relation to neuronal activity and neurotransmitter release within brain circuits. Until recently, limited studies have addressed the *in vivo* trafficking of AVs in the murine CNS (Luo et al., 2024). However, they have not investigated the role of neuronal activity and have not addressed molecular mechanisms of regulation.

The *locus coeruleus* comprises approximately 3,000 neurons in rodents (Sara, 2009) and fewer than 50,000 neurons in humans (Mouton et al., 1994). *LC-NE* neurons are characterized by relatively small cell bodies and extensively branched neuromodulatory projections across diverse brain regions (reviewed in Chandler et al., 2019). These neurons are critically involved in modulating a wide range of functions, including wakefulness and sleep states, arousal, anxiety, attention, pain, mood, and the response to stress, thereby influencing animal behavior as well as various forms of learning and memory processes (Samuels et al., 2008; Sara, 2009; Wagatsuma et al., 2018; Suárez-Pereira et al., 2022; reviewed in Sara & Bouret, 2012; reviewed in Chandler et al., 2019; reviewed in Ross and Van Bockstaele, 2021).

LC-NE neurons among the most burdened cells in the central nervous system, are characterized by their high sensitivity to neurodegeneration in both Alzheimer's and Parkinson's disease. Age- and disease-dependent modifications in their endo-lysosomal system and neuroprotective autophagy suggest that both systems are critically involved in the elimination of polymerized catecholamine derivatives, such as neuromelanin (NM), an early biomarker of Parkinson's disease (Zucca et al., 2018; Yamaguchi et al., 2018; reviewed in Lu et al., 2020; Rubinsztein & Nixon, 2024). In this context, autophagy plays a crucial role in maintaining the viability of noradrenergic neurons under various stressful conditions, including those induced by pharmacological and neurotoxic agents (Ferrucci et al., 2013; reviewed in Limanaqi et al., 2020). Recent snRNA-seq profiling of the neurotypical adult human *LC* aimed at comparing *NE*-positive neurons, identified and characterized by their gene expression signatures in the *LC* versus the surrounding regions, found the autophagy marker mRNA MAP1LC3B among the top 70 significantly elevated genes (Weber et al., 2023), indicating a high demand for autophagy regulation in these neurons.

The activity of *LC* neurons is intricately linked to the modulation of wakefulness and various sleep phases, which, in turn, correlates with the oscillatory amplitude of NE release, which plays a crucial role in shaping the microarchitecture of sleep associated with memory performance (Kjaerby et al., 2022). Some studies address the role of insufficient sleep resulting in cognitive impairments (Tartar et al., 2009; Ramesh et al., 2012; Stickgold et al., 2013) and propose that it affects the clearance of metabolic wastes and disrupts intracellular protein degradation pathways, including endosome-

autophagosome-lysosome (EAL) pathways as evident by accumulation and dysregulation of cellular LAMP1-positive lysosomes (Xie et al., 2013; Xie et al., 2022).

Oscillations of NE released from *LC* synaptic boutons are detected by a family of adrenergic GPCRs, including β2- and α2-adrenergic receptors (AR), each with distinct functions. The activation of β2- and α2AR plays a pivotal role in modulating the wake-sleep cycle, with their activities and downstream signaling being differentially influenced by NE levels (Xu et al., 2003; Wang & Limbird, 2002; 2007; Fig.2). Adrenergic signaling plays a critical role in maintaining wakefulness and regulating REM sleep, during which *LC-NE* neurons exhibit reduced activity (Aston-Jones & Bloom, 1981) and spontaneously released NE predominantly interacts with α2AR, initiating downstream Gαi/o-protein coupled signaling and leading to the inhibition of cAMP/PKA signaling (Saunders et al., 1999). Furthermore, the higher affinity of α2AR for NE compared to β2AR (Lakhlani et al., 1997) suggests that during wakefulness, elevated NE levels primarily activate β2AR, triggering downstream $G\alpha_s$-protein coupled signaling and subsequently activating the cAMP/PKA pathway (Fig. 2).

While the consequences of activating β2AR or α2AR on the membrane of *LC-NE* neurons remain unclear, their influence on autophagy processes in general is increasingly recognized. A growing body of evidence underscores the significance of β2ARs in regulating endo-lysosomal and autophagy-related processes in both non-neuronal cells and neurons. This regulatory role is recognized as vital for maintaining cellular proteostasis (Farah et al., 2014; Campos et al., 2020; reviewed in Giorgi et al., 2017; Lee et al., 2020). For instance, in the context of neurogenic myopathy, the activation of β2ARs by agonists in the skeletal muscles of mice serves as a protective measure against the disturbance of autophagy flux, thereby maintaining the integrity of skeletal muscle proteostasis and contractile properties (Campos et al., 2020). Similarly, the stimulation of β2AR with isoproterenol, norepinephrine, and salbutamol has been found to increase autophagy flux in cardiac fibroblasts (Aránguiz-Urroz et al., 2011). In line with this, in hepatic cells, the administration of the β2AR agonist clenbuterol stimulates autophagy flux, while the β2AR antagonist propranolol produces opposing effects (Farah et al., 2014). Multiple GPCRs, including AR, have been reported to be involved in autophagy regulation, highlighting the integration of several GPCRs and autophagy at both physiological and pathological levels (Reviewed in Öz-Arslan et al., 2024a, b). Remarkably, activation of β2AR by related agonists restores lysosomal proteolysis by re-acidifying lysosomes in the context of PSEN1 familial Alzheimer's disease (FAD). This activation rescues lysosomal hydrolase activity and autophagy flux, effectively reducing the abnormal accumulation of autophagic vesicles (Lee et al., 2020; Lee & Nixon, 2022). Therefore, exploring of the impact of sleep on the regulation of neuronal proteostasis through autophagy is a captivating field of research, with a potential for developing therapeutic applications aimed at enhancing sleep quality in preventing neurodegenerative processes.

It has been proposed that secretory autophagy, mediated by extracellular vesicle particles (EVPs), serves as an alternative mechanism for clearing sequestered material and maintaining proteostasis in case of endo-lysosomal dysfunction (Solvik et al., 2022; Burbidge et al., 2022; Cashikar and Hanson, 2019; Mauthe et al., 2018). This pathway is upregulated in response to endo-lysosomal inhibition and depends on multiple autophagy-related genes essential for autophagosome formation (Leidal et al., 2020), as well as on small GTPase Rab27a, which regulates the secretion process (Solvik et al., 2022). Alterations in the endo-lysosomal system that occur in the *LC* in an age- and disease-dependent manner might trigger secretory autophagy as an alternative mechanism for *LC*-related cargo disposal, potentially involving the SNARE protein complex for secretion, likely regulated by neuronal activity (Ponpuak et al., 2015; Kimura et al., 2017a, 2017b; reviewed in Fahran et al., 2017; Nakamura et al., 2024; Chang et al., 2024; Martinelli et al., 2021; Tan et al., 2022; Hartmann et al., 2023). It is also conceivable that small extracellular vesicles particles mediate **specific inter-neuronal communication** as suggested recently (reviewed in Nieves Torres & Lee, 2023).

Neurons of the mesencephalic trigeminal nucleus (*Me5*), located adjacent to the *LC* and receiving direct projections from *LC-NE* neurons (Takahashi et al., 2010), have been shown to relate to the *LC* both anatomically and functionally through the exchange of membranous compartments (Goto et al., 2020). The neuronal cell bodies of *Me5* are significantly larger than the somata of *LC-NE* neurons, providing a greater capacity to accommodate endo-lysosomal and auto-lysosomal machinery that could potentially kick in on demand, functioning as an accessory digestive system to

maintain *LC* proteostasis. However, it remains unclear whether the vesicular exchange between the *LC* and *Me5* involves secretory autophagy mechanisms, what role heightened *LC* activity plays in this process, and how *D2* receptors, which mediate the restoration of REM sleep in dopamine-depleted animals (Dzirasa et al., 2016; Eban-Rothschild et al., 2016), regulate the exchange of membranous compartments originating from the *LC* and taken up by *Me5*.

2.3 Project- and subject-related list of publications

1. Airan R, *et al.* (2009) Temporally precise *in vivo* control of intracellular signalling. *Nature* **458**, 1025–1029 (2009). **2**. Allen PB, *et al.,* (1997) Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines. Proc Natl Acad Sci U S A. 94(18):9956-61. **3**. Andres-Alonso M, *et al.* (2019) SIPA1L2 controls trafficking and local signaling of TrkB-containing amphisomes at presynaptic terminals. *Nat Commun***10**, 5448. **4**. Andres-Alonso M, *et al.,* (2021) Autophagy and the endolysosomal system in presynaptic function. Cell Mol Life Sci. 78(6):2621-2639. **5.** Aránguiz-Urroz O, *et al.,* (2011) Beta2-adrenergic receptor regulates cardiac fibroblast autophagy and collagen degradation, Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 1812:1,23-31. **6.** Aston-Jones G & Bloom FE. (1981) Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. J Neurosci. 1(8):876-86. **7**. Bademosi AT, *et al.,* (2023) EndophilinA-dependent coupling between activity-induced calcium influx and synaptic autophagy is disrupted by a Parkinson-risk mutation. Neuron. 111(9):1402-1422.e13. **8.** Batlevi Y, *et al.,* (2010) Dynein light chain 1 is required for autophagy, protein clearance, and cell death in Drosophila. Proc Natl Acad Sci U S A.107(2):742-7. **9**. Baucum AJ, *et al.,* (2012) Age-Dependent Targeting of Protein Phosphatase 1 to Ca^{2+}/Calmodulin-Dependent Protein Kinase II by Spinophilin in Mouse Striatum. PLoS ONE 7(2): e31554. **10.** Burbidge K, *et al.,* (2021) LGALS3 (galectin 3) mediates an unconventional secretion of SNCA/α-synuclein in response to lysosomal membrane damage by the autophagic-lysosomal pathway in human midbrain dopamine neurons. *Autophagy*, *18*(5), 1020–1048. **11.** Cai Q, *et al.,* (2010) Snapin-regulated late endosomal transport is critical for efficient autophagy-lysosomal function in neurons. Neuron. 68(1):73-86. **12.** Cai Q & Ganesan D. (2022) Regulation of neuronal autophagy and the implications in neurodegenerative diseases. Neurobiol Dis. 162:105582. **13**. Campos JC, *et al.,* (2020) B2-adrenoceptor activation improves skeletal muscle autophagy in neurogenic myopathy. FASEB J 34:5628–41. **14.** Cashikar AG & Hanson PI. (2019). A cell-based assay for CD63-containing extracellular vesicles. PLoS One. 14(7):e0220007. **15.** Cason SE, *et al.,* (2021) Sequential dynein effectors regulate axonal autophagosome motility in a maturation-dependent pathway. J. Cell Biol. 220, e202010179. **16.** Cason SE, *et al.,* (2022). Spatiotemporal analysis of axonal autophagosome–lysosome dynamics reveals limited fusion events and slow maturation. Mol. Biol. Cell 33(13):ar123. **17.** Cason SE & Holzbaur ELF (2023) Axonal transport of autophagosomes is regulated by dynein activators JIP3/JIP4 and ARF/RAB GTPases. J. Cell Biol. 222, e202301084. **18.** Chang YC & Chang KT. (2024) Secretory autophagy - a new paradigm regulating synaptic plasticity. Autophagy. 30:1-3. **19**. Chandler DJ, *et al.,* (2019). Redefining Noradrenergic Neuromodulation of Behavior: Impacts of a Modular Locus Coeruleus Architecture. J Neurosci. 16;39(42):8239-8249. **20**. Cheng XT, *et al.,* (2015) Axonal autophagosomes recruit dynein for retrograde transport through fusion with late endosomes. J Cell Biol. 209(3):377-86. **21.** Chheda MG, *et al.,* (2001) Phosphorylation of Snapin by PKA modulates its interaction with the SNARE complex. Nat Cell Biol. 3(4):331-8. **22**. Dzirasa, K. *et al.,* (2006) Dopaminergic control of sleep-wake states. J. Neurosci. 26, 10577–10589. **23.** Di Giovanni J, Sheng ZH (2015) Regulation of synaptic activity by snapin-mediated endolysosomal transport and sorting. EMBO J. 34(15):2059-77. **24.** Eban-Rothschild A, *et al.,* (2016) VTA dopaminergic neurons regulate ethologically relevant sleep–wake behaviors. Nat. 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Trends Neurosci. 43(10):767-779. **30.** Fois GR, *et al.,* (2022) Locus coeruleus activation during environmental novelty gates cocaine-induced long-term hyperactivity of dopamine neurons. iScience. 25(4):104154. **31.** Foley K, *et al.,* (2023) Regulation of PP1 interaction with I-2, neurabin, and F-actin. Mol Cell Neurosci. 124:103796. **32.** Fu MM, *et al.,* (2014) LC3 binding to the scaffolding protein JIP1 regulates processive dynein-driven transport of autophagosomes. Dev Cell. 29(5):577-590. **33.** Giorgi FS, *et al.,* (2017) The Neuroanatomy of the Reticular Nucleus Locus Coeruleus in Alzheimer's Disease. Front Neuroanat 11:80. **34.** Glatigny M, *et al.,* (2019) Autophagy Is Required for Memory Formation and Reverses Age-Related Memory Decline. Curr Biol. 29(3):435-448.e8. **35.** Goldsmith J, et al., (2022) Brain-derived autophagosome profiling reveals the engulfment of nucleoid-enriched mitochondrial fragments by basal autophagy in neurons. 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2.4 Project plan

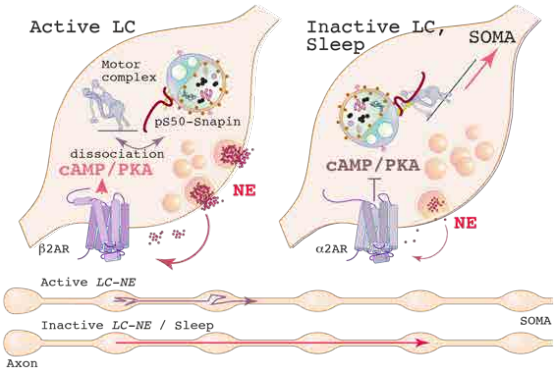
The overarching goal of this project is to describe and understand how **network-related, cellular and molecular mechanisms** regulate the clearance **of *LC-NE* neurons from waste material** through **autophagy** during different states of LC activity**,** various animal behaviors in wakefulness, as well as during sleep. To this end, three work packages (**WP1-3**) are proposed, each building on preliminary findings, to address specific research questions as outlined below.

In **WP1**, we will employ GRAB-NE sensors to monitor norepinephrine release and assess its impact on the velocity of autophagic vesicles within the *LC* axons in the *PFC*. We will further use a hM4d-based chemogenetic approach to test the hypothesis that reducing *LC* activity, as it occurs physiologically during sleep, accelerates the trafficking of autophagic vesicles, thereby facilitating their faster delivery to the *LC* somata, enabling *LC* clearance from autophagic material (hereupon just *LC* clearance). Additionally, we will investigate the molecular mechanisms underlying the local activation of PP1 in the *PFC* initiating movement of LC3B-positive vesicles in conditions related to sleep. In **WP2** we will address the involvement of β2AR in regulating both the long-distance autophagic vesicle trafficking velocity in axons in the *PFC* and their somatic delivery in behaviorally challenged animals by using 2P imaging and optical fiber-mediated photoconversion in conjunction with optogenetic approaches, using OptoXRs as a means to simulate GPCR activation. In **WP3**, we will assess the impact of differential *LC* activity states on autophagosome disposal through cell-to-cell communication, specifically between *LC* and *Me5* neurons, via secretory autophagy mechanisms. This will be evaluated through shRNA-mediated, *LC*-specific targeting of Rab27a and Sec22b, key molecular regulators of secretory autophagy. We will investigate *LC* viability, animal behavior, and learning ability in animals lacking Rab27a and Sec22b in the *LC,* alongside the expression of selected DREADDs for chemogenetic intervention. Finally, in **WP3**, we will also assess the potential modulatory effects of D2R through combined expression of selected OptoXRs on *LC*-to-*Me5* cell-to-cell communication and autophagy induction in *Me5* neurons in slice preparations. If successful, we will apply optical stimulation of appropriate OptoXRs *in vivo* to promote autophagy-

related clearance in the *LC* and evaluate respective animals for improved learning following sleep fragmentation. Altogether, the expected results will provide new insights into the combinatorial roles of *LC* activity and inactivity states in the regulation of **autophagy** and may resolve conflicting evidence related to the role of sleep in autophagy induction, trafficking, and clearance *in vivo*. Understanding the regulation of neuronal autophagy in all its aspects, including autophagosome navigation through distal axons, somatic cargo delivery and disposal could inform the development of intervention strategies to prevent *LC* neurodegeneration.

WP1: How does neuronal activity guide the trafficking of autophagic vesicles in *Locus coeruleus* axons?

Our previous work demonstrates an increased dwell time of LC3B-positive vesicles at synaptic boutons, which critically depends on local cAMP/PKA activation. Conversely, inhibition of PKA activity results in a decreased dwell time and an increased velocity of LC3B-positive vesicles during retrograde transport (Andres-Alonso et al., 2019; Andres-Alonso et al., 2021). The absence of typical synaptic contacts at most NE terminals and the reliance on volume transmission suggest a presynaptic, autocrine mechanism of autophagy regulation in *LC-NE* axons.



Surprisingly little is known about the functional interplay of different GPCRs in *LC* clearance through autophagy and the regulation of autophagy in the *LC* during sleep and wakefulness.

Fig. 2.: *Molecular hypothesis of cAMP/PKA-dependent regulation of LC3B-positive vesicle trafficking in LC axon*

We, therefore, hypothesize that the direct involvement of presynaptic α2AR and β2AR, activation of which leads to down- or upregulation of cAMP/PKA signaling, respectively, offers an intriguing possibility for a tightly regulated cAMP/PKA-dependent mechanism controlling AV trafficking in noradrenergic axons. Specifically, low levels of extracellular NE, as observed during REM sleep, will preferentially activate α2AR, resulting in Gi-dependent inhibition of cAMP production. This will decrease the dwell time of AVs and enhance their ability to reach the somata with minimal stopovers, thus adding to efficient *LC* clearance. Conversely, during wakefulness, which corresponds to tonic and phasic activities of the *LC*, levels of released NE will be sufficiently high to trigger efficient signaling via β2AR, leading to autocrine activation of presynaptic cAMP/PKA. This may impact synaptic function and behavior, favoring multiple immobilizations during AVs transport. Thus, *LC-NE* neurons may meet their high demands for presynaptic proteostasis by **temporally regulating the speed of autophagic vesicle trafficking, accelerating it during *LC* inactivation and sleep, and slowing it during periods of heightened *LC* activity associated with distinct animal behaviors** (Fig. 2). In **WP1**, we will address three fundamental questions: Does NE release affect the trafficking velocity of AVs within *LC* axons in the PFC? How do the activity states of the *LC* correlate with the regulation of AV trafficking? What is the molecular mechanism that triggers the mobilization of AV movements during *LC* inactivation related to sleep?

Methods: To answer these questions, we have established CRE-dependent, *LC*-specific labelling of autophagosomes by viral delivery of mNeonGreen-tagged LC3B of B6.CgDbhtm3.2(cre)Pjen/J Dbh ***cre*** knock-in mice (Fig. 3A), followed by *in vivo* 2P imaging through a transcranial window. Designer Receptor Exclusively Activated by Designer Drugs (DREADDs) have been tested and are ready for use, pending TVA-1638 approval, for chemogenetic neuronal silencing of *LC* (Armbruster et al., 2007; AAV-EF1a-DIO-hM4D(Gi)-mCherry/Addgene #50458) and for increasing neuronal activity in the *LC* (AAV-hSyn-DIO-rM3D(Gs)-mCherry/Addgene #50461) accordingly. *In vivo* technologies and imaging processing are established, and most molecular tools, such a AAV particles and antibodies, are available. Additionally, molecular assays are well-developed. Proof-of-principle experiments using pharmacology will be conducted in neuronal cell cultures.

Our yet unpublished observations indicate that autophagosomes are indeed mobile within *LC* axons in the PFC, and we identified several populations of autophagic vesicles characterized by distinct motility pattern within distal *LC* axons in the PFC (Fig. 3B). Principal component analysis revealed that their trafficking velocities and minimal displacement ratios vary across different axons (Fig. 3C-D). Additionally, *LC*-specific neuronal labelling allowed us to assess the sparse presence of LAMP2-

positive lysosomes in *LC* distal axons projecting into the *PFC* (Fig. 3E-G). This finding suggests that these axons may lack the ability to degrade their cargo locally, requiring autophagic cargos to be transported to the neuronal soma of the *LC* for disposal.

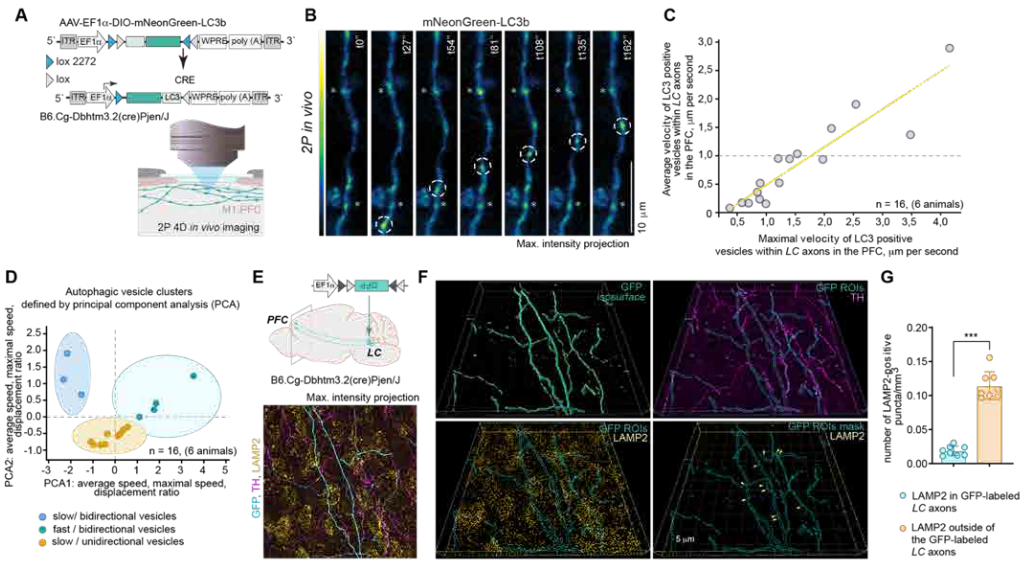


Fig. 3.: *Autophagic vesicles are mobile in Locus coeruleus projections at the PFC A. Schematic representation of Cre-dependent LC-specific labelling of autophagosomes and the implementation of a chronic transcranial window, enabling 4D time-lapse two-photon imaging of AVs motility within LC axons in the M1-PFC region in vivo. B. Representative 2P image frames captured through the transcranial window. C.-D. PCA analysis indicating AV trafficking velocities and defined clusters. E-G. LAMP2-positive lysosomes are sparsely present within the LC axons located at the PFC. 3D reconstructions, volume rendering, and masking were performed using Imaris (Bitplane).*

To assess the role of NE release in *LC* terminals on the mobilization/immobilization of AVs and their trafficking velocity within *LC* axons in the *PFC*, we will employ a newly developed second generation NE sensor (GRAB_NE2h; Feng et al., 2019; Kjaerby 2022; Feng et al., 2023), specifically designed to detect localized NE release and co-express it with an autophagy marker protein fused to mRuby (AAV-DIO-mRuby-LC3B), which is suitable for use in combination with GRAB-NE for 2P-imaging via a transcranial window (Fig. 4 for the experimental workflow). In the next set of experiments, we will chemogenetically modulate neuronal activity in the *LC* to assess the trafficking velocities of AVs by co-expressing mNeonGreen-LC3B with either hM4D(Gi) (Vazey et al., 2014; McCall et al., 2015; Wagatsuma et al., 2018) or rM3D(Gs), an engineered version of the muscarinic acetylcholine receptor accordingly.

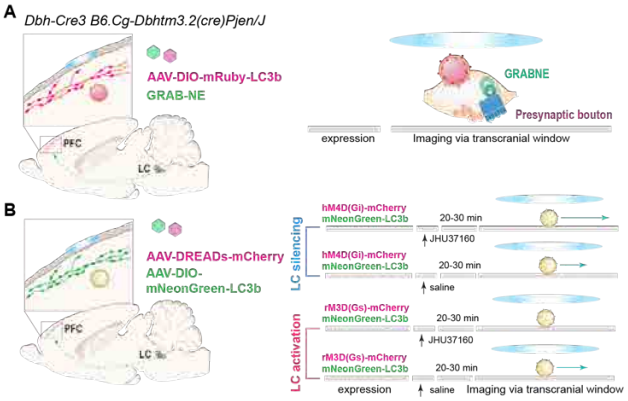


Fig. 4.: *Schematic representation of the experimental workflow in* ***WP1***

We will capture time-lapse videos of the designated field of view through transcranial window positioned above the *M1-PFC* in headplate-fixed animals before and after activating the DREADD receptors by administering the DREADD receptor agonist JHU37160 (Bonaventura et al., 2019). The hypothesis predicts that *LC* silencing, which physiologically occurs during REM sleep, will accelerate the trafficking of autophagic vesicles by reducing their dwell time, thereby facilitating faster delivery to the *LC* somata. *LC* activation may have the opposite effect of increasing dwell time and slowing the delivery of AVs to the somatic region. These results suggest a functional interplay between activity and inactivity states in the regulation of autophagic vesicle delivery to the somata of *LC-NE* neurons for cargo disposal.

Is local activation of protein phosphatase PP1 required to initiate the movement of LC3B-positive vesicles under sleep-related conditions in distal *LC* axons, and what are the molecular mechanisms underlying the local activation of PP1 at LC3-positive vesicles?

Yet unpublished results of heterologous co-immunoprecipitation experiments revealed that both family members, SIPA1L1 and SIPA1L2 immunoprecipitate spinophilin from HEK293T cell extracts (Fig. 5A) and SIPA1L2 recruits spinophilin into vesicular compartment (Fig. 5B) in COS7 cells.

Although phosphorylation of spinophilin by PKA does not alter its direct interaction with recombinant PP1α *in vitro*, it has been shown to preferentially associate with PP1γ (Terry-Lorenzo, 2002).
Our preliminary results show that spinophilin dissociates from PP1γ in axons upon PKA activation, as revealed by a proximity ligation assay (PLA) in hippocampal neurons. The role of spinophilin as a negative regulator of PP1γ in triggering AV mobilization will be assessed using shRNA-mediated knockdown in neuronal cell cultures through plasmid-mediated expression, as AAV-shRNA may pose an oncogenic potential in *in vivo* applications.

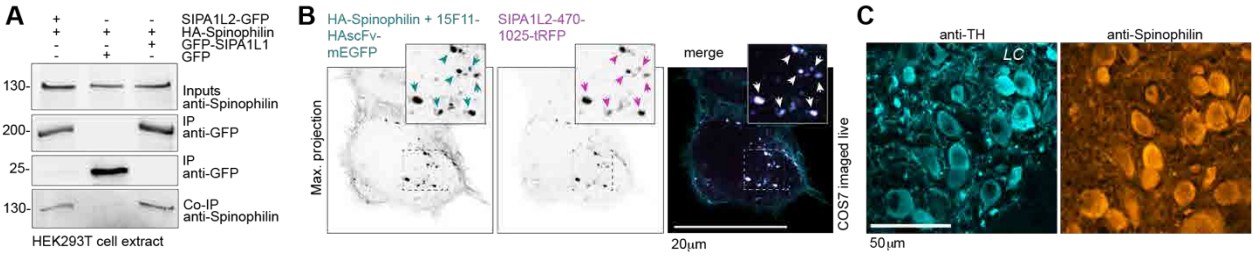
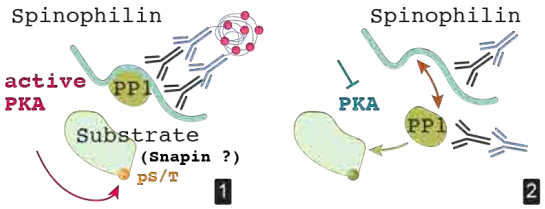


Fig. 5.: *Spinophilin associates with SIPA1L2-containing vesicular compartments and is abundant in LC neurons*

To test the hypothesis that Snapin is a substrate for PP1γ, we will use classical biochemical assays including immunoprecipitations and *in-vitro* phosphatase assay using recombinant proteins, as well as experiments in cell culture and okadaic acid treatments. Immunohistochemical detection of spinophilin in *LC* neurons suggests that the mechanism of PP1γ scavenging may be relevant for regulating the trafficking of LC3-positive vesicles in *LC* axons.

Fig. 6.: *Schematic representation of the PLA between spinophilin and PP1γ*



In the next set of experiments, we will assess whether the spinophilin-PP1γ complex is part of autophagic vesicles in distal axons within the *PFC*. Subsequently, we will evaluate whether the *LC* activity state influences Spinophilin-PP1γ association using a PLA in *LC* axons within the *PFC* labelled with GFP and co-expressing either Gi- or Gs- DREADDs in neuronal somata, following treatment with either saline or the DREADD receptor agonist JHU37160. Three-dimensional reconstructions of *LC* axons (like in Fig. 3) will allow us to quantitatively assess differences in PLA puncta within distal noradrenergic axons under different *LC* activity states.

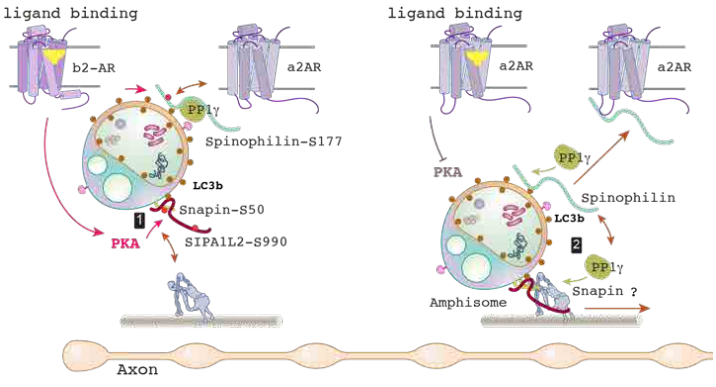


Fig. 7.: *Molecular hypothesis of the mechanism controlling the triggering the mobilization of AVs: 1. PKA activity downstream of β2AR results in the phosphorylation of Snapin and its subsequent dissociation from the dynein motor. 2. The dissociation of spinophilin from PP1γ results in the activation of PP1γ's phosphatase activity, leading to Snapin dephosphorylation and the reassembly of the Snapin-DIC complex*

We will perform a similar assay in brain sections of animals sacrificed during sleep or after a behavioral challenge involving exposure to an open arena and assess the association of spinophilin with PP1γ via PLA. All together, these findings will suggest that spinophilin might be involved in the control of LC3B-positive amphisome trafficking as well as synaptic dwell time in axons in response to the activation of α2AR (Fig. 7).

Expected Results: We will characterize the trafficking velocities of AVs in the distal axons of *LC-NE* neurons in response to NE release and in inactive state of *LC-NE* and we hope to nail down the molecular mechanisms by which *LC* inactivity triggers local PP1 activation in axons, facilitating the retrograde transport of autophagic cargo with the role of sleep in this process.

WP2: Does activation of β2 adrenergic receptors regulate the somatic delivery of AVs in behaviorally challenged animals?

The low abundance of the lysosomal marker LAMP2 in distal axons (Fig. 3E-G) suggests that AVs need to reach *LC* somata for cargo disposal. At present, it is unclear whether the activation of β2AR, resulting in cAMP/PKA signaling, is require for the control of axonal proteostasis in *LC-NE* neurons. Therefore, in **WP2**, we will address fundamental question: What is the contribution of β2AR to the physiological processes required for autophagy in *LC* neurons? Specifically, are AVs originating in the *PFC* transferred back to the *LC* and how are β2AR involved in the regulation of somatic delivery of AVs in *LC* neurons?

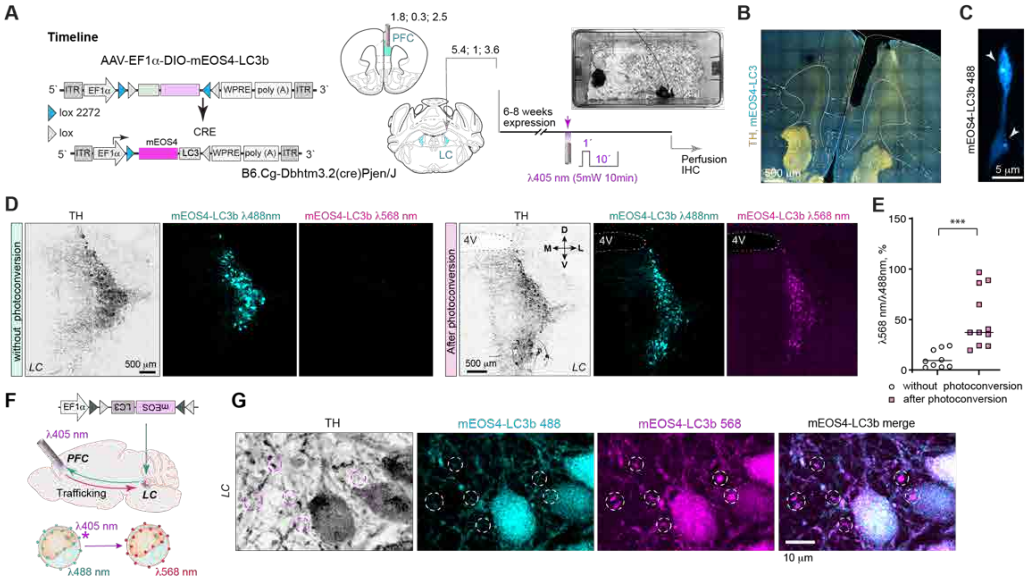


Fig. 8.: *mEOS4-LC3B detection of photoconverted LC3B in the LC-NE. A schematic illustration depicts the timeline of the experimental procedure. Photoconversion of mEOS4-LC3B (ex. at 405nm) within the PFC allows for distinguishing between AVs transported from the distal PFC and other pools of LC3-positive vesicles. Photoconverted mEOS4-LC3-positive vesicles are detected within the somatic compartment of TH⁺ neurons in the LC (dashed circles). Please note that in this experiment, the somatic level is overexposed to highlight puncta in neurites, and image represent the maximal intensity projection from several optical sections).*

Methods: Methodological procedures aimed at labelling AVs with AAV-based delivery of the autophagy marker protein LC3B fused to the mEOS fluorescent protein into the *LC* of *Dbh-Cre3 B6.CgDbhtm3.2$^{(cre)}$Pjen/J* mice have been established. Optical fiber-mediated photoconversion of AVs in the *PFC* and their visualization in the *LC* substantiate the existence of long-distance trafficking of AVs through *LC* axons (Fig. 8). In **WP2**, real-time and *in situ* activation of Optoβ2AR will enable us to directly assess the trafficking velocity of AVs in the *PFC* using 2P imaging through a transcranial window. Our preliminary finding suggests that distal *LC* axons may lack the ability to degrade their cargo locally (Fig.3E-G) due to a sparse presence of LAMP2-positive lysosomes in *LC* distal axons projecting into the *PFC*, requiring autophagic cargos to be transported to the neuronal soma of the *LC* for cargo disposal. *LC*-specific labelling of AVs with mEOS (mEOS4-LC3B), followed by *in vivo* optical fiber-mediated photoconversion at the *PFC*, revealed that LC3-positive organelles indeed reach the soma after their photoconversion in distal axons. Intriguingly, animals exposed to a novel environment, characterized by increased *LC* activity (Takeuchi et al., 2016, Fois et al., 2022; Sciolino et al., 2022) exhibited fewer LC3-positive vesicles in the somata of *LC* neurons after photoconversion at the *PFC* (Fig. 9A-D). Moreover, these vesicles co-localized with SIPA1L2, the neuronal RapGAP involved in trafficking of hybrid organelles, known as amphisomes, and serving as their marker. This co-localization suggests the hybrid nature of LC3B-positive vesicular compartments and involvement of SIPA1L2 in regulating amphisome trafficking in *LC-NE* neurons (Fig. 9E, F). Building upon previous findings, in **WP2** we will address the provocative hypothesis that activation of β2AR, coupled to the Gs/cAMP/PKA pathway (Karnik et al., 2003), in distal axons of the PFC serves as a signal for the immobilization of AVs within *LC* axons in the *PFC*, thereby slowing down the delivery of LC3B-positive amphisomes to somatic regions and delaying somatic cargo disposal (illustrated in Fig.2).

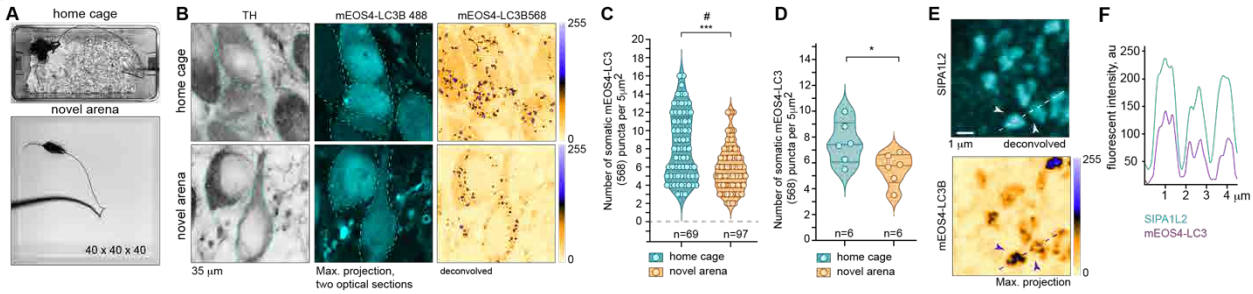


Fig. 9.: *Animals exposed to a novel environment exhibit fewer LC3B-positive vesicles in the somata of LC neurons after photoconversion at the PFC*

To address the hypothesis outlined above and to avoid systemic effects of β2AR agonist action in **WP2**, we will employ chimeric rhodopsin/β2AR (Optoβ2AR), which exhibits dynamics similar to endogenous β2AR *in vivo* (Kim et al., 2005; Airan et al., 2009; Suida et al., 2015; Morri et al., 2018), and apply photostimulation for the specific activation of a downstream signaling. For this, I have subcloned N-terminally V-SVG-tagged Optoβ2AR (Morri et al., 2018, Fig. 10A) into an AAV-DIO plasmid and produced AAV particles to specifically express the probe in *LC* neurons.

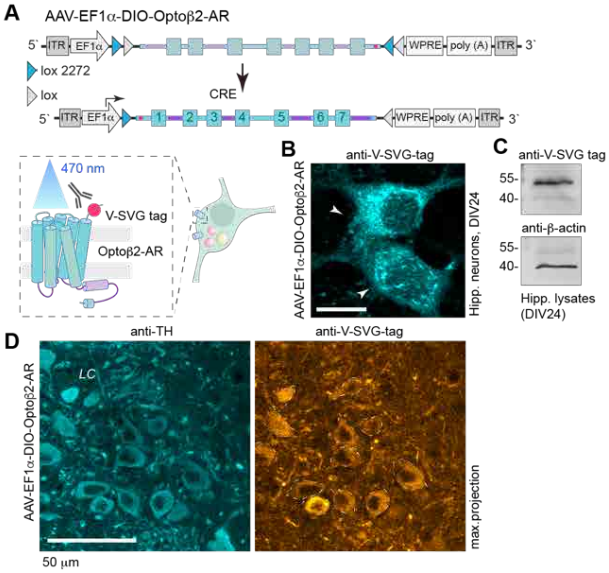


Fig. 10.: *AAV-EF1a-DIO-Opto-β2AR construct details and its expression in culture and in LC neurons*

This approach will be used to assess the role of β2AR activation in controlling the velocity and dwell time of AVs within these axons *in vivo* via a transcranial window. AAV-EF1a-DIO-Optoβ2AR is efficiently targeted to hippocampal cultured neurons (Fig. 10B-C) and is also expressed in the *LC* (Fig. 10D), suggesting that it may play a role in regulating the immobilization of LC3B-containing vesicles in these axons. In the next set of experiments, in **WP2**, we will test synaptic expression of Optoβ2AR *in vivo* and report on that photostimulation enhances cAMP and pERK levels in the *PFC* axons using the red fluorescent protein-based cAMP indicator 'Pink Flamindo' (Pink Fluorescent cAMP Indicator, Harada et al., 2017) and pERK-specific antibodies. Additionally, we will apply *in vivo* photostimulation of Optoβ2AR co-expressed in *LC* neurons along with fluorescently tagged LC3B to assess differences in axonal dwell time before and after photostimulation.

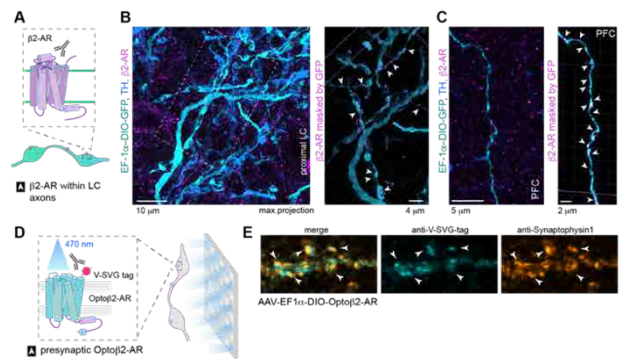


Fig. 11.: *β2AR expressed presynaptically in distal axons in the PFC and Optoβ2AR efficiently expressed in presynapse in neurons in culture*

Collectively, these circuit-specific optogenetic experiments with Optoβ2AR will report on the role of presynaptic β2AR coupled to cAMP/PKA activation in regulating the dwell time of AVs in *LC* inputs targeted to the M1-PFC, thereby elucidating the temporal delay in their somatic delivery and cargo disposal.

Expected Results: We will learn whether the activation of β2AR in the distal axons of *LC-NE* is sufficient for the immobilization of AVs *in vivo* and how this process influences the timing of autophagic cargo delivery into *LC* somata for disposal in animals exposed to novel environment.

WP3: Does the *LC* utilize secretory autophagy for autophagy cargo disposal, and what is the impact of β2AR on membrane exchange between *LC* and *Me5* neurons?

Long-range projecting *NE* neurons in the *LC* are exceptionally vulnerable for degeneration and endo-lysosomal and autophagy pathways are involved in the elimination of polymerized catecholamine derivatives (Zucca et al., 2018; Yamaguchi et al., 2018; Sulzer et al., 2018; Matchet et al., 2021).

Additionally, the *LC* is one of the first areas in the brain affected by the accumulation of beta-amyloid deposits (reviewed in Mather & Harley, 2016). Intriguingly, a recent study suggests a ***functional interplay*** between the *LC* and the adjacent mesencephalic trigeminal nucleus (*Vmes/Me5,* later referred as *Me5*), where *Me5* neurons exhibit enhanced immunoreactivity for beta-amyloid within cytosolic vesicular structures, and the induced cell death of these neurons promotes *LC* neurodegeneration (Goto et al., 2020), suggesting a tight control between adjacent neuronal populations for the exchange of membranous compartments. Concurrently, NE was found to protect against amyloid-induced toxicity via activation of the cAMP/PKA signaling pathway downstream of β2AR (Counts et al., 2010; Liu et al., 2017).
In **WP3**, we will explore the attractive hypothesis that secretory autophagy is involved in *LC-Me5* communication and contributes to *LC* clearance. We will further elaborate on the role of β2AR in the control of this process and a possible transcellular induction of autophagy in Me5 neurons.
Intrigued by the functional interplay between the *LC* and *Me5*, we expressed an acidification-stable version of tagged LC3B in *LC* neurons and observed mRuby-FP fluorescence in the vesicular compartments of anatomically adjacent *Me5* neurons (Fig. 12A, B). These vesicular compartments were ATG16L1-positive, confirming their autophagic nature (Fig. 12C). In **WP3,** we will perform RNA-scope analysis to exclude the possibility that mRuby mRNA is present in the *Me5* following AAV injection in the *LC* and to strengthen our hypothesis that the mRuby-LC3B protein and not mRNA was transferred between the neighboring neuronal populations.

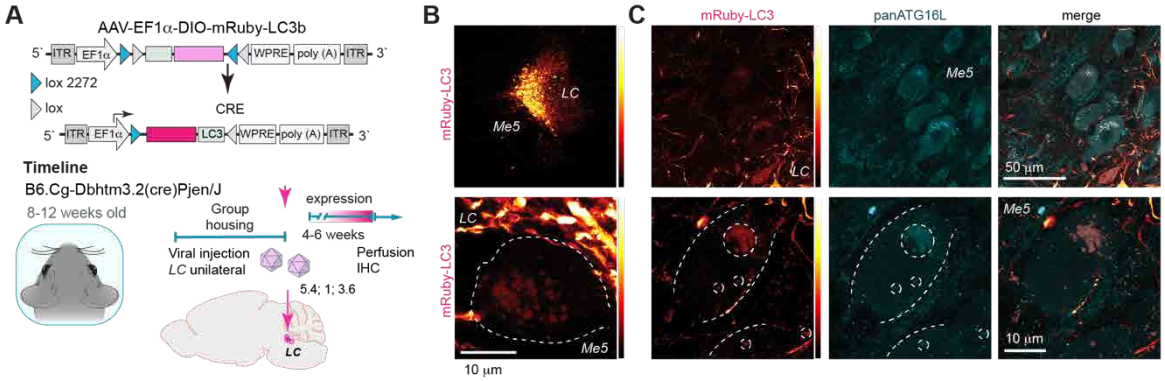


Fig. 12.: *LC3B fused to acidification-stable mRuby-FP, expressed specifically in LC neurons, appears in ATG16L1-positive vesicular compartments of anatomically adjacent Me5 neurons*

The autophagy marker mRNA MAP1LC3B is among the significantly elevated transcripts expressed in human *LC* compared to the neighboring regions (Weber et al., 2023), indicating a high demand for autophagy regulation in these neurons. Our preliminary IHC analysis of endogenous expression of LC3B expression identified a highly abundant population of LC3B-assocoated somatic vesicles, as indicated by the number of LC3B puncta (Fig. 13A). Next, our preliminary comparative analysis revealed that the averaged immunoreactivity for the selective autophagy receptor p62/Sequestosome 1 (p62/SQSTM1) is significantly higher in *LC* neurons, which have relatively small somata, compared to *Me5* neurons (Fig. 13B, C). Conversely, the lysosomal marker LAMP2 was more abundant in *Me5* neurons, suggesting that these neurons may exhibit higher catabolic activity than *LC* neurons (Fig. 13E-F). Therefore, the functional interplay between *LC-NE* neurons and adjacent *Me5* neurons in the exchange of membranous compartments, as suggested in the literature (Goto et al., 2020), along with our preliminary observations, strongly supports the hypothesis of a functional crosstalk between the *LC* and *Me5* for the exchange of membranous compartments containing cargoes for degradation. However, the mechanisms underlying this crosstalk remain largely elusive. In **WP3**, we will address questions such as whether lysosomal acidification in *LC* neurons differs from that in *Me5* neurons, and whether the crosstalk between *LC* and *Me5* for membranous exchange occurs via secretory autophagy mechanisms.
Methods: We plan to utilize a genetic acidification probe, AAV-DIO-RFP-GFP-LC3B (Kimura et al., 2007), to visualize changes in organelle acidification *in vivo* using an advanced, recently developed technology characterized by minimal tissue damage: the 110 µm thin laser-scanning endo-microscope (Stibůrek et al., 2023). This ultra-thin laser-scanning endo-microscope has been shown to be effective in detecting subcellular vesicular structures, such as lysosomes, while minimizing tissue damage (Fig. 14). As an alternative approach, in **WP3** we will perform functional *in vitro*

imaging of *LC* sections dissected from animals expressing GFP, fill them with the pHrodo indicator Red (Invitrogen) to quantify cytosolic pH levels in *LC* neurons *in vitro* and perform time-lapse confocal imaging to monitor changes in acidification. This method will enable us to assess cytosolic acidification in adjacent *Me5* neurons and perform comparative analysis of cytosolic acidification of *LC* vs. *Me5* neurons.

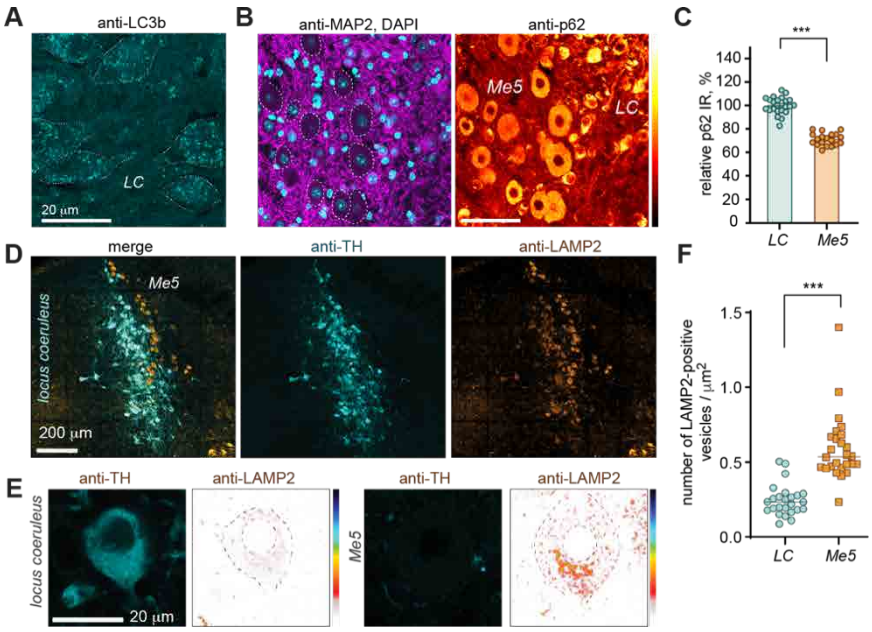
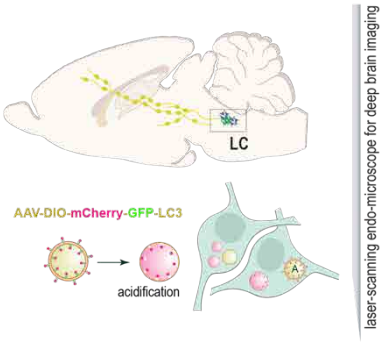


Fig. 13.: *Immunohistochemical analysis of p62 and LAMP2 expression in LC vs. Me5.*

MAP1LC3B family proteins are crucial for both classical and secretory autophagy pathways (Nieto-Torres et al., 2021). The secretory autophagy pathway is upregulated in response to endo-lysosomal inhibition or dysfunction and relies on multiple autophagy-related genes essential for autophagosome formation (Leidal et al., 2020). Reduced LAMP2 immunoreactivity within the *LC*, along with potential changes in acidification indicative of alterations in the endo-lysosomal system, might activate an **alternative secretory autophagy pathway** in *LC* neurons. Additionally, our preliminary IHC experiments indicate that Rab27a is highly expressed in the somata of noradrenergic neurons, though less so in the *Me5* neurons (Fig. 15A, B), suggesting that Rab27a may play a role in *LC-Me5* functional crosstalk and promote secretory autophagy in *LC* neurons.

Fig. 14.: *Schematic representation of the experimental workflow for the assessment of in vivo lysosomal acidification (will be conducted in collaboration with Prof. Dr. T. Čižmár, Jena upon approval of the pending TVA).*



To determine if crosstalk between the *LC* and *Me5* involves secretory autophagy mechanisms, we will use newly generated AAVs, to *LC*-specifically express shRNA to knock down Rab27a (Solvik et al., 2022; adapted to mouse), or SEC22B, respectively (Martinelli et al., 2021), with viral CRISPR/Cas9-knockouts to be considered as an alternative. We will co-express either AAV-shRNAs (both having no hazard potential, ZKBS), as well as a scrambled AAV-shRNA control, with mRuby-LC3B, followed by IHC analysis on different autophagy markers. We expect to observe an absence of mRuby-LC3B in Me5 neurons expressing shRNA for Rab27a or Sec22b compared to the scrambled control, presumably accompanied by concomitant accumulation of mRuby-LC3B in *LC* somata. This would suggest a critical role for secretory autophagy in mediating LC-Me5 membrane exchange (Fig. 15C). In subsequent experiments, we will co-express Optoβ2AR with mRuby-LC3B to perform a comparative analysis of mRuby-LC3B FP signal in *Me5* neurons by applying optostimulation using a LED-array. These experiments will help clarifying, whether a previously observed neuroprotective effect of cAMP/PKA activation via β2AR, described in non-*LC* neurons (Counts et al., 2010; Liu et al., 2017), might be related to the control of axonal AV-pausing and/ or secretory autophagy in *LC* neurons. Next, in a subset of behavioral experiments, we will investigate, whether inhibiting secretory autophagy in the *LC* affects learning, and whether chemogenetic *LC* silencing can improve learning in sleep-deprived animals. Finally, in condition of blocked secretory autophagy, photoconversion of mEOS4-LC3B during *LC* activity *versus* inactivity in behavioral paradigms, will allow to discriminate, whether fewer photoconverted LC3B puncta in *LC* somata (Fig.9), result from slowing down AV trafficking rather than from increased autophagy flux. Preliminary results suggest that the signal for pATG16L, which is known to label nascent autophagosomes (Tian et al., 2020), is enhanced in *Me5*

neurons upon expression of Optoβ2AR in the *LC*, indicating a transcellular induction of autophagy by *LC* neurons (Fig. 16).

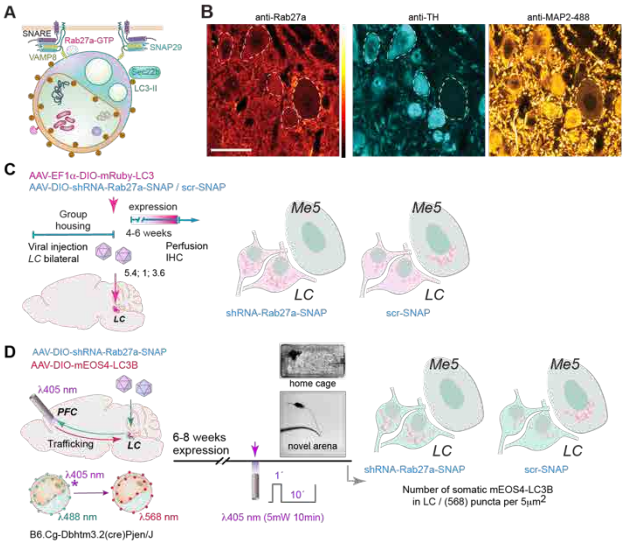
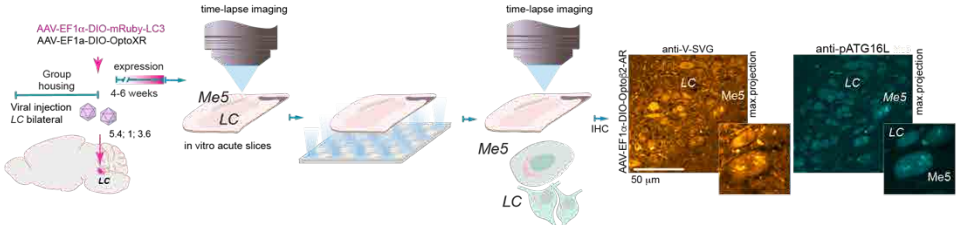


Fig. 15.: *Schematic representation of the experimental workflow with blocking secretory autophagy in LC*

To further address whether enhanced Gs-cAMP signaling via β2AR promotes autophagy in *Me5* neurons we will block secretory autophagy and assess the transcellular autophagy induction. We will also assess the modulatory effect of D2 receptors (OptoD2R), which are known to restore REM sleep in dopamine-depleted animals, on secretory autophagy mechanisms. We will elucidate the cellular mechanisms underlying the functional crosstalk between the *LC* and *Me5* in the exchange of membranous compartments, specifically involving secretory autophagy and we will assess the role of GPCRs in modulating this secretion. If successful, we will apply optical stimulation of OptoD2Rs promoting *LC* clearance *in vivo* to enhance learning in animals subjected to sleep fragmentation.

Fig. 16.: *Schematic representation of the experimental workflow assessing β2AR effects on transcellular autophagy induction in Me5 neurons and potential modulatory effect of D2R.*



Expected Results:

We envisage a clear-cut statement on the relevance of LC-Me5 communication via secretory autophagy for cargo disposal as a neuroprotective means to support *LC* clearance as prerequisite for cognitive function and, eventually, cell survival. We expect to unravel activity-related steps, possibly defining a 'sluice' mechanism mediated by β2AR. We further expect insights on specific role of ARs and modulatory GPCRs in regard to this transcellular mechanism.

Altogether, the proposed work addresses **fundamental molecular and cellular neurobiological principles** of neuronal proteostasis regulation by autophagy in the heavily burdened neurons of the *LC*, exploring its physiology and enhancing therapeutic potential.

(Preliminary work presented in the current project proposal was supported by the local LIN special project and performed by Dr. Anna Karpova and PhD student, Ahmed Adel Ahmed Aly, supervised by Anna Karpova (LIN). I acknowledge M. Prigge (LIN) for sharing the mouse model and H.B. Jia (CNI-LIN) for the help with 2P imaging.)

2.5. Timeline

	2025			2026				2027				2028
	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1
WP1												
WP2												
WP3												

2.6. Requested funding

Funding for	2025		2026		2027		2028	
Staff	Quant.	Sum	Quant.	Sum	Quant.	Sum	Quant.	Sum
Postdoctoral Researcher 100%	1	64,575	1	86,100	1	86,100	1	21,525
Other costs: Consumables, Reagents, HiWi, travel costs		22,425		29,900		29,900		7,475
Animals		3,000		4,000		4,000		1,000
Total		90,000		120,000		120,000		30,000

(All figures in Euros)