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Uruguayan Insect Research: Seven Decades Shaping Neuroscience

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Insect research has significantly advanced neuroscience by addressing fundamental questions. Uruguay has played a pivotal role in this field, contributing to foundational discoveries that comprise neuronal plasticity, circadian rhythms, and molecular neurodevelopment. Uruguayan researchers have explored synaptic activity-induced changes in developmental proteins at the neuromuscular junction and investigated age-related morphological changes in motor terminals influenced by circadian rhythms.

Collaborative efforts have expanded insights into the olfactory glomeruli, revealing synaptic spinules and microcircuitry through advanced microscopy. Molecular investigations have identified temporal expression patterns of synaptic genes during neurodevelopment. Explorations into hypoxic development in the optic lobe have uncovered oxygen-mediated brain size regulation via atypical soluble guanylyl cyclases.

Additionally, Uruguayan researchers have elucidated mechanisms of reversible neurodegeneration in mutants, highlighting the utility of *D. melanogaster* as a model for human neurodegenerative disorders.

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This review underscores seventy years of Uruguayan contributions to insect neuroscience, demonstrating how despite having a limited number of researchers, Uruguay has embraced and promoted interdisciplinary collaborations and innovative methodologies.

1. Introduction

This year, as the Uruguayan Society for Neuroscience (SNU) celebrates its 30th anniversary, there has never been a more opportune moment to revisit and celebrate the legacy of Uruguayan insect research. From its humble beginnings amidst the historical context of World War II, the field of insect neuroscience in Uruguay has flourished, propelled by a spirit of innovation and scientific curiosity.

Uruguayan researchers have long been at the forefront of our understanding of the intricate workings of insect brains. From the pioneering work of Clemente Estable and Eduardo De Robertis to the groundbreaking discoveries of Omar Trujillo-Cenóz, Jacobo Melamed and Rafael Cantera, Uruguayan scientists have made valuable contributions to the field, shaping the landscape of neuroscience both locally and globally.

As we reflect on seven decades of progress, we are reminded of the enduring impact of Uruguayan research on the landscape of neuroscience and the vital role that the SNU plays in shaping the future of the field. We invite readers to share their insights and reflections on the pivotal contributions of past scientists and the implications of their work for contemporary research.

In this article, we will journey through the evolution of Uruguayan insect neuroscience, from its beginnings to its current state. We will explore the pioneering investigations into neuronal structure, revisiting circadian rhythms and synaptic dynamics, as well as the latest research in developmental biology and neurodegeneration.

Glitter years: The historical foundations

Emerging from the historical turmoil of World War II, a period of technological advances and innovation set the technological basis of modern biochemistry and cell biology. In that pivotal moment, Uruguay received its first electron microscope through financial support of the Rockefeller foundation. This technological advance catapulted microscopical analysis and revolutionized our understanding of the cellular mechanisms underlying macroscopical phenomena.

A long standing debate between Camillo Golgi and Santiago Ramón y Cajal, who shared the Nobel Prize in 1906 [1], was still warm. Aiming to understand the ultrastructure of neurons, one of Cajal's disciples, Clemente Estable, recruited the Argentinian cell biologist Eduardo De Robertis and organized a Department of Cell Ultrastructure at the Uruguayan research institution which now bears Estable's name [2]. Discovery of microtubules [3] [4], mitochondria, and centrifugal transport of vesicles, along the axon [4] and the synaptic cleft [5], supported Cajal's view of neurons as discrete entities.

While the contributions of early pioneers such as Cajal and Golgi are widely celebrated, we cannot ignore the upcoming diverse perspectives and methodologies that have shaped the field of neuroscience. How do these historical crossroads continue to influence our understanding of the nervous system today? We hope a partial answer could emerge from this review.

We will now dive into a sea of fascinating discoveries emerging from investigations carried out in Uruguay, largely unknown to most of our students and colleagues. Their key players are briefly presented in Box 1.

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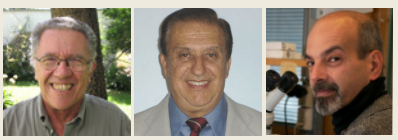
Box 1 | **Key players**

Omar Trujillo-Cenóz (left), Emmeritus Researcher at Instituto de Investigaciones Biológicas Clemente Estable until 2022. Head of the Department of Comparative Neuroanatomy until his retirement in 2005. **Jacobo Melamed** (center), worked at Department of Comparative Neuroanatomy at Instituto de Investigaciones Biológicas Clemente Estable until 1970. Moved to Brazil where he practiced as both a clinical ophtalmologist and researcher. **Rafael Cantera** (right) Emmeritus Professor at Stockholm's University. Head of the Department of Developmental Neurobiology at Instituto de Investigaciones Biológicas Clemente Estable in 2004 until his retirement in 2022.



In the light of evolution: Unraveling neuronal structure and contacts

Forty years before the concept of Unity of life [6], Estable already valued model diversity [7]. A little known aspect of Estable was his work in entomology, where not only he addressed the systematics of some insects of Uruguay, but made important behavioral observations [8]. This early work set the basis for a school of animal behavior, and provided an evolutionary perspective to the ultrastructural observations made afterwards. While Darwin's work was relatively recent, the school of comparative anatomy that inspired him was already well established.

Soon after the first simultaneous observations of synaptic vesicles presented at meetings [9] [10], De Robertis adopted the comparative approach to generalize his ideas. Consequently, the earliest mention of arthropods in Uruguayan neuroscience literature is no other than the general description of the chemical synapse, *"In view of these observations it is postulated that the simple contact of membranes between nerve endings does not constitute a synapse and that the presence of the submicroscopic vesicular component, in addition to the membrane contact, is the specific character of most synaptic junctions"* [11]. Then, their landmark paper was published [12].

One of De Robertis' former apprentices, Omar Trujillo-Cenóz, conducted seminal work using the lepidopteran *Eumorpha labruscae*. He made the groundbreaking observation that glial cells ensheath nerve fibers and proposed that synaptic vesicles arise from the endoplasmic reticulum [13], a concept that had only recently been suggested [14]. Subsequent observations in an isopod crustacean revealed a linear arrangement of mitochondria and vesicles along nerve fibers [15].

Aiming to understand the fundamental features of the arthropod nervous system, a comparative study of ganglia ultrastructure revealed conserved structures across taxa. This led to the proposition that arthropods have pear-shaped monopolar neurons insulated by a glial capsule, with interneuronal contacts being restricted to the neuropil [13] [16].

An innovative step ahead was the idea that highly-complex behavior could emerge from integrative processes within a relatively simple system like the insect brain. As synaptic contacts were spatially-restricted, understanding their relations promised insight into the mechanisms underlying insect behavior. Once again, a comparative study was undertaken, this time on the organization of the glomeruli of coleoptera and hymenopterans [17].

The synaptic relationship of glomeruli proved far more difficult to understand, and had to wait another 50 years for concepts and technology to develop [18] [19], as we will discuss later.

2. Making sense out of sensory systems

A long trip for a short distance: Mapping the insect visual pathway

Arthropods have remarkable optical systems that arose early in evolution [20]. Some groups exhibit amazing eyes that have captivated scientists for centuries [21]. The growing field of visual neuroscience that gained momentum since the 1950s [22] was intrigued by a fundamental question: How do insects, exhibiting a relatively simple visual system and few neurons, achieve extraordinary flight performances?

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Electron microscopy revolutionized our understanding of the insect visual system, revealing that each ommatidium consists of (six to) eight photoreceptor cells (R1-R8), arranged in three peripheral duets and a one in the center [23] [24] [25]. This arrangement, conserved among species, uncovered evolutionary and functional significance to color vision, with outer photoreceptors expressing long wavelength-sensitive opsins and the inner ones generally sensitive to UV-wavelengths [26] [27].

A comprehensive comparative study of sensory organs across various phyla, including photoreceptors of bees and sensory cells in the distal portion of the palps of lepidopterans [28] provided further evolutionary insight into functional anatomy. So, how was visual information processed in these systems? Pioneering connectomics studies within the compound eye were undertaken, following Kenyon and Cajal's seminal work [29] [17]. Cajal's recognition of the convergence of visual systems from cephalopods, insects and vertebrates [30], probably guided early ultrastructural investigations.

Distinctive synaptic arrangements, such as ribbon synapses resembling those found in vertebrates [31] [32] were identified in dipterans [33]. The discovery of a T-shaped structure at the presynapse in flies [29] marked a remarkable advance, now widely recognized as presynaptic active zones [34]. Additionally, capitate projections, an invaginating process from glial cells at the synapsis, were described in the fly visual system [29], now thought to be involved in synaptic vesicle recycling [35].

Comparative studies with *Lucilia sp.* flies supported the idea that the optical cartridge, rather than the ommatidia, serves as the sensory unit [36]. Using a combination of outstanding electron micrography (Figure 1A) and an important work of three-dimensional conceptualization, they produced a model of the apical portion of the ommatidium (Figure 1B).

Mapping connections within the insect visual system, it was proposed that the first synaptic plexus of horizontal fibers of the medulla serves as a movement detection system [33]. Thus, Trujillo-Cenóz proposed that the regular arrangement of second-order synapses acts as a formal screen for the visible world of the fly. These were the first descriptions of the neural superposition eye, a relatively modern type of compound eye providing high resolution and sensitivity to some dipterans [37] [38]. This sophisticated eye captures each point of the visual space by multiple photoreceptors from different ommatidia which converge on the same synaptic unit in the brain. Furthermore, different photoreceptors within the same ommatidium capture different points in visual space [39].

Further investigations elucidated the cellular basis of polarized light perception in dipterans [23] [40]. It was found that long-legged flies have two different orientation patterns of microvilli in central cells of the ommatidium providing the filter system. Thus, vertically-oriented microvilli diminish absorption of horizontally-polarized light (such as that being reflected by horizontal surfaces as water), enhancing prey-detection against glossy surfaces or over the water. The second type has an orthogonal arrangement of microvilli that provides a two-channel polarization analyzer [41].

This remarkable body of work on the visual system of dipterans was crowned by a behavioral study regarding the visual discrimination of color patterns in the parasitic robber fly *Mallophora ruficauda*. It was found that patterns of dark color and vertical position can trigger the male sexual program, thus suggesting that the nervous system has an oversize-biased wide-band filter [42].

Cutting-edge technologies, such as quantum computing and artificial intelligence, are still promising to offer insights into some fundamental questions. How and which emergent properties [43] arise from a given anatomical blueprint? How are cellular connections hard-wired into the developing organism in order to achieve stereotyped outcomes?

A nonlinear touch: Mechanoreceptors of the cockroach

Insect sensory systems not only provide a cost-effective model for research, but also for educational purposes due to their simplicity and availability. A novel laboratory experiment using minimal open hardware and free open source software has recently been proposed for

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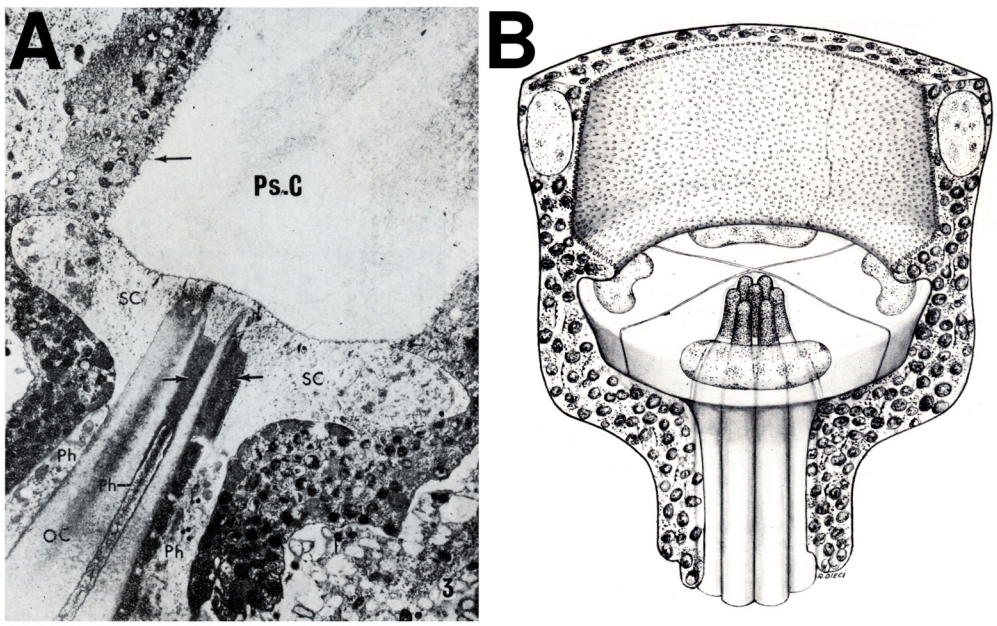

Figure 1. Ultrastructure of the apical portion of the fly ommatidium. (A) Longitudinal section through the apical portion of one ommatidium. The pseudocone cavity (PsC) contains a fine granular material which represents the liquid of gelatinous substance forming *in vivo* the pseudocone of Grenacher. The wall of the pseudo cone cavity is formed by the cytoplasm of the corneal pigment cells. Prolongations of these cells surround the apical segments of the photoreceptors. Note the presence of short microvilli arising from the surface of the corneal pigment cells and projecting into the pseudocone cavity (arrow). The Semper cells (SC) form the floor of the pseudocone cavity. The superior surface of the Semper cells shows small numerous irregularities; the inferior surface has deep indentations occupied by cylindrical rods consisting of a dense homogeneous substance (arrows). Three photoreceptor cells (Ph) can be seen in the lower left corner of the plate (two of them show their rhabdomeres). OC, ommatidial cavity. Magnification 4,700X. (B) This drawing is a three-dimensional representation of the apical portion of one ommatidium. The wall of the pseudocone cavity consists of two pigment cells (corneal pigment cells). The floor of the cavity is formed by four Semper cells which are represented as the transparent elements. Note the presence of seven rods which prolongate the apical segment of the photoreceptors. These rods lie partially recessed in the interior surface of the Semper cells. Reproduced from [36], with permission.

teaching electrophysiology with cockroaches, making scientific inquiry accessible to middle and high school students [44].

Early explorations of the mechanoreceptors of the cercal system of the cockroach *Periplaneta americana* revealed distinct properties of the wind-sensitive bristle-like and the contact-sensitive thread-like sensilla [45] [46]. Bristle-like sensilla are non-directional, but stimuli moving towards its base proved to produce maximal response [46]. It was found that the afferents that innervate thread-like sensilla have spontaneous activity [45], while those of bristle-like do not [46]. Gain increases were found to be evoked by increasing inputs, which could be explained by membrane characteristics providing a nonlinear gain element [45].

3. Hiatus: the 1990s

From that point forward, Uruguayan insect neuroscience had to wait until the eve of the new century to see significant advancements. Then, Cantera and Trujillo-Cenóz detailed the association between glial cells and elements of central neuropils in different insect species of the Lepidoptera, Diptera and Odonata orders. They observed that some glial cells extend intricate

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arborizations into synaptic neuropils, circumscribing distinct groups of synaptic terminals, while others reside entirely within the neuropil, enwrapping fiber bundles in a manner akin to mammalian oligodendrocytes. Moreover, they described that some glial processes envelop the tracheoles of neuropils, resembling the way endfeet of astrocytes surround blood capillaries in the mammalian central nervous system [47].

4. Developmental milestones in insect neurobiology

Early morphogenetic studies

The cornerstone was set by a study of the ultrastructure of the eye-antennal imaginal discs now using *Drosophila melanogaster* and *Lucilia* where the morphogenetic furrow was described as the leading edge of morphogenesis among other remarkable findings [48]. Furthermore, the first description of ommatidial development revealed the ability of undifferentiated disc tissue to organize ommatidia [49]. Using a partial disc transplantation approach to investigate the development of the dorso-ventral symmetry of the ommatidial rhabdomeric pattern, these researchers found that even undifferentiated disc cells are spatially determined [49].

Notably, using transplanted imaginal discs to generate ectopic eyes in *Lucilia*, researchers discovered that photoreceptor axons can form presynaptic structures with glial cells [50]. A controversial finding that provided an integration bridge between the theories of Cajal and Golgi findings. Now recognized as neuronal-glial circuits, these integrative structures led to the development of a unified theory of brain organization [51].

A bachelor's thesis supervised by Trujillo-Cenóz and authored by Fernández in the mid-1980s, examined morphological aspects of cellular differentiation in honeybee photoreceptors. Despite its scholarly value, the study was only published as a thesis and did not appear in an academic journal, which may have constrained its reach and impact.

The research traced the photoreceptor's development from the epithelial cell stage to full maturity, covering circuit maturation within the system. Images obtained in the larval stage revealed the presence of microvilli on proto-photoreceptors, which differed from those observed during the nymphal period, specifically where the rhabdom grows (Figure 2A). In larvae, the microvilli were found to be longer and more regular compared to those found in nymphal stages of dipterans, where the rhabdom originates from the growth of such microvilli along the lateral-medial surface of each photoreceptor. Ultrastructural analysis of the adult rhabdom (Figure 2B) revealed that microvilli are oriented perpendicularly to the longitudinal axis of each cell (Figure 2C). While not definitive, this pioneering study identified differences from earlier research exploring phasmids [52].

This work reflects Uruguay's progress in keeping up with the advancing role of women in neuroscience. Remarkably, more than 40% of the scientific staff at Instituto de Investigaciones Biológicas Clemente Estable were women in the mid 1950s, as observed in pictures taken at that time (as in [53]). To the best of our knowledge, none of them authored any of the papers regarding insect models during the first thirty years! A notable and encouraging aspect of 21st-century Uruguayan insect neuroscience research is the increasingly prominent role of women in authorship.

Neuronal plasticity and circadian changes in motor terminals

A landmark study made the first contribution to Uruguayan insect neuroscience of the century. Conducted in 2003, and comprising visits to Cantera's lab in Stockholm, this study explored synaptic dynamics at the neuromuscular junction of *D. melanogaster*. It revealed that synaptic activity, such as electrical stimulation of the nerves and exposure to glutamate, the neurotransmitter used at the insect neuromuscular junction, led to a decrease in the levels of two developmental- and signal transduction-relevant proteins, Cactus (homolog to IκB-α) and Dorsal (homolog to NF-κB), at the post-synapse [54].

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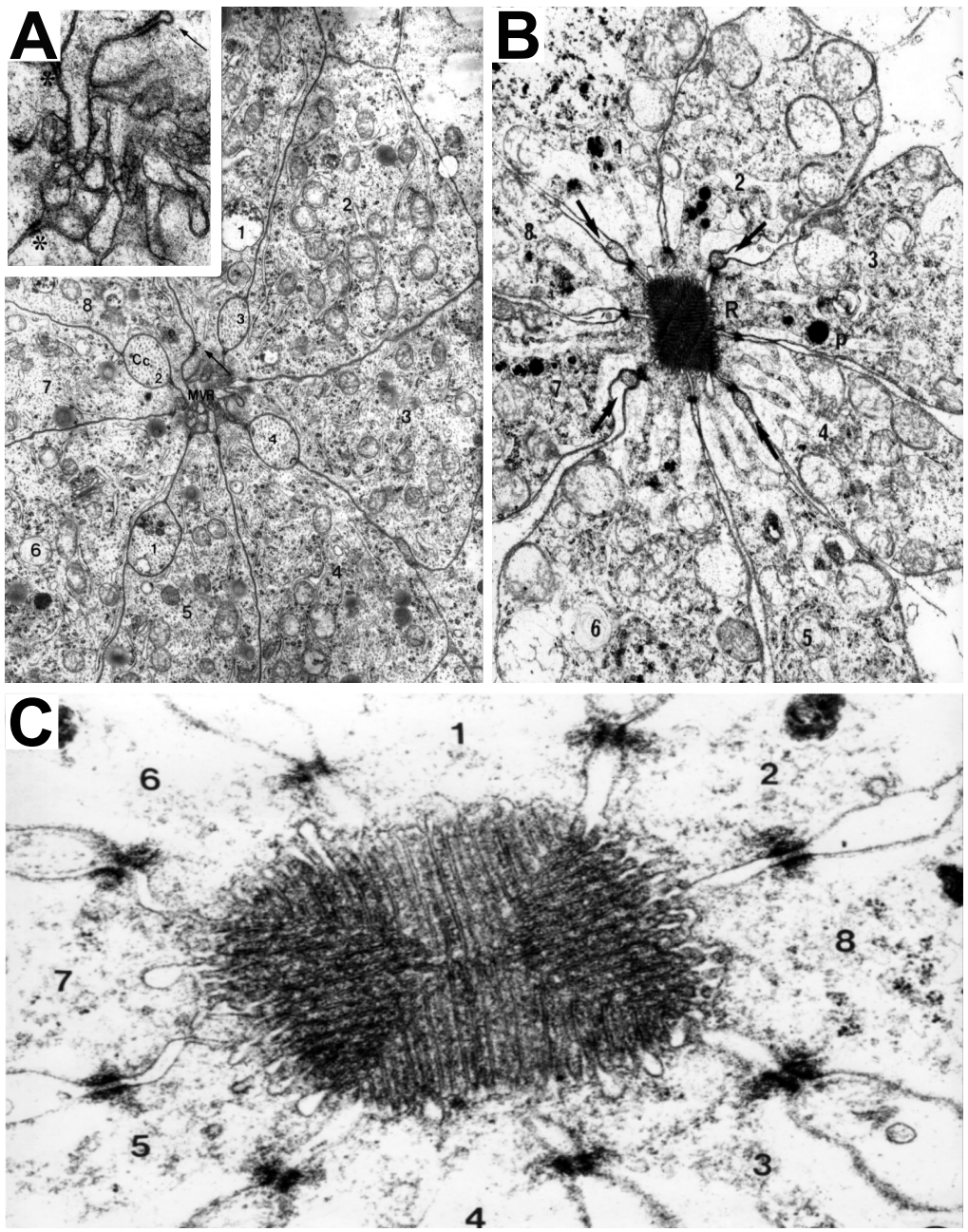

Figure 2. Ultrastructural changes of microvilli during nymphal and adult stages in *Apis mellifera* ommatidia.
(A) Transverse section of a nymphal ommatidium showing the circular arrangement of photoreceptors (1-8). A ninth cell is not visible as it is located at a deeper level near the basal membrane. The differentiation process of the rhabdom is visible in the central area of the ommatidium. The immature rhabdomeric microvilli (MVR) are irregular and unordered. Desmosomal junctions (asterisks) can be seen, and it is common at this stage to find "omega" figures (arrow and inset at higher magnification). The extensions of the crystalline cone cells (Cc 1-4) exhibit a larger diameter than in the adult. Magnification 17,300X. Inset magnification 51,800x. (B) Transverse section of the ommatidium in an adult individual showing the arrangement of the photoreceptor cells (1-8). The rhabdom (R) appears as a compact electron-dense structure. Arrows indicate the extensions emanating from the crystalline cone. The photoreceptors contain abundant pigment granules (P) that are more numerous in the more apical regions of the cells. Magnification 17,000X. (C) Micrograph of a mature rhabdom showing its "paracrystalline" structure. The rhabdomeric microvilli are oriented in two perpendicular directions. The photoreceptors are numbered (1-8). Magnification 19,000X. Reprinted from [52], with permission.

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In 2004, the Uruguayan researcher Rafael Cantera, who was then Professor of Functional Zoomorphology at the Department of Zoology at Stockholm's University, joined the Instituto de Investigaciones Biológicas Clemente Estable and established the Department of Developmental Neurobiology. Cantera's groups in both Uruguay and Sweden used the neuromuscular junction of *D. melanogaster* as the model to study neuronal plasticity.

Furthermore, under Cantera's guidance, the Uruguayan research group initiated or contributed to studies on circadian rhythm-associated processes, antennal lobe glomeruli connectomics, neurodevelopment and neurodegeneration using the fruit fly.

Using confocal and electron microscopy, they explored the rhythmically-changing morphology of the neuromuscular junction on abdominal muscles throughout *D. melanogaster* adulthood. Age-related ultrastructural, morphological and functional changes within these synaptic structures were found. Notably, a progressive enlargement of boutons, variations in nerve branch length and diameter, and discernible ultrastructural traits of impaired endocytosis and vesicle dynamics during aging were observed. These findings were further validated by comparative analysis with individuals carrying a temperature-sensitive mutation in the dynamin homolog shibire. Intriguingly, they noted that age diminishes the time required for the establishment of the complete paralysis phenotype in these individuals, while prolonging the recovery period [55].

Cantera's group first observed a rhythmic fluctuation in a specific *D. melanogaster* flight motor neuron morphology, characterized by thicker branches and larger boutons during the day. This rhythmicity persists for two days under constant darkness, indicative of a true circadian cycle driven by a molecular clock. The morphogenetic rhythm is indeed disrupted in individuals carrying arrhythmic mutations in clock genes timeless and period, and it is lost as the flies age [56].

From this work, two pertinent questions arose: Are these morphological changes in the terminals directly controlled by the biological clock, independently of synaptic activity? Is the central clock (network of brain clock neurons) responsible for regulating the rhythmic changes in bouton size?

In a subsequent study, researchers explored these questions using paralysis and sleep deprivation to manipulate synaptic activity levels and observed that bouton size rhythmicity remained unaffected under both conditions. Notably, they found that the cycle in bouton size lasts for two days in decapitated flies, further supporting the idea that this rhythmicity is largely independent of synaptic activity. Altogether, they suggested the existence of an alternative –peripheral– pacemaker, distinct from the main central clock [57].

Building on their previous studies, Cantera's group discovered that *D. melanogaster* motor synapses undergo circadian reorganization, evident in both vesicle size and distribution, with distinct patterns emerging under different lighting conditions [58]. Additionally, they observed a rhythmic fluctuation in the number of motor synapses, noting an increase in synapsis assembly during the dark phase compared to the light phase [59].

Aiming to understand the developmental reach of these rhythmic phenomena, Ruiz et al., conducted an investigation regarding clock gene expression in the embryonic central nervous system of *D. melanogaster*. They found that *per* is absent in embryonic glia, suggesting delayed or postembryonic onset, while *tim* is expressed in glia at lower levels than in neurons. Both *per* and *tim* expression diminishes in neurons and glia during late embryonic stages.

Despite close proximity in the nerve cord, *per* and *tim* neurons originate from diverse neuroblasts, with expression induced in individual progeny cells rather than lineage-inherited. These findings suggest potential non-clock functions for *per* and *tim* in neuronal development [60].

The antennal lobe olfactory glomeruli: synapse ultrastructure and connectomics

In a study on synaptic spinules in the olfactory circuit of *D. melanogaster* using focused ion beam-scanning electron microscopy, Gruber et al., observed these structures penetrating

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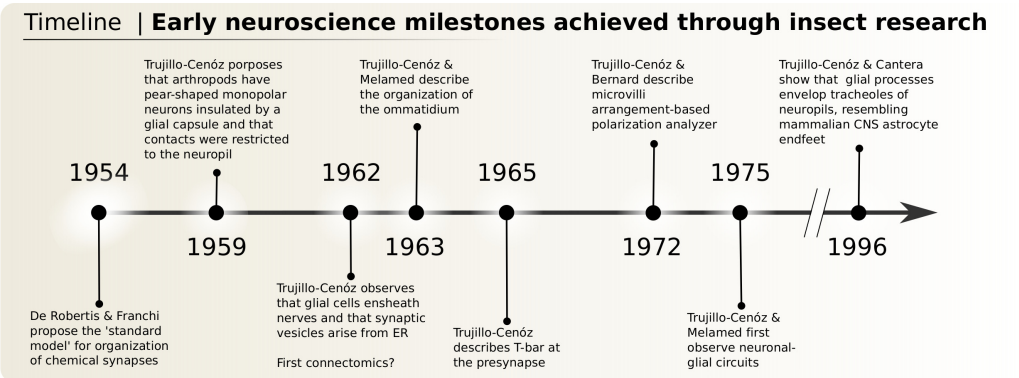

synaptic boutons and emanating from other neurons, akin to mammalian synaptic spinules. Detailed neuronal tracing revealed that spinules are frequent in all three major types of neurons innervating DA2 glomerulus, with olfactory sensory neurons receiving a significantly higher number of spinules compared to other olfactory neurons. Additionally, double-membrane vesicles, believed to represent material pitched-off from spinules, were most abundant in presynaptic boutons of the olfactory sensory neurons. The study also highlighted a close association between spinules, the endoplasmic reticulum, and mitochondria within the host neuron, suggesting a potential role for synaptic spinules in synapse tagging, synapse remodeling, and synaptic plasticity [61].

In collaboration with Cantera's group, involving several stays in Montevideo, Rybak et al. studied the synaptic microcircuitry of olfactory sensory neurons, projection neurons and antennal lobe glomeruli local interneurons in *D. melanogaster* utilizing electron microscopy. By ectopically expressing a membrane-bound peroxidase and conducting serial reconstructions of projection neuron dendrites, the researchers identified, counted, and mapped synaptic input and output sites, revealing the segregation of synapses along the dendrites of projection neurons. Through the integration of confocal and electron microscopy, 3D models of the antennal lobe were generated, elucidating the synaptic microcircuits formed by projection neurons with olfactory sensory and local interneurons [62]. This comprehensive approach provided detailed insights into the ultrastructural organization of synaptic contacts within the olfactory circuitry, enhancing the understanding of the synaptic architecture and functional interactions in the antennal lobe of the fruit fly.

Using volume-based focused ion beam-scanning electron microscopy, researchers have uncovered that in *D. melanogaster*, narrowly tuned glomerular circuits, responsible for processing specific odorants with remarkable precision, display distinct ultrastructural and microcircuitry features in contrast to broadly tuned circuits, which are capable of detecting a diverse array of odorants. By examining the connectomes of two narrowly tuned olfactory glomeruli, the researchers identified several potentially universal characteristics of this kind of glomeruli. These include unique synaptic compositions, the lateralization of sensory input, and distinctive synaptic circuitry [63]. In recent years various connectomes of a whole fly (*D. melanogaster*) brain emerged [64] [65] [66].

5. Molecular insights into neural development

The tyranny of time: temporal expression patterns

On the verge of the big data revolution, a transcriptomic analysis was conducted on mRNA-Seq data from *D. melanogaster* embryos. Aiming to understand the genetic basis of big developmental transitions like neural development and differentiation, these researchers elaborated catalogs of

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biological functions representing early and late phases of neurodevelopment, finding a global switch in their expression profiles that closely mirrored the temporal sequence of biological processes. Simultaneous changes in transcript levels of many genes within each catalog were observed, consistent with their functions. Through a genome-wide systematic search, they identified genes enriching catalogs associated with specific temporal frames, demonstrating predictive capability for application in other tissues or developmental processes [67]. The fascinating question of how interrelated are the different dimensions of gene expression, motivated the training of machine learning algorithms to build a list of several hundred putative synaptic genes based only on their temporal expression pattern [68]. It proved to have predicted 35% of the genes experimentally identified in the next four years, and motivated an improved training scheme [69] thus obtaining a new refined catalog of putative synaptic genes.

By studying the neuroectodermal gene *Patched-related* (*Ptr*), which encodes an uncharacterized transmembrane protein, they approached the developmental role of a gene differentially expressed during gastrulation. During early embryogenesis Ptr localizes to cell membranes, experiencing an expression increase during mid-embryogenesis when hemocytes, the phagocytic cells differentiate, and then localizes in migrating hemocytes [70]. Hemocytes clear apoptotic debris generated during nervous system development. It was found that *Ptr* is necessary for the proper nervous system development as *Ptr* knockdown produces alterations in its morphology, such as reduced glia and axonal disarray [71]. Most *Ptr* mutant embryos fail to hatch, and when they do, their larvae are lethargic and most die before reaching adulthood, a phenotype consistent with neuromuscular defects [72].

A breath of fresh air: Understanding hypoxic development of the optic lobe

A highly remarkable topic in biology during the last decades has been the molecular mechanisms cells use to sense and adapt to changes in oxygen availability. Kaelin, Semenza and Ratcliffe have been granted the Nobel Prize in Physiology or Medicine in 2019 for their advances in this field.

Insect respiratory system consists of a series of hollow tubes called tracheoles that carry out gas exchange directly between the internal milieu and the environment through openings in the body wall called spiracles [73]. Simple enough, this mechanism could be regarded as the most successful among metazoans as insects hold an enormous number of species [74].

However, in the developing larval brain of flies tracheoles are not homogeneously distributed. They are abundant in the central brain (CB) where most cells are postmitotic, but scarce in the proliferative optic lobe (OL) where most cells are still undifferentiated. A multicentric collaboration was needed to develop and validate a genetically-encoded ratiometric biosensor that allows imaging hypoxia states with cellular resolution [75].

With this hypoxia sensor, we were able to demonstrate that the neurogenic OL experiences a state of physiological hypoxia relative to the CB, which increases with larval development. Neuroblasts were the cell type experiencing the most severe hypoxia. Furthermore, even CB neuroblasts were kept, although to a lesser extent, within a hypoxic microenvironment [76].

A major breakthrough was accomplished when they found that the distance between a cell and its closest respiratory tube defines its oxygenation state [76], thus demonstrating with single cell resolution what Malpighi deduced and others tried to refine [77].

Despite being in a physiological hypoxic state, they found evidence that OL cells were not showing a HIF-mediated hypoxic response. Aiming to understand a possible pathway involved in a noncanonical hypoxic response, using genetic tools we performed loss-of-function and targeted gain-of-function experiments and found that atypical soluble guanylyl cyclases (asGCs) regulate brain size [78].

asGCs are oxygen sensitive and upon hypoxia produce cGMP, a second messenger that is known to be a neurogenic stimulus. To further characterize cGMP signaling in the OL, we developed a transgenic fly [79] able to express the highly sensitive cGMP FRET biosensor CUTie2 [80] in another multicentric consortium based mostly in Uruguay.

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Erase and rewind: Novel avenues in degeneration/regeneration

Despite being small, the vibrant Uruguayan neuroscientific community set their own milestones in nervous system injury, exemplified by the first ultrastructural study of regenerating nerves [14]. Using a model of nerve transection in the grasshopper *Laplatracris dispar*, researchers established the similarities of the early reactive phenomena to mechanical injury in insects [81] and vertebrates [14] [82].

Using a *D. melanogaster* transgenic model of Transthyretin-associated amyloidosis, researchers investigated the formation of aggregates and amyloid filaments in specific tissues and examined the relationship between toxicity and various forms of soluble or aggregated Transthyretin proteins. This model featured retinal expression of a secreted form of mutated Transthyretin detectable in hemolymph. Through ultrastructural analysis, they identified intracellular aggregates of Transthyretin nanospherules, as well as amyloid filaments in the thoracic adipose tissue and brain glia of transgenic flies carrying two copies of the mutated Transthyretin gene. Functional analysis revealed that the formation of aggregated nanospherules within large, membrane-bound cytoplasmic structures, in adipose tissue was correlated with reduced toxicity, and that this phenomenon was more pronounced in old flies [83]. A multicentric collaboration led by Cantera while still in Sweden, uncovered an exceptional phenomenon of reversible neurodegeneration in *D. melanogaster* mutants lacking functional spalt genes (*sal* homologs). These genes encode transcription factors that regulate gene expression within the nervous system. At embryonic stage 16, mutants exhibit features of degeneration in the central nervous system, which intriguingly undergo histological reversion by stage 17 [84]. Researchers hypothesized that the reversal of neurodegeneration might be mediated by a reorganization of the transcriptome, effectively counteracting the lack of function in the spalt gene. To test this hypothesis, they conducted an analysis using mRNA-Seq to compare the transcriptomes of *spalt* mutant embryos with those of wild-type organisms at the same embryonic stages. It produced a list of differentially expressed genes between the two stages. Remarkably, this list was enriched with genes previously associated with neurodegeneration or the reversal of neurodegenerative phenotypes, termed neuroprotection, in both D. melanogaster and humans. This finding reinforced the model's potential to identify novel genes implicated in neurodegeneration or neuroprotection [85]. Through histological and functional approaches, researchers observed that *D. melanogaster* mutants carrying a loss-of-function mutation in the gene *white*, previously identified as a candidate gene to prevent neurodegenerative processes and commonly used as a control strain, experience retinal degeneration and exhibit shorter lifespans, reduced motor abilities, and decreased tolerance to dietary and cellular toxicity stressors compared to wild-type individuals [86]. This study highlighted significant issues with using this strain as a control, emphasized the need to include wild-type controls in future research, and advised caution when interpreting previous results and planning new experiments.

Another gene identified as a candidate by Ferreiro et al., 2012 was *pretaporter*, which was downregulated during the reversal of the histological neurodegeneration phenotype. Pretaporter is thought to be located not only in the endoplasmic reticulum and plasma membrane during apoptosis, as previously reported, but also in the nuclei, membranes, and apical granule-like structures of the *D. melanogaster* salivary gland (Silvera and Ferreiro, unpublished). We hypothesized that the lack of *pretaporter* expression could restrain features of the neurodegenerative phenotype observed in a specific Parkinson's Disease model with flies carrying a mutation in the *parkin* gene, another degenerative context. *Parkin* mutants exhibit a well-characterized phenotype, including the loss of specific dopaminergic neurons, reduced survival, and motor defects [87] [88] [89] [90]. We found that the absence of pretaporter alleviates some features of the phenotype caused by the mutation in *parkin*. It does so by preventing the reduction of the number of the specific dopaminergic neurons typically seen in *parkin* mutants, improving survival rates in both male and female *parkin* flies, and reducing motor ability decay in female *parkin* flies (Silvera et al., unpublished). Altogether, these findings prompted a reevaluation of Pretaporter's functions and encouraged future research aimed at uncovering novel roles that

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could help us understand the mechanisms by which Pretaporter mitigates degenerative traits. In essence, the study by Cantera et al., 2002 not only highlighted the first reported case of reversible neurodegeneration, but also provided a valuable framework for discovering genes that might have an influence on several neurodegenerative backgrounds, potentially offering insights relevant to both insect and human biology.

6. Conclusion and outlook

In conclusion, our review provides a comprehensive overview of Uruguayan insect neuroscience, celebrating its rich history, diverse research themes, and ongoing contributions to our understanding of the nervous system.

We would like to end as we started, with some unanswered questions. What key questions do you believe will define the next chapter of insect neuroscience research? What emerging technologies hold the greatest promise for disentangling the complexities of neurobiology, and what obstacles must be overcome to unleash their potential? How can a scientific society bind together to create something that is more than the sum of its parts?

We invite you to engage actively with our work beyond the limits of this manuscript. Whether through further exploration of the topics discussed herein, consideration of new ideas and methodologies, or application of our research findings in your own work. This version is intended to be a live preprint, that will be kept up to date.

We strongly encourage you to comment on our preprint at the repository website, or to prompt interactions through our social media accounts.

The future awaits, and it is ours to shape. Thank you for embarking on this journey with us.

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