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Review

Lighting Up Ca^{2+} Dynamics in Animal Models

Nelly Redolfi [1], Paloma García-Casas [1], Chiara Fornetto [1], Sonia Sonda [1], Paola Pizzo [1,2] and Diana Pendin [1,2,*]

[1] Department of Biomedical Sciences, University of Padua, 35131 Padua, Italy; nelly.redolfi@unipd.it (N.R.); paloma.garciacasas@unipd.it (P.G.-C.); chiara.fornetto@unipd.it (C.F.); sonia.sonda@studenti.unipd.it (S.S.); paola.pizzo@unipd.it (P.P.)

[2] Neuroscience Institute, National Research Council (CNR), 35131 Padua, Italy

* Correspondence: diana.pendin@unipd.it

Abstract: Calcium (Ca^{2+}) signaling coordinates are crucial processes in brain physiology. Particularly, fundamental aspects of neuronal function such as synaptic transmission and neuronal plasticity are regulated by Ca^{2+}, and neuronal survival itself relies on Ca^{2+}-dependent cascades. Indeed, impaired Ca^{2+} homeostasis has been reported in aging as well as in the onset and progression of neurodegeneration. Understanding the physiology of brain function and the key processes leading to its derangement is a core challenge for neuroscience. In this context, Ca^{2+} imaging represents a powerful tool, effectively fostered by the continuous amelioration of Ca^{2+} sensors in parallel with the improvement of imaging instrumentation. In this review, we explore the potentiality of the most used animal models employed for Ca^{2+} imaging, highlighting their application in brain research to explore the pathogenesis of neurodegenerative diseases.

Keywords: calcium imaging; calcium indicators; GECI; animal models; nervous system

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1. Introduction

Ca^{2+} signaling pathways are involved in virtually every aspect of cellular physiology, and their dysregulation have often been documented in pathological situations. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) must be tightly controlled in terms of both space and time to generate regulated signals. This dynamic control is obtained through the orchestrated interplay of Ca^{2+} transport systems, localized in the plasma membrane (PM) and the membranes of the intracellular Ca^{2+} stores and the Ca^{2+} buffering systems.

Ca^{2+} generates versatile signals in the nervous system [1]. In neurons, fast Ca^{2+} influx occurs via voltage-gated Ca^{2+} channels upon membrane depolarization, while slower Ca^{2+} signals come from intracellular stores, which work together sin generating the dynamic regulation of brain activity. Here, Ca^{2+} signals operate over a wide temporal range to control a plethora of phenomena [2], spreading from neurotransmission [3] to plasticity [4] and gene transcription [5].

Besides regulating fundamental neuronal functions, Ca^{2+} signals also sustain processes relevant to the occurrence and progression of neurodegeneration [6–9]. Common, early Ca^{2+} alterations have been described across different neurological conditions, setting the basis for the hypothesis of a shared upstream Ca^{2+} dysregulation in their pathogenesis. Nevertheless, in different neurodegenerative diseases, e.g., Alzheimer's, Parkinson's, and Huntington's disease, specific defective Ca^{2+} signals have been reported, originating from distinct neuronal compartments, organelles, or subdomains. These distinctive features are supposed to initiate or sustain disease pathogenesis, often in selected neuronal subtypes (e.g., dopaminergic neurons in Parkinson's disease [7,10] and striatal neurons in Huntington's disease [11]) or specific brain regions (e.g., the hippocampus in Alzheimer's disease, AD [12]), that are likely more vulnerable to the defect. Thus, investigating Ca^{2+} signaling regulation and dysregulation represents a key to obtaining a comprehensive picture of

disease pathogenesis, with the final aim of developing effective and specific therapeutic strategies [13]. Many of these Ca^{2+}-mediated pathological features have been successfully explored in animal models. Indeed, relevant genes and fundamental biological pathways are mostly conserved through evolution [14–16]. Notably, most of the cellular processes involved in the pathogenesis of human disorders are conserved in animal models [17], making the latter valuable tools to investigate the mechanisms that take part in aging and neurodegeneration.

In the 1980s, the development of the first Ca^{2+} indicators enabled the possibility to measure Ca^{2+} dynamics in living cells, opening the route for deciphering fundamental intracellular signaling pathways [18,19]. Later, genetically encoded Ca^{2+} indicators (GECIs) [20] also allowed the implementation of imaging techniques in vivo in multi-cellular animals. This represented a breakthrough for brain research since GECIs enabled the indirect measure of neuronal activity by recording the fast and transient variations in $[Ca^{2+}]_i$. The investigation of Ca^{2+} signals through fluorescence imaging is now of utmost importance in order to gain information regarding not only single-cell, activity-dependent $[Ca^{2+}]_i$ variations, but to also map neuronal population activity and functional connectivity [21,22] as well as localized Ca^{2+} dynamics from specific subcellular regions, e.g., dendritic spines [23].

In this review, we will analyze the applications of Ca^{2+} imaging in some of the most used animal models, highlighting their role in unraveling important aspects of the pathogenesis of neurodegenerative diseases.

2. Fluorescent Indicators for Ca^{2+} Imaging in Animal Models

Over 30 years ago, the design of the first organic fluorescent Ca^{2+} indicators represented a revolution in the field of Ca^{2+} investigations, initiating the fruitful season of cellular Ca^{2+} imaging [19]. A variety of probes have been developed since then that now cover a wide range of Ca^{2+} affinities and kinetic properties [24].

There are two major families of indicators that have been developed, i.e., chemical probes and GECIs. Chemical indicators (e.g., fura-2, indo-1, fluo-4), small fluorescent molecules based on BAPTA (1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) chelating moiety, are widely used in cultured and isolated cells [25–27] but have been poorly exploited in live animal models [28–31], which is mainly due to tissue permeation issues. On the contrary, GECIs have been widely exploited in live animals due to the techniques available to express the engineered Ca^{2+} sensing proteins that they encode in vivo [32–34]. Notably, GECIs allow the monitoring of Ca^{2+} not only in the cytosol, but also in specific organelles, thanks to the addition of specific targeting moieties to their sequences [35–37].

GECIs include single fluorescent protein-based indicators (e.g., GCaMP), bioluminescent probes (e.g., aequorin) and fluorescence (or Förster) resonance energy transfer (FRET)-based indicators (e.g., cameleons) [38]. Among single fluorescent protein indicators, the most widely used is the GCaMP family of probes [32–34,39,40]. They consist of a circularly permuted variant of Green Fluorescent Protein (GFP), whose N-terminus is connected to the M13 fragment of the myosin light chain kinase. On the other side of the protein, the C-terminus ends with the Ca^{2+}-binding region of calmodulin (CaM). In the presence of Ca^{2+}, M13 wraps around Ca^{2+}-bound CaM, and this leads to a conformational change that increases the fluorescence intensity of the molecule [41]. In recent years, efforts have been devoted to ameliorating the brightness, dynamic range, and Ca^{2+} affinity of the first indicators. As a result, a palette of GCaMP indicator variants have been developed, with a range of Ca^{2+} affinity that allows the measure of $[Ca^{2+}]_i$ not only in the cytosol, but also in the subcellular compartments [24]. Importantly, other fluorescent protein variants (e.g., XCaMPs) have been developed, making the GCaMP family a useful tool to measure Ca^{2+} dynamics simultaneously in different neuronal types or subcellular regions [42] (Table 1).

Table 1. Examples of the most common GCaMP sensors used for Ca^{2+} imaging and relative affinity for Ca^{2+} (K_d).

	Reference	Characteristics	Variants	K_d (nM)
GCaMP3	Tian et al., 2009 [40]	• Adequate fluorescence at resting cell $[Ca^{2+}]$ for the detection of expressing cells. • Unable to detect single action potentials (APs) in vivo.	GCaMP3	345 ± 17 [43]
GCaMP5	Akerboom et al., 2012 [44]	• Improved dynamic range (triplicated in GCaMP5G, compared to GCaMP3). • Variants can be chosen according to their properties (e.g., affinity for Ca^{2+}) to fit particular experimental requirements.	GCaMP5A	307 ± 12
			GCaMP5D	730 ± 18
			GCaMP5G	460 ± 11
			GCaMP5K	189 ± 5
			GCaMP5L	390 ± 18
GCaMP6	Chen et al., 2013 [45]	• Able to detect single APs (GCaMP6f presents the shortest half-decay time). • Variants can be chosen according to their properties (e.g., affinity for Ca^{2+}) to fit particular experimental requirements. • Used to image large groups of neurons, as well as tiny synaptic compartments. • Adequate for imaging experiments over multiple weeks. • Adequate fluorescence at basal cell $[Ca^{2+}]$.	GCaMP6s	144 ± 4
			GCaMP6m	167 ± 3
			GCaMP6f	375 ± 14
jGCaMP7	Dana et al., 2019 [46]	• Enhanced signal-to-noise ratio allows detection of individual spikes over wide fields of view (jGCaMP7s,f). • Used for imaging of small processes thanks to the high brightness (jGCaMP7b).	jGCaMP7s	68 ± 5
			jGCaMP7f	174 ± 9
			jGCaMP7c	298 ± 5
			jGCaMP7b	82 ± 6
XCaMP	Inoue et al., 2019 [42]	• Multiple color variants (blue, green, yellow, red). • Improved signal linearity. • Adequate fluorescence at basal cell $[Ca^{2+}]$ (XCaMP-G). • Enables measurement of fast-spike trains in individual neurons.	XCaMP-G	200 ± 8
			XCaMP-Gfo	128 ± 5
			XCaMP-Gf	115 ± 4
			XCaMP-R	97 ± 10
			XCaMP-Y	81 ± 6
			XCaMP-B	71 ± 3

Although of undiscussed value, single protein-based GECIs suffer from some sensitivity to focal plane shifts, which are caused by the movement of samples, photobleaching, and other artifacts that could lead to a change in fluorescence that is not linked to a change in $[Ca^{2+}]_i$. To overcome this limitation, fluorescence emitted upon excitation at the isosbestic point of the GCaMP excitation spectrum, which is not susceptible to $[Ca^{2+}]_i$ variations, can be used to normalize the GCaMP signal [47]. Alternatively, the indicator can be co-expressed together with a fluorescence protein, with which fluorescence signal can be used as a normalizer for movement-linked artifacts [48].

An interesting alternative to single-protein sensors consist of using ratiometric indicators, in which variations of the ratio of the fluorescence emitted by two fluorescent proteins are correlated to $[Ca^{2+}]_i$. One example of these sensors is the FRET-based cameleon family of probes [49], in which the $[Ca^{2+}]$ responsive elements M13 and CaM are bound to a cyan (CFP) and a yellow (YFP) variant of the GFP, respectively. Upon excitation, the CFP emits at 480 nm in the absence of Ca^{2+}, while in the presence of Ca^{2+}, the interaction between CaM and M13 brings CFP and YFP closer, and the energy released from the CFP is absorbed (and thus emitted at 535 nm) by the YFP. By calculating the ratio of the YFP/CFP fluorescence emissions, it is possible to infer $[Ca^{2+}]_i$ with no interference from focal plane shifts, photobleaching, or dye leakage.

Efforts for the amelioration of existing GECIs and for the development of new ones are continuously ongoing. This path precedes in parallel with the improvement of imaging instrumentation [50]. Indeed, early Ca^{2+} imaging experiments in live tissues/animals were performed with epifluorescence microscopy equipped with charged coupled detec-

tor (CCD)-based cameras using synthetic dyes. However, the application of wide-field microscopy is limited by light scattering across the z-axis and resolution and contrast fall as the imaging plane goes deeper into the tissues. As a result, this technique can only be successful with small and transparent invertebrates [51]. These limitations were overcome by the development of confocal microscopy, which takes advantage of a focused illumination light and a pinhole that rejects scattered out-of-focus light. However, the increase in excitation necessary to compensate the signal loss causes photobleaching and photodamage [52], thus once again limiting the application of confocal imaging to in vitro or very thin preparations [22,53]. The development of two-photon (2P) microscopy [52] allowed the study of Ca^{2+} dynamics within cells localized deeper in tissues. Importantly, this permitted to move from cell cultures and isolated tissues to the imaging of the intact brain of an entire transgenic animal, exploring neuronal circuitry in vivo [51,53–55]. The continuous implementation of the system, e.g., the use of gradient refractive index (GRIN) lenses and microendoscopy, recently allowed imaging of deeper subcortical structures in awake mice [56–59]. Moreover, to overcome the limitation of the restricted field of view of high-resolution microscopy, which is usually smaller than a mouse brain, mesoscale 2P microscopes have been developed, allowing the imaging of extended brain areas with subcellular resolution [60]. Miniaturized, head-mounted microscopes with a large field of view have been also developed in order to expand the activity mapping in the dorsal cortex in freely behaving mice [61].

Thanks to this parallel development of fluorescent indicators and dedicated instrumentation, Ca^{2+} imaging has developed into a powerful tool for the imaging of the brain in living animals. As technology advances, upgraded systems could be designed to map less accessible brain areas in order to obtain a larger view of the nervous system with increased spatio-temporal resolution.

3. Ca^{2+} Imaging in Mice

Among species, mice are the most common animal models used in research. This is mainly due to their similarity to humans at the genetic and physiology level as well as their relatively short generation time and lifespan, which make them useful for aging studies [62,63]. Moreover, thanks to the advancement of technologies, new genetic tools [64] and a large number of transgenic mouse models of disease, including neurodegenerative disorders (see, e.g., http://www.alzforum.org/research-models (accessed on 17 August 2021)), are now available.

Since their development, GECIs are the elective probes for ex vivo/in vivo Ca^{2+} imaging in mice. GECIs can be expressed through in utero electroporation [65] or by injecting viral vectors, such as adeno-associated virus (AAV) or lentivirus, which are known to have efficient tissue transduction and a low immunogenicity [66]. However, the delivery of viral vectors presents some limitations, such as invasive surgery and the uncontrolled level, duration, and specificity of transgene expression [67]. Moreover, the restricted spread of the virus can represent a limit in experiments requiring wide-field imaging, such as mesoscale functional imaging, and multiple injections are often required to guarantee the adequate, widespread expression of the indicator [34].

To overcome these limitations and to reach a more stable, long-term expression of Ca^{2+} probes, GECI-expressing transgenic mice have been developed [32,33,68–70]. These, in combination with the evolution of appropriated optical imaging techniques, i.e., multiphoton or wide-field systems, led in vivo Ca^{2+} imaging studies, especially those investigating processes related to aging and neurodegeneration in old mice, to a rapid improvement.

The most employed GECIs for live imaging in mice are the GCaMPs. In particular, GCaMP6 [32,39] has been used to study Ca^{2+} dynamics in cellular and subcellular compartments. Improved versions of GCaMP6 have been recently generated, i.e., the enhanced version GCaMP7 [46] and the multicolor XCaMPs, characterized by high signal-to-noise ratio and high-frequency spike resolution and optimized for studying complex Ca^{2+} dynamics in vivo [42,71] (Table 1).

Although commonly used, GCaMPs could present side effects when expressed chronically. Cytotoxicity and abnormalities in brain activity have been reported [72] due to the interference of CaM with the gating and signaling of L-type Ca^{2+} channels (Ca_V1). To overcome this drawback, a new GCaMP-X variant was generated by protecting Ca^{2+}-free CaM to avoid Ca_V1 perturbation [73]. Alternatively, inducible/tissue-specific expression of Ca^{2+} indicators can be used, e.g., via Cre/LoxP-mediated recombination [33] or by the tetracycline-controlled transactivator (tTA)/tetracycline response element (TRE) system [74]. Modern genetic approaches, such as the use of tightly regulated genomic locus TIGRE [75] improved these strategies [68] and also expanded the repertoire of driver and reporter transgenic mouse lines for in vivo Ca^{2+} imaging [64].

Another limitation of GCaMPs is represented by their excitation emission spectra. Indeed, traditional GCaMPs cannot be used in mice that concomitantly express other GFP-based proteins. Similarly, their simultaneous use with optogenetic tools is prevented, due to the overlap of a GCaMP excitation spectrum with those of light-sentitive ion channels, such as channelrhodopsin (ChR2). To overcome this weakness, improved red-shifted GECIs with a sensitivity comparable to GcaMP6 were generated: mRuby-based jRCaMP1a and jRCaMP1b, mApple-based jRGECO1a [76], and a near-infrared Ca^{2+} sensor iGECI [77]. The new probes allow deep-tissue imaging and dual-colour imaging together with GFP-based probes and the use of optogenetic tools in Ca^{2+} imaging experiments.

Organelles play a fundamental role in shaping cellular Ca^{2+} signals. Given their importance in physiology and their involvement in the pathogenesis of neurodegenerative diseases [8,78–80], specific Ca^{2+} probes have been developed to target them [38,81]. Nowadays, techniques to measure intra-organelle Ca^{2+} at the cellular level are available and are used routinely. However, this type of measurement is still poorly exploited in living animals.

As far as the mitochondria are concerned, a large number of probes, both chemical [82] and genetically encoded [83–85], are available to monitor organelle Ca^{2+} dynamics in cultured cells. Until recently, however, measurements of mitochondrial Ca^{2+} dynamics in vivo were limited to the use of the virus-mediated delivery of GECIs (see above). Mice lines have only been generated for the expression of mitochondria-targeted bioluminescence-based sensors [86,87] which, however, present a limited spatial resolution. Very recently, we reported the generation of a new transgenic mouse line expressing the mitochondria-targeted cameleon Ca^{2+} sensor 4mtD3cpv [35], which would represent a new valuable tool to investigate mitochondrial Ca^{2+} handling in vivo.

Thanks to the tremendous improvement in Ca^{2+} sensor expression in mice and imaging techniques, Ca^{2+} signalling alterations in a variety of diseases have been described [6,88,89]. For example, a number of studies support the notion of a correlation between Ca^{2+} dysregulation and AD [8,90]. AD is the most common neurodegenerative disorder, characterized by cognitive and behavioral impairment that heavily hinders daily activities. Its diagnosis is confirmed only on post-mortem brain tissue by assessing the presence of senile plaques, formed by extracellular deposits of amyloid β (Aβ) peptides, and intracellular neurofibrillary tangles (NFT) of the microtubule-binding protein tau. The most common forms of AD are sporadic (SAD) without any inheritance, whereas only a small percentage is familial (FAD). The genetic causes of FAD are mutations in the amyloid precursor protein (*APP*), presenilin-1 (*PSEN1*), and presenilin-2 (*PSEN2*) genes. In the late 1980s, Ca^{2+} homeostasis alterations were proposed to be responsible for AD-linked neurodegeneration [91]. Indeed, in the following years, experiments performed in cell cultures, ex vivo and in vivo highlighted that Ca^{2+} handling impairment is an early event in AD pathogenesis [12,90].

In this context, the development of several transgenic AD mouse models (https://www.alzforum.org/research-models (accessed on 17 August 2021)), along with new tools for Ca^{2+} imaging, allowed the investigation of Ca^{2+} dynamics at different levels, ranging from in vivo multiphoton imaging of Ca^{2+} dynamics within organelles [92] to Ca^{2+} imaging in awake mice [93,94]. As an example, advanced Ca^{2+} imaging techniques

have recently been applied to explore hippocampal activity in a knock-in AD mouse model carrying mutated APP. These mice were crossed with a transgenic mouse line that co-expresses the Ca^{2+} indicator GCaMP7 and a Ca^{2+}-insensitive red fluorescent protein (DsRed2) in neurons [95], and two-photon microscopy was applied to image the activity of 1000 neurons in the dorsal hippocampal CA1 region of awake mice. Neuronal activity was detected in the form of Ca^{2+} transients monitoring GCaMP7 fluorescence, while the red fluorescent protein, showing stable fluorescence independent on neuronal activity, was used to precisely align the imaging field during subsequent acquisitions. Ca^{2+} signals were followed together with Aβ deposition for several months while the animals were subjected to specific tasks that allowed the estimation spatial and temporal representations in the hippocampus separately, revealing that place cells were mostly impaired in AD mice, especially in the proximity of Aβ deposition [93].

4. Ca^{2+} Imaging in *Drosophila melanogaster*

The fruit fly *Drosophila melanogaster* is an established model organism in scientific research that offers the advantages of fast and robust genetic manipulation techniques, s lack of genetic redundancy compared to vertebrates, and a completely annotated genome since 2000 [96]. Fly handling is procedurally easy and cheap, and macroscopic phenotypic features allow for the quick selection of individuals [97]. *Drosophila* life cycle starts with the laying of eggs that develop into embryo and then into larvae, which after passing three instar stages undergo pupation followed by metamorphosis, eclose in the adult form. A single fly can lay hundreds of eggs, and the whole cycle lasts about 10 days at 25 °C, making it relatively easy to obtain a large number of experimental individuals.

The lack of genetic redundancy and the easy genetic manipulation via well-consolidated knock-down and knock-out strategies make the characterization of protein function in *Drosophila* straightforward. Noteworthy, relevant genes encoding Ca^{2+} toolkit players and conserved Ca^{2+} signaling pathways were first described in flies, e.g., the first member of Transient Receptor Potential (TRP) channel superfamily [98] and the Store-Operated Ca^{2+} entry (SOCE) molecular players stromal interacting molecule 1 (STIM1) and ORAi1 [99,100]. Indeed, the basic building blocks of the Ca^{2+} signaling toolkit are conserved between mammals and flies [101]. Single homologs of key molecular players are present in *Drosophila*, such as voltage- and ligand-gated PM Ca^{2+} channels [102], the Ca^{2+} release channels the inositol 1,4,5-trisphosphate receptor (IP_3R, named itpr) [103] and the Ryanodine Receptor (RyR) [104,105], the Ca^{2+} ATPases located on PM (PMCA) [106], Sarco/ER (SERCA) [107], the secretory pathway membranes (SPCA, named SPoCk) [108], the Na^+/Ca^{2+} exchanger NCX (Calx) [109], Ca^{2+} interacting proteins, e.g., Calcineurin (CaN), Calmodulin (Cam), Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII), the and Ca^{2+}/Calmodulin-dependent serine protein kinase (CaSK).

The ease of transgenesis in *Drosophila* allowed the generation of several lines for the expression of GECIs. Most frequently, the expression of indicators relies on the Gal4-UAS binary expression system [110,111] as follows: One fly strain carries the cDNA coding for the Ca^{2+} sensor under the control of an upstream activating sequence (UAS) so that the gene is silent in the absence of the transcription factor Gal4. A second fly strain expresses Gal4 under the control of a cell specific promoter. Upon mating, the resulting progeny will express the probe in the specific cell type determined by the Gal4 line promoter. UAS lines for the controlled expression of both cytosolic and organelle-targeted GECIs have now been developed in flies and exploited to unravel questions of physiological and pathological relevance.

Although the anatomic divergence between the fruit fly and humans is evident, the *Drosophila* nervous system proved particularly helpful in the study of neuronal function in physiology and pathology. In the adult fly, both a central and a peripheral nervous system can be recognized. The central brain and the optic lobes are lodged in the head capsule. Neurons from the brain descend in the ventral nerve cord, and motor neurons project towards muscles (for a complete description of *Drosophila* nervous system anatomy,

the reader can refer to [112]). The adult fly brain comprises about 200,000 neurons [113]. In the central brain alone, over 20 million connections have been mapped [114]. Similar to mammals, these neurons are organized in distinct, highly interconnected regions, indicated as neuropils, that are able to mediate complex behaviors, such as learning, smell and vision, circadian rhythms, courtship and mating, aerial navigation, and memory [115,116]. Importantly, as is also the case for humans, behavioral performance in flies decline with age [117]. Moreover, the molecular mechanisms that govern neuronal functionality, including axon pathfinding, synaptogenesis, membrane excitability, and neurotransmission are conserved between *Drosophila* and mammals [16].

From the experimental point of view, the great advantage of using *Drosophila* as a model organism is the high accessibility of its nervous system at all developmental stages. Ca^{2+} imaging procedures have been adapted or specifically developed for the study of Ca^{2+} dynamics in vivo in the nervous system in embryos [118,119], larvae [120–123], and also in adult flies [124]. A critical issue is to obtain adequate excitation light access to the structure of interest. *Drosophila* larvae are partially transparent, and the GECIs expressed in superficial tissues, such as central nervous system (CNS) or sensory neurons, can be excited through the intact animal's cuticle, provided that the fluorescence is strong enough. The main obstacle to larval Ca^{2+} imaging is the sample movement due to continuous body wall muscle contraction, which can be partially avoided using sticky tape [125] or chambers [126] and corrected by using ratiometric GECIs (see above). Adult CNS can be imaged across the cuticle using 3P microscopy [127] or, most frequently, accessed upon surgery [125,128]. Single-wavelength GECIs, such as GCaMPs, are mostly used in adult brains since these preparations are less subjected to movement-linked artefacts. The ease of genetic manipulation in flies allows the combination of Ca^{2+} imaging with other genetically encoded tools, e.g., optogenetics [129–131] and genetically encoded voltage indicators (GEVIs) [132,133]. GECIs expressed in flies can also be exploited to perform in vitro, i.e., primary neuronal culture [26,27,134], or ex vivo Ca^{2+} imaging experiments, e.g., larval neuromuscular preparation [36] or the isolated larval brain [135].

Optical Ca^{2+} imaging techniques can be applied to explore a wide range of biologically relevant questions in *Drosophila* [136]. Mechanisms of sensory processing have been extensively explored in flies using Ca^{2+} imaging techniques to investigate the response of specific brain regions to various sensory stimuli. These studies mainly investigated olfaction [137–142], visual circuits [143], auditory stimuli [144], taste [145], mechanosensation [146], and thermosensation [125]. Moreover, Ca^{2+} imaging using GECIs has been successfully applied to the *Drosophila* brain to investigate the neuronal plasticity underlying associative learning and memory formation [139] and the large-scale mapping of functional brain connectivity [147]. Whole-brain imaging during adult fly open field behavior represents an interesting task that would allow Ca^{2+} imaging to be performed in freely walking flies during sensorial and social behaviors [148]. Long-term (>24 h) chronic preparations, which are fundamental to dissect the mechanisms of plasticity, neurodegeneration, and aging, can be exploited, although they are quite critical from the experimental point of view due to fly fragility and their opaque exoskeleton [128].

The potential of the described approaches makes *Drosophila* a powerful model system to elucidate the Ca^{2+} dynamics involved in pathogenic processes in neurodegenerative diseases. Remarkably, over 60% of known human disease-causing genes have a fly homolog [149], and a wide collection of fly models of neurodegenerative disorders have been developed so far. Disease models have been created in flies by expressing human disease-associated proteins (or their fly homologs). In the case of loss of function disease mutations, knock out/knock down approaches have also proved successful in mimicking the pathology. Neurodegeneration has been observed in many of these models, confirming their reliability.

As an example, *Drosophila* AD models have been generated, and neuronal Ca^{2+} homeostasis has been evaluated using different approaches, e.g., loading the chemical indicator fura-2 in dissociated neurons [150,151] or imaging the whole brain of adult flies expressing

GECIs ex vivo [152] or in vivo through a cranial window [153]. AD fly models expressing mitochondria-targeted GECIs were also exploited, and mitochondrial Ca^{2+} levels have been measured in dissected third instar larval brains [154].

5. Ca^{2+} Imaging in Zebrafish

Zebrafish (*Danio rerio*) is a small freshwater teleost native of Southeast Asia belonging to the cyprinid family. In recent years, it has become a prominent vertebrate model in neuroscience as well as in other fields of biological research. The reasons for its increasing popularity stem from characteristics that allow it to overcome many limitations that emerge when working with other vertebrates, especially tissue opacity and the large size and complexity of the brain. Given its small size, rapid development (precursors of the internal organs appear by 36 h post fertilization) and the short generation time (generally 3–4 months), zebrafish are easy to manipulate and to raise in the laboratory [155].

Notably, the zebrafish genome has been completely sequenced. Interestingly, 70% of zebrafish genes share homology with the human genome, and 84% of genes associated with diseases in humans are present in zebrafish [14,15]. Moreover, a large homology in brain structure and neurochemistry is present between zebrafish and the other vertebrates [156], making e the investigation of neuronal physiology and pathology possible.

Despite the small size of the zebrafish brain (~300 µm thick, 400 µm wide, and 800 µm long), it maintains the basic vertebrate brain organization, although at a lower-level complexity. Moreover, the transparency of the embryos and larvae makes the entire nervous system optically accessible, allowing the brain-wide monitoring of neuronal activity throughout the whole CNS in real time and at single-cell resolution [157–163]. A total of 100,000 neurons have been counted in larval CNS, which is relatively simple yet capable of handling a wide array of activities, e.g., visually guided behaviors such as optomotor and optokinetic response, phototaxis, and prey capture [164–167]. Altogether, these characteristics make zebrafish larvae powerful models to study the neuronal mechanisms underlying complex behaviors at the synaptic, cellular, and circuit level [162].

Zebrafish disease models can be generated in order to explore the progression of pathologies and the alterations of related signaling pathways in vivo [168–171]. Reverse genetic techniques can be employed to transiently manipulate target genes, e.g., by mRNA or morpholino antisense oligonucleotide injection into fertilized eggs at one- to four-cell stage to induce the overexpression or knock down of specific disease-associated proteins [171,172] or by using most recent genome editing techniques such as CRISPR-Cas9 [173]. Notably, due to the low-cost availability of large samples, zebrafish are also amenable for high-throughput screening of large compound libraries [174,175], allowing the very effective selection of new drug candidates without the costly and time-consuming processes linked to targeted drug design.

One of the great advantages of zebrafish is the ease of application of techniques ranging from advanced Ca^{2+} imaging, electrophysiological recordings, and optogenetics, allowing the investigation of neuronal activity in vivo at single cell resolution as well as full circuit reconstruction.

Dextran-conjugated and membrane-permeant chemical indicators have been employed to image Ca^{2+} in zebrafish. However, they require injection procedures and do not allow the selective loading of specific cell types. For these reasons, the GECIs represent the elective choice for Ca^{2+} imaging experiments in zebrafish. Transgenic lines can be generated by microinjecting [176] the GECI cDNA flanked by tol2 recombination recognition sites into fertilized eggs (at one-cell stage) together with Tol2 transcript. This results in the random recombination of the transgene with genomic DNA [177]. Transgenes are usually placed under the control of cell-specific gene enhancers or promoters, e.g., HuC promoter (referred to as elavl3), which labels the whole nervous system, to guarantee GECI expression in specific cell types or at a specific time during development.

GECIs, such as aequorin, cameleons, and GCaMPs, have been exploited in zebrafish, allowing Ca^{2+} imaging experiments to be performed in intact, free moving animals [23,178].

Neuronal activity can be evaluated in specific cellular types in which the reporter is expressed [44,157–161] in response to multiple stimuli and/or conditions. The advantage of expressing fluorescent Ca^{2+} sensors in specific cell types, in combination with appropriate imaging techniques (i.e., confocal, 2P, light-sheet microscopy), is that it allows to image neuron functional dynamics at early stages of zebrafish development as well as during neurodegeneration. As a result, a morphological and functional reconstruction of the pathways underlying brain function and dysfunction can be obtained with a high spatial and temporal resolution, a task that is currently only achievable in transparent zebrafish larva.

6. Ca^{2+} Imaging in *Caenorhabditis elegans*

Caenorhabditis elegans is a nonparasitic nematode firstly described as a model organism in 1963. Only a decade later, it was clear that worms could be used as a genetic tool to study aging and neurodegeneration [179]. The success of this model organism is linked to some of its experimental highlights, including (i) their short lifespan, around 20 days; (ii) their large homogeneous progeny, as hermaphrodites, they normally reproduce by self-fertilization; (iii) their simple anatomy, which still includes nervous, muscular, digestive, and reproductive systems [180]. Moreover, the *C. elegans* genome was completely sequenced in 1998 [181], and posterior genomic and proteomic comparative analysis showed that around 83% of the worm proteome has predicted human homologous genes [182,183].

The *C. elegans* nervous system is extremely simple when compared to mammals yet complex enough to consider this model suitable to study neurodegeneration. Notably, the full connectome of the 302 neurons comprising the hermaphrodite worm nervous system has been mapped [184–186]. Despite the reduced number of neurons, *C. elegans* presents standard glutamatergic [187], GABAergic [188], cholinergic [189], dopaminergic [190], and serotoninergic [191] transmission, and subpopulations of sensory neurons, interneurons, and motor-neurons have been identified.

The availability of the full genomic sequence of *C. elegans* led to the identification of homologs of Ca^{2+} signalling components, including PM [192,193] and intracellular Ca^{2+} channels, such as IP_3R [194] and RyR [195,196]; the Ca^{2+} pumps SERCA [197] and PMCA [198]; transporters, such as NCX [199]; and SOCE molecular players [200]. Furthermore, a plethora Ca^{2+} signalling modulators are present in *C. elegans*, including CaM, kinases, and phosphatases [201–203].

One of the features that make *C. elegans* an advantageous animal model is its genetic manipulability. Forward and reverse genetic approaches have been largely exploited in worms, and a considerable number of transgenic lines have been generated over time [204,205]. Moreover, the availability of a genome-wide RNAi library [206] allows reverse genetics screening. The approach is particularly relevant since it provides partial downregulation of the expression of proteins whose complete loss would result in early lethality [197,207]. Pharmacological high throughput screenings can also be performed in *C. elegans* models of neurodegenerative diseases to search for small molecules as possible therapeutic treatments [208,209].

Consistently, *C. elegans* models carrying gain/loss of function mutations of genes involved in neurodegenerative diseases have been generated, recapitulating many of the characteristics of the disease at the cellular level [210,211]. These models provide interesting possibilities for the study of disease mechanisms.

Ca^{2+} sensors can be expressed in *C. elegans* in a tissue specific manner. A fundamental feature for Ca^{2+} imaging experiments is the transparency of the worms throughout their lives. It is thus possible to record Ca^{2+} sensor fluorescence in vivo in the intact animal. The two most broadly used Ca^{2+} sensors in *C. elegans* are GCaMPs and FRET-based cameleons, the most frequently expressed in neurons or muscular cells [44,212–214], alone or under the optogenetic manipulation of neuronal activity [215]. Although the majority of the studies in *C. elegans* have been performed using untargeted probes expressed in the cytosol, relevant examples of Ca^{2+} sensors expressed in organelles exist, e.g., mito-

chondria [216–218]. Recently, advanced approaches allowed whole-brain Ca^{2+} imaging, in which about 100 neurons were simultaneously monitored in moving worms [219,220].

The application of these powerful techniques to *C. elegans* models have provided critical information on the role of Ca^{2+} signaling in aging [221]. As an example, the dynamics of cytosolic and mitochondrial Ca^{2+} during aging have been recorded in live, non-anesthetized worms [216,222].

7. Final Remarks

Here, we depicted four commonly used animal models used to study neurodegeneration, highlighting their characteristics and potentiality in the field of Ca^{2+} imaging. The combination of genetic accessibility and methodological advances has enabled the exploration of Ca^{2+} dynamics not only inside single cells but also among a population of neurons. As a result, cellular and circuit neurophysiology can be explored in physiopathological conditions.

Many relevant examples of mouse and *Drosophila* models of neurodegenerative diseases exist where Ca^{2+} dynamics have been successfully explored. Additionally, Ca^{2+} probes can be expressed in *C. elegans* and zebrafish, two models that have great potential in terms of genetic manipulation. We foresee that these organisms will be profitably used to explore Ca^{2+} dynamics in models of neurodegenerative disorders [223,224].

Thanks to the improvement of both Ca^{2+} sensors and imaging instrumentation, the study of Ca^{2+} dynamics is progressively moving from in vitro samples to animals in vivo, providing an ever-growing number of strategies for investigating neural function and dysfunction. It is however worth to mention that in recent years, three-dimensional (3D) brain organoids from patient-derived, inducible pluripotent stem cells (iPSCs) have been developed, showing themselves to be useful models for studying different aspects of aging and neurodegeneration [225–227].

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