Annex 11.- Publications Screenshots

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REVIEW ARTICLE

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An atlas of nano-enabled neural interfaces

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Advances in microscopy and molecular strategies have allowed researchers to gain insight into the intricate organization of the mammalian brain and the roles that neurons play in processing information. Despite vast progress, therapeutic strategies for neurological disorders remain limited, owing to a lack of biomaterials for sensing and modulating neuronal signalling in vivo. Therefore, there is a pressing need for developing material-based tools that can form seamless biointerfaces and interrogate the brain with unprecedented resolution. In this Review, we discuss important considerations in material design and implementation, highlight recent breakthroughs in neural sensing and modulation, and propose future directions in neurotechnology research. Our goal is to create an atlas for nano-enabled neural interfaces and to demonstrate how emerging nanotechnologies can interrogate neural systems spanning multiple biological length scales.

Understanding the working principles of the nervous system has been a major goal of modern biomedical research. The brain is a high-dimensional functional network that accommodates around 100 billion neurons (in humans) which form local and long-range connections that drive processing of information in specialized brain centres and establish communication across systems, respectively. The complexity of the mammalian brain arises from three important phenomena. First, the high dimensionality of the vertebrate brain creates a 'big data problem' for neuroscience research. Second, neuronal subcellular structures span various length scales, such as tens of nanometres for synapses to centimetres for axonal projections, making it difficult to probe these components simultaneously with current technologies. Finally, approximately another 100 billion non-neuronal cells, called glia, reside in the brain and regulate neural activity through mechanisms that are still poorly understood.

Importance of nanoscale for neuronal interfaces

Historically, breakthroughs in neuroscience research have been tied to technological advances. For instance, bioelectric phenomena were first explored in the 1790s by Luigi Galvani, but it was not until the advent of electrophysiological recordings in the mid-twentieth century that theories of membrane excitability and ion channels were properly formulated. More recently, the design of new molecular tools for neuromodulation has provided scientists with the ability to control the activity of defined populations of neurons and to establish links between neural pathways and behaviour[1]. Nevertheless, our understanding of brain function remains poor, and our ability to prevent and treat neurological disorders is limited, in part because of the lack of minimally invasive tools capable of probing neuronal systems across the range of spatiotemporal scales. Currently, medical professionals rely on bulky implantable metal electrodes to pinpoint the origin of seizures, alleviate symptoms of Parkinson's disease and stimulate nerve growth following injury. The large size of these electrodes relative to individual cell bodies and nerve fibres leads to the activation of surrounding neurons during stimulation, creating unwanted side-effects[2]. Additionally, mechanical insertion of these bulky microelectrodes combined with micromotions within the skull[3] leads to immune activation and glial scar formation[4], which encapsulates the electrode, causes displacement from neurons of interest, decreases the overall device performance and even remodels the structure and function of the neural network surrounding the electrode.

Motivated by increasing demand, academic, federal and private sectors have expanded their research efforts in neural technologies. For instance, the BRAIN Initiative[5] in the United States, the Human Brain Project[6] in the European Union and the China Brain Project[7] are all multi-billion dollar projects that aim to develop new technologies, build seamlessly integrated neural interfaces for the interrogation of neural signals in freely moving and socially active subjects (Fig. 1a), and design personalized electronic medicine.

Nanoscale biomaterials can circumvent the limitations of current technologies, representing a potential imperceptible platform (Fig. 1a) for interfacing the nervous system at unprecedented spatiotemporal scales. First and foremost, the use of nanoscale building blocks means that electrodes can be packed together more densely, improving the resolution of current recording and neuromodulatory systems. Furthermore, given that bending stiffness scales with the cube of material thickness[8], rigid materials become soft, flexible, stretchable and more biocompatible as their feature size reaches the nanoscale (Fig. 1a–c), reducing the mechanical mismatch between brain tissue and engineered materials. Therefore, although the overall size of implantable devices may remain at the macroscopic scale, nanoscale building blocks that mimic structural and mechanical properties of neural tissue can be seamlessly integrated with brain tissue, without being recognized by the immune system[9]. Indeed, multiple lines of evidence have demonstrated that neural tissue exhibits negligible glial activation and scar formation when implanted with devices fabricated from nanoscale mechanically compliant subunits[10] (Fig. 1b, right). Additionally, scaling down

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Competing interests

The authors declare no competing interests.

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Chapter 11

Methods for Investigating TRP Channel Gating

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Abstract

A complete characterization of temperature -and voltage-activated TRP channel gating requires a precise determination of the absolute probability of opening in a wide range of voltages, temperatures, and agonist concentrations. We have achieved this in the case of the TRPM8 channel expressed in *Xenopus laevis* oocytes. Measurements covered an extensive range of probabilities and unprecedented applied voltages up to 500 mV. In this chapter, we describe animal care protocols of patch-clamp pipette preparation, temperature control methods, and analysis of ionic currents to obtain reliable absolute open channel probabilities.

Key words TRPM8, Xenopus care, Oocytes extraction, Patch clamp, Pipette pulling, Temperature control, Noise analysis, TRP channels, Gating mechanism

1 Introduction

Since TRP channels are polymodal receptors with allosteric gating, the researchers find experimental limitations or barriers trying to characterize channel behavior under the influence of different stimuli. Such as how to obtain an accurate determination of the absolute maximum open probability in a wide range of agonist concentration, temperatures, and voltages. The low voltage dependence of TRP channels forces investigators to apply extremely depolarized voltages to obtain saturation of the conductance–voltage curves. Instantaneous tail current measurement is the ideal procedure to obtain conductance–voltage curves. However, deactivation of TRP channel is often very fast in comparison with the voltage clamp dynamics. How to get an accurate measurement of the time course of deactivation process? How to avoid clamp errors when channels are overexpressed? One of the most satisfying achievements of our group has been to be able to measure the

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seal [10]. (A good Glup should not change the original pipette resistance.).

13. Pulse duration must be as short as possible, to preserve membrane stability. We use pulse protocol with different durations: short pulses for extreme depolarization.
14. The pipette electrode is at room temperature, but the bath electrode is at different controlled temperatures. Therefore, the applied potentials need correction for temperature since the standard potential, E^0, of the Ag/AgCl electrode is temperature dependent [26]. $E^0 = 0.23695 - 4.8564 \cdot 10^{-4} t - 3.4205 \cdot 10^{-6} t^2 - 5.869 \cdot 10^{-9} t^3$. E^0 is in volts and the equation is good for $0 < t < 95$ °C. According to this equation the electrode asymmetry will be −11 mV at 0 °C, −6 mV at 10 °C, 0 mV at 20 °C, +7 mV at 30 °C, and +14 mV at 40 °C.
15. We calculate the variance using a method based on the differences between successive points along the isochrones [10, 27, 28]. This procedure is coded in analysis a computer program [2] gently provided by Dr. Francisco Bezanilla (University of Chicago).
16. Curve fitting minimizes the sum of squares of the residuals, that is, the difference between the experimental and the calculated values. To make sure to represent all the mean current values equally, we separated the mean current axis in 0.1 nA intervals and counted the number of samples in each bean. The weight applied to each squared error is the reciprocal value of the number of samples on each bean times the theoretical value of the parabola (*see* also Fig. 6).
17. We measure the G/G_{max} from the tail currents after depolarization to different voltages rather than from the steady-state condition. As shown by Diaz et al. and Cox et al. [29, 30] the tail current method avoids the artifacts introduced by slow and fast blocks.

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cancers

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Review

Direct Intercellular Communications and Cancer: A Snapshot of the Biological Roles of Connexins in Prostate Cancer

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Abstract: Tissue homeostasis is the result of a complex intercellular network controlling the behavior of every cell for the survival of the whole organism. In mammalian tissues, cells do communicate via diverse long- and short-range communication mechanisms. While long-range communication involves hormones through blood circulation and neural transmission, short-range communication mechanisms include either paracrine diffusible factors or direct interactions (e.g., gap junctions, intercellular bridges and tunneling nanotubes) or a mixture of both (e.g., exosomes). Tumor growth represents an alteration of tissue homeostasis and could be the consequence of intercellular network disruption. In this network, direct short-range intercellular communication seems to be particularly involved. The first type of these intercellular communications thought to be involved in cancer progression were gap junctions and their protein subunits, the connexins. From these studies came the general assumption that global decreased connexin expression is correlated to tumor progression and increased cell proliferation. However, this assumption appeared more complicated by the fact that connexins may act also as pro-tumorigenic. Then, the concept that direct intercellular communication could be involved in cancer has been expanded to include new forms of intercellular communication such as tunneling nanotubes (TNTs) and exosomes. TNTs are intercellular bridges that allow free exchange of small molecules or even mitochondria depending on the presence of gap junctions. The majority of current research shows that such exchanges promote cancer progression by increasing resistance to hypoxia and chemotherapy. If exosomes are also involved in these mechanisms, more studies are needed to understand their precise role. Prostate cancer (PCa) represents a type of malignancy with one of the highest incidence rates worldwide. The precise role of these types of direct short-range intercellular communication has been considered in the progression of PCa. However, even though data are in favor of connexins playing a key role in PCa progression, a clear understanding of the role of TNTs and exosomes is needed to define their precise role in this malignancy. This review article summarizes the current view of the main mechanisms involved in short-range intercellular communication and their implications in cancer and delves into the biological, predictive and therapeutic role of connexins in PCa.

Keywords: intercellular communication; connexins; cancer; prostate cancer

invasion through GJIC-dependent and -independent processes. In this regard, it would be of great interest to verify whether communication between PCa cells and non-malignant TME cells through gap junctions allows cancer cells to acquire a more aggressive phenotype in vitro and in vivo. In summary, further studies on the specific role of Cxs in the biology of PCa cells as well as the contribution of these proteins in the pro-tumorigenic promoting effects of the TME cells could provide new potential biomarkers and or therapeutic target that could help to counteract PCa. This is especially important in advanced/metastatic stages, since current treatments for this disease produce mostly non-curative responses. In addition, efforts should be focused to understand how other types of intercellular communication such as TNTs and exosomes are involved in PCa progression.

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Defects in G-Actin Incorporation into Filaments in Myoblasts Derived from Dysferlinopathy Patients Are Restored by Dysferlin C2 Domains

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Abstract: Dysferlin is a transmembrane C-2 domain-containing protein involved in vesicle trafficking and membrane remodeling in skeletal muscle cells. However, the mechanism by which dysferlin regulates these cellular processes remains unclear. Since actin dynamics is critical for vesicle trafficking and membrane remodeling, we studied the role of dysferlin in Ca^{2+}-induced G-actin incorporation into filaments in four different immortalized myoblast cell lines (DYSF2, DYSF3, AB320, and ER) derived from patients harboring mutations in the *dysferlin* gene. As compared with immortalized myoblasts obtained from a control subject, dysferlin expression and G-actin incorporation were significantly decreased in myoblasts from dysferlinopathy patients. Stable knockdown of dysferlin with specific shRNA in control myoblasts also significantly reduced G-actin incorporation. The impaired G-actin incorporation was restored by the expression of full-length dysferlin as well as dysferlin N-terminal or C-terminal regions, both of which contain three C2 domains. DYSF3 myoblasts also exhibited altered distribution of annexin A2, a dysferlin partner involved in actin remodeling. However, dysferlin N-terminal and C-terminal regions appeared to not fully restore such annexin A2 mislocation. Then, our results suggest that dysferlin regulates actin remodeling by a mechanism that does to not involve annexin A2.

Keywords: dysferlin; actin; C2 domains; annexin A2; dysferlinopathy

1. Introduction

Dysferlin is a transmembrane protein containing seven cytosolic C2 domains, which bind Ca^{2+} and acidic phospholipids with different affinities [1,2]. Its most well-known function is to facilitate Ca^{2+}-dependent aggregation and fusion of vesicles at wounded plasmalemma [3–5] by a mechanism

4.6. G-actin Incorporation into Filaments and Immunofluorescence

Non-transfected or transfected myoblasts were incubated 6 min at 37 °C in KGEP buffer (139 mM K^+ glutamate, 20 mM PIPES, 5 mM EGTA, 2 mM ATP-Mg^{2+}, 0.3 μM Alexa Fluor 488-G-actin conjugate, and 20 μM digitonin, pH 6.9) in the absence or presence of 10 μM free Ca^{2+} [27,40]. The online software Ca-EGTA Calculator v1.2 (University of California, Davis, CA, USA; https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/CaEGTA-NIST.htm) was used to estimate the Ca^{+2} and EGTA concentrations to achieve 10 μM free Ca^{2+} with parameters of pH of 6.9 and temperature equal to 25 °C. Next, samples were fixed with 4% p-formaldehyde (PFA), stained with 5 mg/mL 4,6-diamidino-2-phenylindole (DAPI), and visualized by confocal microscopy. In cells transfected with dysferlin-HA, after PFA fixation, samples were incubated with the anti-dysferlin antibody (1:50), washed three times, and incubated with a Cy3-conjugated anti-rabbit secondary antibody (1:100). For immunodetection of annexin A2, after PFA fixation, samples were incubated with the anti-annexin A2 antibody, washed three times, and incubated with Cy2-conjugated anti-rabbit secondary antibody (1: 250). Images were captured at the equatorial plane of the cells using identical exposure settings between compared samples. To quantify G-actin incorporation, cell fluorescence of each individual cell was divided by the cell area, which was determined by manually drawing the cell outline using the differential interference contrast. For annexin A2 distribution, annexin A2 immunostaining was analyzed also in a single focal plane, and data are shown as the ratio of the mean fluorescence intensity of the cytosol/whole cell of annexin A2. All images were analyzed and processed using ImageJ software (NIH, Bethesda, USA).

4.7. Statistics

Results were expressed as means ± SEM. Normality of data was checked using the Kolmogorov–Smirnov test. Statistical comparisons were performed using a *t*-test. All statistical analyses were performed using InStat3 software (GraphPad Software Inc, La Jolla, CA, USA).

4.8. Ethics Statement

The investigators declare to know the Manual of Biosafety Regulations stipulated by CONICYT (Chile), version 2008; CDC (USA) Biosafety Manual 4th Edition; and Laboratory Biosafety, WHO, Geneva, 2005 (mainly in reference to experiments with recombinant DNA and RNA and the manipulation of cell lines). This research was approved by the Biosafety and Bioethics committees of Universidad de Valparaíso (Chile), approval identification numbers BS002/2016 and BEA080-216, respectively.

Supplementary Materials: The following are available online at http://www.mdpi.com/1422-0067/21/1/37/s1; Figure S1: High Ca^{2+} concentrations increase G actin incorporation into filaments; Figure S2: Intensity profiles of G-actin in C25 and dysferlin-deficient myoblasts; Table S1: Incorporation GST- amphiphysin-1 SH3 domain fusion protein in permeabilized C25 and DYSF3 myoblasts; Figure S3: Dysferlin expression in RCMH cells with stable dysferlin knockdown; Table S2: mCherry fluorescence intensity in C25 and DYSF3 myoblasts; Figure S4: Intensity profiles of annexin A2 (red) and G-actin (green) in C25 and DYSF3 myoblasts.

Author Contributions: X.B.-M., C.F.-C., H.A-S., and C.A. performed experiments and analysis. M.J.G. performed experiments and contributed to sample preparation. A.M.G.-J. performed experiments and critically revised the manuscript. A.B. and V.M. provided the cell lines used in this study as well as technical help and critical reading of the manuscript. M.C.M. and P.C. critically revised the manuscript and contributed to writing the manuscript. A.M.C. conceived the study, designed experiments, and drafted the manuscript. All authors contributed to interpretation of data and revised the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Metal Bridge in S4 Segment Supports Helix Transition in *Shaker* Channel

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ABSTRACT Voltage-gated ion channels play important roles in physiological processes, especially in excitable cells, in which they shape the action potential. In S4-based voltage sensors voltage-gated channels, a common feature is shared; the transmembrane segment 4 (S4) contains positively charged residues intercalated by hydrophobic residues. Although several advances have been made in understating how S4 moves through a hydrophobic plug upon voltage changes, the possible helix transition from α- to 3_{10}-helix in S4 during the activation process is still unresolved. Here, we have mutated several hydrophobic residues from I360 to F370 in the S4 segment into histidine, in *i*, *i* + *3* and *i*, *i* + *6* or *i*, *i* + *4* and *i*, *i* + *7* pairs, to favor 3_{10}- or α-helical conformations, respectively. We have taken advantage of the ability of His to coordinate Zn^{2+} to promote metal ion bridges, and we have found that the histidine introduced at position 366 (L366H) can interact with the introduced histidine at position 370 (stabilizing that portion of the S4 segment in α-helical conformation). In the presence of 20 μM of Zn^{2+}, the activation currents of L366H:F370H channels were slowed down by a factor of 3.5, and the voltage dependence is shifted by 10 mV toward depolarized potentials with no change on the deactivation time constant. Our data supports that by stabilizing a region of the S4 segment in α-helical conformation, a closed *(*resting or intermediate*)* state is stabilized rather than destabilizing the open (active*)* state. Taken together, our data indicates that S4 undergoes α-helical conformation to a short-lived different secondary structure transiently before reaching the active state in the activation process.

SIGNIFICANCE Conformational transitions between α-helix and 3_{10}-helix in the segment 4 (S4) of *Shaker* potassium channel during gating has been under debate. This study shows the coordination by Zn^{2+} of a pair of engineered histidine residues (L366H:F370H) in the intermediate region of S4 in *Shaker*, favoring α-helical conformation. In the presence of 20 μM of Zn^{2+}, the activation currents of L366H:F370H channels become slower, with 10 mV positive shift in the voltage dependence and no effects on deactivation time constants suggesting a stabilization of a closed state rather than destabilization of an open (active) state. Collectively, our data indicate that S4 undergoes secondary structure changes, including a short-lived secondary structure transition when S4 moves from the resting to the active state during activation.

INTRODUCTION

Voltage-gated ion channels (VGIC) play several roles in physiological processes, especially in excitable cells, shaping the generation and propagation of action potentials (1). It has been shown by biochemical, electrophysiological, and structural biology experiments that VGIC are formed by four subunits (or domains), each one containing six transmembrane segments (S1–S6) (2,3). In each subunit, segments 1–4 (S1–S4) constitute the so-called segment 4 (S4)-based voltage sensor domain (VSD), and the segments S5 and S6 form a fourth of the pore domain. In S4-based VSDs, S4 contains positively charged residues (mainly constituted by arginine side chains), every three residues between hydrophobic residues. These charged residues sense the electric field across the membrane and move upon depolarization, conferring to the pore the ability to conduct ions (4). Segment S2 contains a negative residue (E283) that forms salt bridges with Arg (R) residues from the S4 segment stabilizing and guiding their movement (5). Also, it has been demonstrated that S1, S2, and S3 segments contain a set of hydrophobic residues that form the "hydrophobic plug," through which S4, the side chains of the R

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His:His interactions using Zn^{2+} to coordinate them could help to probe transitions in other S4-based voltage sensors, which have been structurally and functionally well characterized, such as Na^+ channels and other K^+ channels.

SUPPORTING MATERIAL

Supporting Material can be found online at https://doi.org/10.1016/j.bpj.2019.08.035.

ATHOR CONTRIBUTIONS

F.B., C.A.Z.B., and J.L.C.-d.-S. contributed to the conception and design of the project. C.A.Z.B. performed research and analyzed the data. C.A.Z.B. and J.L.C.-d.-S. wrote the manuscript with inputs from F.B.

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Controlling ionic conductivity through transprotein electropores in human aquaporin 4: a non-equilibrium molecular-dynamics study†

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Electroporation is a matter of intensive ongoing research interest, and a much-neglected topic in trans-membrane proteins, particularly in view of such promising potential applications in medicine and biotechnology. In particular, selected such novel and exciting applications are predicated on controlling ionic conductivity through electro-pores. Here, we scrutinise the mechanisms of ions' electric conductivity, by means of structural rearrangements, through quasi-stable electro-pores through human-AQP4 as a well-representative prototype of trans-membrane ionic conduction, achieving exquisite control over ionic permeability manipulated by the application of intense static electric fields.

Introduction

Biological membranes consist predominantly of lipids and proteins in mass ratios ranging from 0.25 to 4; in most membranes the lipids are the predominant molecular species representing the so called phospholipid bilayer[1,2] which acts as an isolating partition dividing two electrolyte solutions with quite different concentrations inside and outside of the cell.

Experimental evidence indicates that the application of an external electric field to biological cells affects transiently or permanently the integrity of their plasma membrane.[3] This effect has been rationalized demonstrating that an electric-field exposure exceeding a threshold strength induces a large change in the transmembrane voltage resulting in rearrangements of the phospholipid bilayer, leading to the formation of aqueous transmembrane pores: lipid membrane 'electro-pores'.[4–6] This leads to direct connection of the two systems on each side, with the dramatic possibility of molecular permeability across the membrane through the electro-pores, forbade previously by transport-selective endogenous channels in bilayer-embedded proteins.[7,8] For electric-field pulses below a critical intensity or duration, the membrane 'heals', generally over time-scales greatly exceeding nanoseconds.[9,10]

Now, an exciting, and as-yet elusive, technological application arising from the notion of a quasi-stable, long-lived electro-pore delivering permeability over extended periods[11] forms the intellectual basis of the idea of controlling a drug-release course, 'ferried' perhaps by field-responsive ions if not ionic *per se*. Indeed, a potentially promising means for achieving such a desideratum hinges on aquaporins (AQPs) as prototypical water-conducting trans-membrane proteins.

Recently, Marracino *et al.* witnessed at first hand the novel phenomenon of electro-pore formation within trans-membrane proteins, as opposed to the phospholipid bilayer,[12] observing stabilised transprotein electro-pores in human-AQP4 by means of non-equilibrium molecular dynamics (NEMD) for extended periods of tens of nanoseconds. This 'fifth pore' forms because of the coupling with the intrinsic dipoles of protein residues: in ref. 12, each residue of the protein in which the electropore is embedded has been analysed, computing the dipole moment with respect to its centre of mass. In the top-end of the electropore, the contribution of residue ASP69 seemingly triggers the mechanism of membrane poration.[12] In an unexposed trajectory, an angular flip of the dipole associated with the residues occurs; in contrast, during the porating-field application, this flip does not happen at all.[12] Observing the probability distribution function of the ASP69 dipole, in particular, it populates only one of the two states initially available,[12] leading the way to permeability enhancement. As reported fleetingly,[5] under conditions of high electro-pore metastability, ionic-conduction events have been observed through lipid membrane electro-pores; here the challenge is to observe

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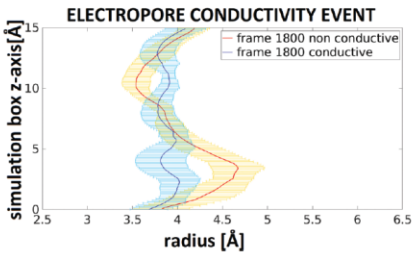

Fig. 7 Approximate schematic of the electropore-channel radial shape as obtained through the HOLE program. The resulting shape is obtained by averaging over five different configurations during events of conduction (red) through the electropore when the sustaining field is applied after 1800 ps; in blue, in contrast, we present the shape of a non-conductive electropore.

Table 2 Sodium and chlorine ions' energy parameters extracted from the employed force field

Element	ε [kJ mol^{-1}]
Na	0.49
Cl	6.72

indicates that the optimum width for the channel to be in its metastable state is 4.6 ± 0.3 Å.

As mentioned briefly above, a single sodium-ion conductivity event was recorded instead, and none within the stable induced pore. The main reason behind this peculiar behaviour is to be found in the Lennard-Jones parameters: the well depth of chlorine and sodium ions differs a lot, as reported in Table 2.

In view of these energies' ratio, $\varepsilon \sim 13.71$, a plausible rationale is as follows. The sodium energy parameter hints at the fact that Na atoms bind more strongly, in general, compared to Cl atoms, according to, *e.g.*, Lorentz–Berthelot[49] combining rules $\left(\varepsilon_{ij} = \sqrt{\varepsilon_{ii}\varepsilon_{jj}}\right)$. The resulting scenario provides for a strong interaction of the cations with POPE, preventing the atoms reaching the very bottom of the electropore from passing through easily. The binding is very tight and also very unlikely to be overcome given the narrow radius (*cf.* Fig. 2), and the sustaining electric field's extent.

Conclusion

In closing, we have used NEMD in external electric fields to observe and control, for the first time, Cl^- conductivity through novel electro-pores formed in transmembrane proteins and observe the exceedingly rare and curious event of Cl^--passage through an endogenous channel. This field-based control over ionic passage constitutes a delicate 'balancing act', in that the strength of the sustaining electric field needs to be modulated carefully to sustain transprotein electro-pores in a quasi-stable state and control the permeability flux without compromising the cell-wall structural stability.

The present study points to the tantalising possibility of achieving, *via* careful application of external electric fields, such fine-grained and precise control over ionic conduction into cells using novel electric-field nano-pulse technology; here, recruiting ionic species as carrier agents for drug-delivery applications is but one technological application. This kind of exquisite system response – and control – of water and ionic flux/behaviour to electric fields in nanoscale geometries embedded in phospholipid bilayers, studied extensively in ref. 50–52, is also promising for other pore geometries, *e.g.*, artificial ones such as carbon nanotubes.[51,52]

Conflicts of interest

There are no conflicts to declare.

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RESEARCH ARTICLE

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Physiological assessment of high glucose neurotoxicity in mouse and rat retinal explants

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Abstract

One of the tissues of the central nervous system most affected by diabetes is the retina. Despite a growing understanding of the biochemical processes involved in glucose toxicity, little is known about the physiological consequences of chronic high glucose (HG) on individual neurons and neuronal circuits. Electroretinogram recordings suggest that retinal bipolar cells (BCs), which filter and transmit photoreceptor output to the inner retina, are among the first cells affected by diabetic conditions, and may therefore serve as sensitive early biomarkers for incipient neuronal damage caused in diabetes. Here, we comparatively assessed retinal integrity, calcium responses, and the electrophysiological profiles of specific BC types of mouse and rat organotypic retinal explants after 1 to 3 weeks in tissue culture, under moderate glucose (MG) and HG conditions. While the retinal layers of both rodent species displayed a progressively reduced thickness in culture, BCs retained their electrophysiological profiles and remained morphologically identifiable for up to 2 weeks. Responses to glutamate and endogenous inhibitory responses were routinely observed, indicating that the retinal circuitry remained intact during this period. Significant physiological differences between MG and HG conditions were evident in calcium signals and in the time course of responses to glutamate, but the voltage-gated current profiles of BCs displayed only minor variations. Overall, rat retina appeared slightly more sensitive to HG levels compared with mouse. In conclusion, electrophysiological analysis of neuronal function in rodent retinal explants is useful for the study of early damage due to HG neurotoxicity.

KEYWORDS
bipolar cells, diabetes, diabetic retinopathy, glucose, patch clamp, retina, retinal explant, RRID: SCR_014199, RRID: SCR_003070, RRID: AB_10013382, RRID: AB_2576217, RRID: SCR_002798

1 | INTRODUCTION

One of the most frequent and debilitating complications of diabetes is diabetic retinopathy, which eventually affects about one third of all diabetics (Ruta et al., 2013). Diabetic retinopathy has traditionally been diagnosed and staged according to ophthalmologic eye fundus evaluation, which reveals characteristic and progressive alterations of the retinal blood supply (Cunha-Vaz, 2011). However, before the first signs of vascular damage are visible in fundus imaging, sensitive methods, such as multifocal electroretinography (ERG), reveal initial functional changes in the diabetic retina. Notably, a significant reduction of the amplitude of the ERG b-wave, which reflects the electrical

A17 amacrine cells. Ca^{2+}-permeable AMPA receptors mediate synaptic input from RBCs onto A17 amacrine cells, which provides reciprocal GABAergic feedback to the RBC (Chávez, Singer, & Diamond, 2006). The latter corresponds likely to the current response observed at 0 mV. This is in line with reports of alterations in the structure and function of AMPA receptors and GABA receptors in the diabetic retina (Castilho et al., 2012; Castilho, Ambrosio, Hartveit, & Veruki, 2015; Castilho, Ambrósio, Madsen, Hartveit, & Veruki, 2015; Ramsey, Ripps, & Qian, 2007). Hence, the delay in the inactivation component of the glutamate-induced inhibitory response in RBCs supports the notion of an initial impairment in the rod pathway under diabetic conditions. This may also correspond to the observed thinning of the OPL, which is mostly composed of synapses between rods and BCs.

On the other hand, OFF BC-3a displayed no electrophysiological difference between MG and HG conditions and after 7 and 14 days in culture. BC-3a responds to glutamate exclusively through kainate receptors (Vielma & Schmachtenberg, 2016), and its inhibitory input is GABAergic and glycinergic (Ivanova, Muller, & Wassle, 2006; Mazade & Eggers, 2013).

Regarding the effect of the HG condition on calcium signaling, previous studies have suggested an altered calcium homeostasis in diabetics animals (Ahn, Kang, & Jeung, 2017; Pereira et al., 2014; Sorrentino et al., 2016). In cardiomyocytes, a deregulation of Ca^{2+} cycling and diminished Ca^{2+} influx have been shown under diabetic conditions (Bergh, Hjalmarson, Sjögren, & Jacobsson, 1988; Lu et al., 2007; Pereira et al., 2006). This effect is due to a reduction in the expression of L-type Ca^{2+} channels in the cell membrane (Lu et al., 2007; Lun Lee, Ostadalova, Kolar, & Dhalla, 1992) and of the intracellular ryanodine receptor (Pereira et al., 2014; Yaras et al., 2005), which generates an overall increase in basal Ca^{2+} levels (Yaras et al., 2005). On the other hand, purinergic receptors are expressed throughout the entire retina, in retinal neurons and prominently in Müller glia cells (Ho et al., 2016; Ho, Vessey, & Fletcher, 2014; Puthussery & Fletcher, 2004; Ventura, Santos-rodrigues, Mitchell, & Paula, 2018). We observed an increase of calcium responses to ATP stimulation under HG compared with MG conditions, which could be interpreted as a consequence of glial activation under prolonged HG conditions. However, the reported effects of diabetic conditions on purinergic receptor expression in different tissues are multiple and complex. An alteration in purinergic receptor subtype expression patterns has been observed in diabetes in the retina and other tissues (Ho et al., 2016; Kneer, Green, Meyer, Rich, & Minns, 2018; Mankus, Rich, Minns, & Trinkaus-randall, 2011; Seref-ferlengez, Maung, Schaffler, & Spray, 2016; Vindeirinho, Santiago, Cavadas, Ambrósio, & Santos, 2016). For example, in diabetic animals, an increase in levels of $P2X_7R$ has been reported in corneal epithelium (Kneer et al., 2018; Mankus et al., 2011), although a decrease in the expression of these receptors also was shown in osteocytes under HG conditions (Seref-ferlengez et al., 2016). Likewise, the metabotropic receptors $P2Y_2R$ and $P2Y_4R$ suffer an overexpression in osteoblasts under diabetic conditions (Seref-ferlengez et al., 2016), as do some adenosine receptors in the retina (Vindeirinho, Costa, Correia, & Santos, 2013). Our results are in line with these reports, suggesting that an upregulation in the expression of purinergic receptors, presumably in Müller cells, induces an increase in the calcium response, which has already been observed in osteoblasts under HG conditions (Seref-ferlengez et al., 2016). It should be noted that our experimental setup did not allow for a differentiation of the purinergic receptor and cell types involved. Moreover, explant culture affects purinergic receptor function compared with ex vivo retina irrespective of the glucose condition (Figure 6e').

Altogether, our results support the idea that the HG condition affects calcium signaling in the retina, suggesting a general deleterious effect of diabetic conditions on calcium homeostasis in excitable cells. We conclude that electrophysiological analysis of neuronal function in rodent retinal explants is useful for the study of incipient glucose neurotoxicity, and may reveal the mechanisms leading to decreased visual function before the onset of clinical diabetic retinopathy. The main limitations of the model is the comparatively short time that the tissue can be exposed to diabetic conditions, weeks compared with decades in vivo, which does not allow to mimic the slowly deleterious processes involved in chronic pathologies like diabetes. Another limitation is the absence of a functional vascular system, whose cells are the most directly affected by elevated glucose levels and might trigger downstream pathologic events in the neuroretina. Notwithstanding these caveats, rodent retinal explants have the advantage of allowing precisely controlled conditions, and remain a useful tool for the study of diabetic retinopathy and other retinal diseases.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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PLOS | GENETICS

RESEARCH ARTICLE

Diapause induces functional axonal regeneration after necrotic insult in *C. elegans*

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Abstract

Many neurons are unable to regenerate after damage. The ability to regenerate after an insult depends on life stage, neuronal subtype, intrinsic and extrinsic factors. *C. elegans* is a powerful model to test the genetic and environmental factors that affect axonal regeneration after damage, since its axons can regenerate after neuronal insult. Here we demonstrate that diapause promotes the complete morphological regeneration of truncated touch receptor neuron (TRN) axons expressing a neurotoxic MEC-4(d) DEG/ENaC channel. Truncated axons of different lengths were repaired during diapause and we observed potent axonal regrowth from somas alone. Complete morphological regeneration depends on DLK-1 but neuronal sprouting and outgrowth is DLK-1 independent. We show that TRN regeneration is fully functional since animals regain their ability to respond to mechanical stimulation. Thus, diapause induced regeneration provides a simple model of complete axonal regeneration which will greatly facilitate the study of environmental and genetic factors affecting the rate at which neurons die.

Author summary

Diapause entry and hibernation have the striking ability to protect the nervous system from diverse types of damage. Here we show that the diapausing dauer larvae of *C. elegans* regenerate broken mechanosensory neurons that were damaged by the hyperactivation of degenerins (MEC-4d) or by axotomy during diapause. This regeneration is complete and functional, rendering neurons capable of responding to touch after three days in diapause. Genetic inactivation of the insulin receptor DAF-2 promotes regeneration of *mec-4d* axons in non-dauer animals. Overexpression of the downstream transcription factor DAF-16 promotes neuronal protection in *mec-4d* neurons while loss of *daf-16* accelerates *mec-4d* induced degeneration. Temperature sensitive activation of DAF-2 during diapause induces the loss of axonal integrity. This indicates that the insulin signaling pathway is an important underlying factor in regeneration. Additionally, we show that complete morphological regeneration depends on DLK-1, a conserved protein required for axonal

S7 Table. Embryonic cells in dauer at 25˚C. Measurements of individual *mec-4d* ALM and PLM neurons on the first and third day of diapause. F, Number of animals counted on each category with a summary of their growth.
(XLSX)

S8 Table. Embryonic cells in development at 25˚C. Measurements of individual *mec-4d* ALM and PLM neurons at hatching, 24 and 48 hours after hatching. F, Number of animals counted on each category with a summary of their growth.
(XLSX)

S9 Table. Measurements of individual *dlk-1; mec-4d* AVM neurons on the first and third day of diapause. Number of animals counted on each category with a summary of their growth.
(XLSX)

S10 Table. Pharynx size of *dlk-1; mec-4d* animals. Width of the pharyngeal bulb measured on days 1 and 3 of diapause of animals illustrated on Fig 8D.
(XLSX)

S11 Table. Touch responses not reported in Fig 2B.
(XLSX)

S1 File. Raw data with replicas of all the experiments shown in the manuscript.
(XLSX)

S2 File. Statistical analysis of the experiments contained in the manuscript.
(XLSX)

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Author Contributions

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Methodology: Mauricio Caneo, Andrea Calixto.

Writing – original draft: Andrea Calixto.

Writing – review & editing: Mauricio Caneo, Alexandra B. Byrne, Mark J. Alkema, Andrea Calixto.

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nature COMMUNICATIONS

ARTICLE

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Noncanonical mechanism of voltage sensor coupling to pore revealed by tandem dimers of Shaker

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In voltage-gated potassium channels (VGKC), voltage sensors (VSD) endow voltage-sensitivity to pore domains (PDs) through a not fully understood mechanism. Shaker-like VGKC show domain-swapped configuration: VSD of one subunit is covalently connected to its PD by the protein backbone (far connection) and non-covalently to the PD of the next subunit (near connection). VSD-to-PD coupling is not fully explained by far connection only, therefore an additional mechanistic component may be based on near connection. Using tandem dimers of Shaker channels we show functional data distinguishing VSD-to-PD far from near connections. Near connections influence both voltage-dependence of C-type inactivation at the selectivity filter and overall PD open probability. We speculate a conserved residue in S5 (S412 in Shaker), within van der Waals distance from next subunit S4 residues is key for the noncanonical VSD-to-PD coupling. Natural mutations of S412-homologous residues in brain and heart VGKC are related to neurological and cardiac diseases.

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Author contributions

J.L.C.-d.-S. and F.B. conceived the project. J.L.C.-d.-S. performed research, analyzed data and wrote the manuscript, receiving inputs from F.B.

Additional information

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Cholesterol Functionalization of Gold Nanoparticles Enhances Photoactivation of Neural Activity

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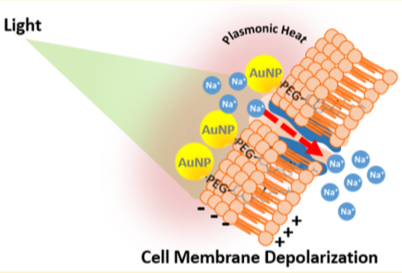
Supporting Information

ABSTRACT: Gold nanoparticles (AuNPs) attached to the extracellular leaflet of the plasma membrane of neurons can enable the generation of action potentials (APs) in response to brief pulses of light. Recently described techniques to stably bind AuNP bioconjugates directly to membrane proteins (ion channels) in neurons enable robust AP generation mediated by the photoexcited conjugate. However, a strategy that binds the AuNP to the plasma membrane in a non protein-specific manner could represent a simple, single-step means of establishing light-responsiveness in multiple types of excitable neurons contained in the same tissue. On the basis of the ability of cholesterol to insert into the plasma membrane, here we test whether AuNP functionalization with linear dihydrolipoic acid-poly(ethylene) glycol (DHLA-PEG) chains that are distally terminated with cholesterol (AuNP−PEG−Chol) can enable light-induced AP generation in neurons. Dorsal root ganglion (DRG) neurons of rat were labeled with 20 nm diameter spherical AuNP−PEG−Chol conjugates wherein ∼30% of the surface ligands (DHLA-PEG-COOH) were conjugated to PEG−Chol. Voltage recordings under current-clamp conditions showed that DRG neurons labeled in this manner exhibited a capacity for AP generation in response to microsecond and millisecond pulses of 532 nm light, a property attributable to the close tethering of AuNP−PEG−Chol conjugates to the plasma membrane facilitated by the cholesterol moiety. Light-induced AP and subthreshold depolarizing responses of the DRG neurons were similar to those previously described for AuNP conjugates targeted to channel proteins using large, multicomponent immunoconjugates. This likely reflected the AuNP−PEG−Chol's ability, upon plasmonic light absorption and resultant slight and rapid heating of the plasma membrane, to induce a concomitant transmembrane depolarizing capacitive current. Notably, AuNP−PEG−Chol delivered to DRG neurons by inclusion in the buffer contained in the recording pipet/electrode enabled similar light-responsiveness, consistent with the activity of AuNP−PEG−Chol bound to the inner (cytofacial) leaflet of the plasma membrane. Our results demonstrate the ability of AuNP−PEG−Chol conjugates to confer timely stable and direct responsiveness to light in neurons. Further, this strategy represents a general approach for establishing excitable cell photosensitivity that could be of substantial advantage for exploring a given tissue's suitability for AuNP-mediated photocontrol of neural activity.



KEYWORDS: *Gold nanoparticles, cholesterol, nanoparticle functionalization, photosensitivity, neural photoactivation, action potential, optocapacitance, dorsal root ganglion cell*

INTRODUCTION

The interfacing of photophysically active nanomaterials with biological tissue is a rapidly growing area of research in fundamental and clinically relevant biomedicine. Gold nanoparticles (AuNPs), in particular, possess unique electronic and

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CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, WI).

DRG Neuron Preparation. Dorsal root ganglia were removed from 1 to 3 day old Sprague–Dawley rats following euthanasia and were immediately placed in ice-cold DMEM. Ganglia were rinsed multiple times with modified EBSS before digestion with 0.25% trypsin (in EBSS) for 20 min at 37 °C under gentle shaking. The trypsin-digested, softened ganglia were then centrifuged and resuspended in EBSS supplemented with 10% v/v fetal bovine serum (FBS). Next, mechanical trituration with Pasteur pipettes of decreasing tip sizes was performed to yield monodisperse DRG neurons in suspension. Following a final centrifugation, cells were resuspended in DMEM containing 5% v/v FBS. Cells were seeded into sterilized poly-L-lysine solution-treated glass-bottom culture dishes and allowed to sit for 30 min to facilitate DRG neurons adhesion to the glass. A 2.5 mL volume of DMEM added with 5% v/v FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (supplemented DMEM) was then added to the dish, and the cells were incubated at 37 °C with 5% CO_2 until use.

Experimental Setup for Photoactivation of DRG Neurons. Dishes containing DRG neurons and bath solution were mounted on a Zeiss IM 35 microscope (Carl Zeiss Microscopy, Thornwood, New York) and visualized through objective lenses ranging from 10× (0.25 NA) to 40× (0.55 NA). Patch pipettes were pulled on a Sutter Instruments P-2000 CO_2 laser micropipette puller (Novata, California) and flame-polished to produce approximately 2 MΩ resistances when filled with internal pipet solution. An analog waveform from an AD/DA converter board (Innovative Integration SBC-6711-A4D4, Simi Valley, CA) drove the amplifier (Axopatch 200B, Molecular Devices, Sunnyvale, California) to clamp the current through the cell's membrane. The amplifier output, the membrane voltage, was digitized at 16-bit resolution and sampled at 20 kHz by the same AD/DA converter board and stored in a personal computer for analysis. The AD/DA converter board was controlled by customized software. A 532 nm laser system (532 nm DPSS laser, UltraLasers, Ontario, Canada) was mounted and aligned to the central axis of the microscope objective, previously measured power by neutral density filters (Thorlabs Inc., Newton, NJ, United States). TTL-controlled acousto-optic-modulator was used (NEOS Technologies, Gooch & Housego, PLC., Melbourne, Florida) to enable presentation of the faster laser pulses. The focused laser beam, a spot with diameter of about 5 μm (about $^1/_5$ of the cell diameter), was allowed to move relative to the cell under investigation by an independent manual X–Y micromanipulators system mounted on the microscope. For experiments labeling the inner membrane leaflet with AuNP–PEG–Chol conjugates, the pipet solution was amended with the AuNP conjugates to a final concentration of 1.5 nM. Positive pressure was not applied to the inside of the pipet until contact was made with the target cell.

DRG Neuron Labeling with AuNP–PEG–Chol. Dishes containing DRG neurons were incubated overnight after the addition of ∼260 μL of AuNP-containing preparation (∼1.5 nM AuNP in Tyrode's solution containing 5% FBS) or Tyrode's solution alone. On the day of experiment, the supplemented DMEM (containing AuNPs or not) was flushed out and the cells by washing with 10 mL plain Tyrode's solution. A final volume of about 0.5 mL was left in the dish and served as the bathing extracellular solution during the electrophysiological tests to probe photosensitization. Unless otherwise indicated, procedures for the presentation of laser pulses to the cells were similar to those previously described.[4,5] The cytotoxicity of DRG neurons labeled with AuNPs (with and without photoactivation) was determined using the LIVE/DEAD Viability kit (ThermoFisher).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.8b00486.

TEM and spectral analysis of AuNPs and AuNP conjugates, analysis of plasma membrane labeling with AuNP–PEG–Chol, and theoretical analysis of predicted AuNP temperature changes (PDF)

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J.L.C. performed the DRG photoactivation experiments. O.K.N. made the AuNP–PEG–Chol conjugates and performed cell labeling and toxicity tests. E.O. synthesized and characterized the AuNPs and, along with A.L.H. and I.V., calculated the theoretical AuNP photothermal responses. J.L.C., D.R.P, F.B., and J.B.D. jointly conceived of the AuNP bioconjugation and plasma membrane tethering concept. All authors contributed to the preparation of the manuscript.

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Notes

The authors declare no competing financial interest.

■ DEDICATION

We dedicate this manuscript to the memory of coauthor David R. Pepperberg who helped conceive of the cholesterol functionalization strategy.

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Research Article

Lack of Association between the *IL6R* Gene Asp358Ala Variant (rs2228145), IL-6 Plasma Levels, and Treatment Resistance in Chilean Schizophrenic Patients Treated with Clozapine

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Alterations in neuroinflammatory processes have been suggested to contribute to the development of Schizophrenia (SZ); one component of the inflammatory system that has been linked to this disorder is interleukin-6 (IL-6). The minor allele of rs2228145, a functional polymorphism in the IL-6 receptor gene, has been associated to elevated IL-6 plasma levels and increased inflammatory activity, making it an interesting candidate to study as a possible factor underlying clinical heterogeneity in SZ. We studied a sample of 100 patients undergoing treatment with clozapine. Their symptoms were quantified by Brief Psychotic Rating Scale; those with the lowest scores ("remitted") were compared with the highest ("clozapine treatment resistant"). We determined allelic frequencies for rs2228145 and IL-6 plasma levels. Our results do not support a role of IL-6 in response to treatment with clozapine. Further studies accounting for potential confounding factors are necessary.

1. Introduction

Schizophrenia (SZ) may be etiologically related to a chronic neuroinflammatory process, with greater inflammatory activity in affected individuals with active psychosis as well as in treatment-resistant cases [1–3]. In this regard, most evidence comes from the observed higher risk of presenting SZ in subjects whose mothers suffered infections during pregnancy, with activation of the maternal immune response [4], and from findings of elevated levels of immune system components in SZ-affected individuals [5]. Variations in complement activity, in turn, have been linked to changes in synaptic pruning in mice, providing an explanatory mechanism for the role of inflammation in SZ [6].

Several inflammatory mediators have been proposed to be implicated in SZ, including interleukin 6 (IL-6) [7, 8]. IL-6 exerts its proinflammatory effects via trans-signaling, binding to the soluble form of the IL-6 receptor (sIL-6R), which is generated by mRNA alternative splicing and partial proteolysis [9]. The coding single nucleotide polymorphism (SNP) rs2228145 of IL-6R gene leads to a substitution of aspartic acid to alanine in position 358 (Asp358Ala variant). The minor allele of rs2228145 (Ala) has been associated with higher sIL-6R levels and circulating/plasma IL-6 levels [9–11].

of our knowledge, this is the first study in Chilean population addressing genetic factors affecting IL-6 levels and treatment resistance in SZ individuals. Ancestry genetic factors that could be specific to our population should also be included in future studies, to allow proper comparison with studies performed worldwide.

Likewise, important factors affecting plasma IL-6 levels that were not controlled in this study should be considered, especially those of metabolic origin, frequently altered in people with SZ [21]. Also, it is known that clozapine administration may indeed affect circulating interleukin levels, which could nullify an initial elevation of interleukin [22]. Similarly, it has also been reported that treatment with other neuroleptics may decrease the levels of sIL-6R [23].

In conclusion, our study does not provide support to a role for the IL-6R rs2228145 (Asp358Ala) variant of the *IL6R* gene in the responsiveness to clozapine in SZ affected individuals. Efforts should be made to increase our sample size and control additional factors affecting interleukin levels, as discussed above.

Data Availability

IL-6 plasma levels, rs2228145 genotype, and BPRS status data can be requested from the corresponding authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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De novo expression of functional connexins 43 and 45 hemichannels increases sarcolemmal permeability of skeletal myofibers during endotoxemia

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ABSTRACT

Endotoxemia caused by bacterial lipopolysaccharides (LPSs) leads to severe skeletal muscular deterioration, starting with higher membrane permeability and decline in resting membrane potential (RMP). However, the molecular mechanism of such changes remains unclear. Here, we evaluated the possible involvement of connexin43- and connexin45-based hemichannels (Cx43 and Cx45 HCs, respectively) as putative mediators of sarcolemmal dysfunctions induced by LPS in control ($Cx43^{fl/fl}Cx45^{fl/fl}$) and Cx43/Cx45 expression-deficient ($Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre) skeletal mice myofibers. At 5 h of endotoxemia, control myofibers presented Cx43 and Cx45 proteins forming functional HCs. Additionally, myofibers from endotoxic control mice showed dye uptake *in vivo*, which was inhibited by carbenoxolone, a Cx HC blocker. A similar increase in membrane permeability was observed in myofibers freshly isolated from skeletal muscle of mice treated for 5 h with LPS, which was blocked by the Cx HC blocker and was absent in myofibers from mice simultaneously treated with LPS and boldine, which is a Cx HC blocker. The increase in sarcolemmal permeability was mimicked by isolated myofibers treated with pro-inflammatory cytokines (TNF-α and IL-1β) and occurred at 5 h after treatment. Endotoxemia also induced a significant increase in basal intracellular Ca^{2+} signal and a drop in RMP in control myofibers. These two changes were not elicited by myofibers deficient in Cx43/Cx45 expression. Therefore, sarcolemmal dysfunction characterizing endotoxemia is largely explained by the expression of functional Cx43 and Cx45 HCs. Hence, current therapy options for individuals suffering from endotoxic shock could be greatly improved with selective Cx HC inhibitors avoiding the underlying skeletal muscle dysfunction.

1. Introduction

Systemic infection with bacteria produces a severe condition clinically known as sepsis associated-intensive care unit acquired weakness (ICUAW). One prominent characteristic of this condition is sarcolemmal injury in both respiratory and peripheral skeletal muscles [1]. At the cellular level, significant depolarization (reduction in resting membrane potential: RMP) and permeabilization of the sarcolemma to small fluorescent dyes have been described [2], but the molecular explanation of these changes remains largely unknown. Recently, it was demonstrated that sepsis induces the expression of proteins that form non-selective channels in skeletal muscle sarcolemma, and it was proposed that they might form functional channels, explaining the previously described channelopathy of the septic condition [3]. However, functional evidence has not been reported to date.

Several changes observed under the septic condition also occur in endotoxemia induced with lipopolysaccharides (LPSs) from Gram negative bacteria [4]. LPSs located in the outer membrane of these

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part, results from compensatory mechanism such as Na^+/K^+ ATPase and Ca^{2+}/Na^+ exchangers. In addition, as observed in sepsis 3, it remains to studied whether other non-selective channels such as $P2X_7$ receptors, TRPV2 channels and Panx1 channels co-expressed with Cxs, are also expressed in endotoxemia.

In agreement with previous work [2], we found a reduction in RMP of myofibers in response to *E. coli* LPS-induced endotoxemia. This response was due to the expression of functional Cx43/Cx45 HCs, since it did not occur in Cx43/Cx45 expression deficient myofibers and was prevented by Cbx and boldine, which are two Cx HC blockers [27–29]. The latter was also observed in freshly dissociated myofibers from LPS treated control mice, in which the acute application of boldine inhibited Etd^+ uptake. On the other hand, the reduction in Cx HC activity induced by boldine in myofibers of control mice treated with LPS was associated with a drastic reduction of Cx HCs immunoreactivity located in the sarcolemma and significant accumulation of intracellular Cx immunoreactive spots that could correspond to down regulation of Cx HCs, which are internalized and traffic from the sarcolemma to a degradation pathway. A similar mechanism has been described for pannexin 1 channels upon high extracellular ATP concentration that first block the channels and after about 15 min causes down regulation of the channel [39]. Alternatively or simultaneously, the long term inhibition of Cx HC by boldine over the LPS induced expression of Cx43 and/or Cx45 HCs might prevent the activation of an intracellular signaling pathway that promotes the expression of these Cxs. In support to this interpretation a similar response has been previously reported in LLC-PK1 cells transfected with Cx43 and treated with Cd^{2+} that induces opening of Cx43 HCs and activation of Cx43 expression *via* a JNK-dependent pathway [40]. Future studies are required to demonstrate the precise mechanism of action of boldine treatment over the cell signaling mediated by Cx HCs.

It is noteworthy that RMP reduction in myofibers of rabbits occurs before deep hypotension, suggesting that RMP reduction is produced before septic shock and not as a consequence of it [6]. It also suggests that this change is an early predictor of successive sepsis-associated changes. Possible mediators of RMP reduction are pro-inflammatory cytokines released by macrophages and/or as outcomes of the activated local inflammasome [41]. Accordingly, TNF-α is known to reduce RMP of rat myofibers in a concentration-dependent manner within the first two hour post-treatment *ex vivo* and *in vivo* [9]. Moreover, we found that freshly isolated myofibers treated with TNF-α or IL-1β for just 5 h presented high sarcolemmal permeability to Etd^+ and the intracellular Ca^{2+} signal likely to be mediated by Cx HC since they were blocked by La^{3+}, a Cx HC blocker [29]. In addition, the expression of Cx HCs has been described to reduce skeletal myofibers RMP [24] and that of HeLa cells transfected with Cxs [35]. RMP reduction is likely due to HC permeability to Na^+ [32], K^+ [33] and Ca^{2+} [34]. Thus, the elevated Cx HC activity found in myofibers of septic mice most likely plays a relevant role in reducing the electrochemical gradient across the sarcolemma. Low excitability of myofibers during sepsis might result from long lasting membrane depolarization, which increases the number of inactivated Na^+ channels. On the other hand, a consequence of stronger intracellular Ca^{2+} signals could be the activation of Ca^{2+}-dependent calpains, which in turn activates the ubiquitin-proteasome system leading to muscular atrophy that characterizes severe sepsis [42].

LPS is known to elevate the serum concentration of several intermediary molecules, including GCs and pro-inflammatory cytokines, which are dramatically up-regulated by LPS. Both of them could be possible candidates mediating skeletal muscle responses. For instance, GCs like dexamethasone induce the expression of functional Cx HCs in skeletal muscles [24]. However, these molecules were discarded in the present work, since the inhibition of GCRs with MIFE did not prevent the expression of Cx HCs. Moreover, skeletal muscles of endotoxic animals have been shown to present abundant infiltration of inflammatory cells [43], and activated innate immune cells are known synthesize and release proinflammatory cytokines in response to LPS [44]. Thus, as mentioned in the previous paragraph, the effect of LPS in the muscle is likely to be mediated by TNFα and/or IL-1β. Consistent with this possibility, it has been suggested that LPS activates the inflammasome signaling pathways in macrophages [45]. Additionally, Cbx, which is a Cx HC and Panx1 channel blocker [29], decreases the release of pro-inflammatory molecules in an animal model of polymicrobial sepsis and significantly increases animal survival rates [46].

All alterations observed in myofibers of mice treated with LPS could explain the channelopathy of skeletal myofibers in endotoxic or septic subjects. In this sense, it was reported that sepsis induces the expression of non-selective channels in the sarcolemma, and it was proposed that channelopathy in skeletal muscles could result from a reduction in the electrochemical gradient across the cell membrane [3]. In support of this hypothesis, we showed in the present work that LPS-induced endotoxemia is associated with a reduction in RMP and an increase in the intracellular Ca^{2+} signal of skeletal myofibers. These two changes where due to Cx HC expression, because their absence or blockade prevented skeletal muscle changes, indicating that these channels could be the main cause of channelopathy in sepsis. Therefore, Cx HCs could be a novel target to efficiently reduce or prevent channelopathy in skeletal muscles, and perhaps the inflammation of the biggest organ (skeletal muscles) that is affected very early during multi organ failure. Skeletal muscle protection could increase life expectancy of septic subjects.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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Chapter 16

Use of *C. elegans* Diapause to Study Transgenerational Responses to Pathogen Infection

Francisco P. Chávez and Andrea Calixto

Abstract

We present a protocol for the study of inter and transgenerational behavioral responses to pathogenesis in *C. elegans*. Transgenerational and intergenerational effects of microbes are best studied in model organisms with short life cycles, large progenies, and quantifiable cellular and behavioral outcomes. This chapter encompasses basic techniques used to study the consequences of bacterial infection in *C. elegans*, including worm growth, quantification of dauer larvae, and quantification of bacterial population dynamics within individual animals. Specific methods for studying transgenerational effects and their duration are also described.

Key words *C. elegans*, Infection, Bacterial population dynamics, Dauer, Transgenerational, Behavior

1 Introduction

Intergenerational transmission implies the direct exposure to the stressor of the parental (F0) and subsequent generation (F1) by means of the developing germ cells or embryos [1]. In the method presented here the formation of the dauer larvae is the readable effect of transmission of information about the pathogenic nature of bacteria [2]. If animals are continuously exposed to pathogenic bacteria, dauer formation is observable in the second generation. The effect is intergenerational starting from the F1 but can only be observed in the F2.

A transgenerational transmission is present when effects of the ancestral exposure to an environment when animals are gravid, are still present in the F3 generation, the first generation that is not directly exposed [3, 4]. In the protocol presented here, a transgenerational effect requires animals to be exposed to nonpathogenic bacteria for two generations (F3 and F4). To correctly eliminate remaining pathogenic bacteria, embryos are extracted by hypochlorite treatment from the uterus of their parental hermaphrodites. If animals retain a transgenerationally transmitted information, a

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4 Notes

1. Leave plates at room temperature for 2–3 days before use to allow for detection of contaminants, and to allow excess moisture to evaporate. Plates stored in an air-tight container at room temperature will be usable for several weeks.
2. Plates should be allowed to grow for no longer than 18–24 h to have effective killing by UV. If plates are overgrown, some bacteria may be protected and grow after several days of culture.
3. To directly follow the process and avoid damage of the embryos, remove a small aliquot every minute and observe on a glass slide, under the scope. Once hermaphrodites are dissolved and embryos released from uterus, immediately centrifuge the Eppendorf tube. The entire process should take no longer than 5 min.
4. Plates must be grown on *E. coli* OP50 and devoid of contamination for several generations before the beginning of the experiment. Because bacterial exposure creates olfactory memory and trigger transgenerational responses, it is of utmost importance that worms are grown for several generations on the control bacteria without any other contaminants.
5. We illustrate the case in which worms are shifted between live bacteria and UV killed bacteria. This protocol can be used to shift animals between any two given conditions.

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REVIEWS

Preserving the balance: diverse forms of long-term GABAergic synaptic plasticity

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Abstract | Cellular mechanisms that regulate the interplay of synaptic excitation and inhibition are thought to be central to the functional stability of healthy neuronal circuits. A growing body of literature demonstrates the capacity for inhibitory GABAergic synapses to exhibit long-term plasticity in response to changes in neuronal activity. Here, we review this expanding field of research, focusing on the diversity of mechanisms that link glutamatergic signalling, postsynaptic action potentials and inhibitory synaptic strength. Several lines of evidence indicate that multiple, parallel forms of plasticity serve to regulate activity at both the input and output domains of individual neurons. Overall, these varied phenomena serve to promote both stability and flexibility over the life of the organism.

The ability of an organism to successfully interact with its environment requires that the nervous system exhibit sufficient functional malleability to adapt to changing contingencies. Learning, in various forms, is thought to rely on the plasticity of chemical synapses formed between neurons. Studies of synaptic plasticity have largely focused on changes to excitatory glutamatergic connections, which exhibit a wide range of modifications over many timescales, including both long-term depression and potentiation[1,2]. By contrast, considerably less is known about the plasticity of inhibitory GABAergic synapses in the mammalian brain. However, a growing body of literature demonstrates that these connections also exhibit varying forms of long-term plasticity that may play critical roles in establishing the proper dynamic operating range of neural circuits, particularly in areas such as the neocortex, hippocampus and cerebellum[3–5].

Excitation and inhibition are often said to be in balance with each other, allowing for both directionally and recurrently wired networks that promote information processing while preventing runaway activity[6]. Moreover, disruption of this balance has been linked to a number of neuropsychiatric disorders, including schizophrenia, autism and epilepsy[6–8]. However, the lack of precise definitions for balance presents challenges to fully appreciating the interactions between glutamatergic and GABAergic signalling. For example, over short timescales (for example, seconds), the average excitatory and inhibitory inputs received by a neuron are approximately but not exactly equal, driving fast fluctuations in the membrane potential that allow precisely timed generation of action potentials[9–12]. Over longer timescales (for example, hours to days), excitation and inhibition appear to track each other, with alterations in the magnitude of one leading to similar alterations in the magnitude of the other, potentially leading to stable modification of the average firing rate of a neuron[13,14]. This latter view suggests a level of homeostasis, whereby cellular mechanisms exist to regulate the interplay of excitation, inhibition and neuronal firing[13]. Together, these perspectives suggest that slower mechanisms of synaptic plasticity may provide the fine tuning necessary for preservation of fast network dynamics across varying environmental constraints. In addition to the temporal relationship between excitation and inhibition, variable spatial distributions along the somatodendritic axis of individual neurons may also play a key role in shaping cellular signalling[15–17].

In the present Review, we focus on the long-term modification of inhibitory GABAergic synapses as a mechanism for controlling neuronal activity. A number of recent studies have shown that changes in both neuronal output (that is, action potential generation) and input (that is, glutamatergic excitation) can drive functionally compensatory alterations of inhibitory inputs. These relationships may be mediated by plasticity at synapses formed by distinct populations of GABAergic interneurons (INs) in a number of brain regions and involving a wide array of molecular signalling pathways. This diversity provides a rich and robust framework by which inhibitory plasticity acts as a key player in establishing balanced network activity in the brain.

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"POLIZONES AÉREOS": VIDA MICROBIANA SOBRE Y A TRAVÉS DE LAS OLAS

"AIR STOWAWAYS": MICROBIAL LIFE ON AND THROUGH THE WAVES

Scarlett E. Delgado* & Camila González-Arancibia**

RESUMEN: Esta revisión analiza el efecto que tienen los microorganismos sobre la recepción olfatoria y el reconocimiento de su hábitat en el caso de las aves marinas. Reconocer dónde estamos y con quién es importante en todas las especies, pero en mayor medida en aquellas que migran grandes distancias y cuya vida transcurre en solitario. Un caso destacable entre este grupo de animales son las aves pelágicas, como los albatros, quienes pueden migrar kilómetros, pero siempre regresan al mismo nido con la misma pareja. En 2017 un grupo de investigadores propuso que las bacterias que pueblan los animales pueden influenciar el reconocimiento olfatorio, idea aún nueva y que puede dar luces para entender el olfato más allá de las aves.

PALABRAS CLAVE: Olfato, Microbiota, Migración, Aves, Mar

ABSTRACT: This review analyzes the effect that microorganisms have on the olfactory reception and recognition of their habitat in the case of the seabirds. Recognizing where we are and with whom is important for all species but, to a greater extent, in those who migrate great distances and whose life is spent in solitude. A notable case in this group of animals is the pelagic birds such as albatrosses, which may migrate for miles but always return to the same nest with the same partner. In 2017, a group of researchers proposed that the bacteria that populate animals can influence olfactory recognition, an idea that is still new and that can shed light on the understanding of olfaction beyond the case of birds.

KEYWORDS: Sense of smell, microbiota, migration, birds, sea.

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Desde el punto de vista de la conservación, entender la influencia de la microbiota en el reconocimiento social y espacial puede contribuir a generar consciencia que no solo cómo nuestra basura afecta, sino que también lo harían los microorganismos que desechamos en el mar, complicando aún más la conservación de aves que se encuentran amenazadas por el actuar humano (Bolton, 2004). A la fecha, los esfuerzos de conservación de las aves en Chile han estado centrados en las aves costeras que vemos con mayor regularidad, estos han promovido la reducción de la perturbación de las áreas costeras e islas donde este tipo de aves nidifican. Este ha sido el centro y también ha mejorado las condiciones de vida de las aves de alta mar, sin embargo, la mayor tasa de muerte de los albatros y petreles ocurre en alta mar a manos de las embarcaciones que realizan la conocida pesca de arrastre (Birdlife, 2004; Burgos, 2012).

En base a esto, la protección que hemos generado hasta la fecha es insuficiente y por lo demás tiene poca relación con lo que ahora conocemos de las aves *Procellariformes*. Si deseamos mantener la estabilidad de la fauna de nuestras costas este es un punto que debemos considerar con el fin de generar reglamentos y protocolos más estrictos para pesca industrial. Este factor es de vital importancia en una región como Valparaíso que es una zona importante para la vida y conservación de estas especies, y al mismo tiempo es un foco de actividad pesquera. Todo lo anterior, vuelve importante la identificación de nichos susceptibles a ser regulados para mejorar la sobrevida de estas aves y del mar (Burgos, 2012). Las aves *Procellariiformes* cumplen una función ecológica en la conservación de nuestras costas, sin embargo, la actividad humana es su principal causa de muerte, quizás si disminuimos los efectos negativos que la pesca tiene sobre ellos, no solo estas especies se verán favorecidas sino que podría contribuir a evitar el colapso de nuestros mares, esfuerzos que no solo contribuirán a la conservación de las aves, sino de toda la cadena trófica (Burgos, 2012). No en vano los petreles fueron indicados como la materialización del patrono de la pesca, San Pedro (Foley, 2005).

CONCLUSIÓN

En la actualidad, si bien la información existente nos sugiere un efecto de la microbiota en la regulación de conductas en aves, este es un campo abierto para investigar en profundidad la neuroanatomía, fisiología sensorial y ecología de las aves *Procellariiformes* en pos del cuidado de estas especies y de nuestras costas, ya que la microbiota no solo regula este tipo de conductas en el hospedero, sino que también, es capaz de regular procesos fisiológicos (tanto metabólicos como neurológicos), por lo que podría tener una importante participación en conductas de distintos animales y podría guiarnos a comprender la naturaleza de estas en una gran variedad de especies migratorias.

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ADVANCED REVIEW

Integration of target discovery, drug discovery and drug delivery: A review on computational strategies

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Most of the computational tools involved in drug discovery developed during the 1980s were largely based on computational chemistry, quantitative structure-activity relationship (QSAR) and cheminformatics. Subsequently, the advent of genomics in the 2000s gave rise to a huge number of databases and computational tools developed to analyze large quantities of data, through bioinformatics, to obtain valuable information about the genomic regulation of different organisms. Target identification and validation is a long process during which evidence for and against a target is accumulated in the pursuit of developing new drugs. Finally, the drug delivery system appears as a novel approach to improve drug targeting and releasing into the cells, leading to new opportunities to improve drug efficiency and avoid potential secondary effects. In each area: target discovery, drug discovery and drug delivery, different computational strategies are being developed to accelerate the process of selection and discovery of new tools to be applied to different scientific fields. Research on these three topics is growing rapidly, but still requires a global view of this landscape to detect the most challenging bottleneck and how computational tools could be integrated in each topic. This review describes the current state of the art in computational strategies for target discovery, drug discovery and drug delivery and how these fields could be integrated. Finally, we will discuss about the current needs in these fields and how the continuous development of databases and computational tools will impact on the improvement of those areas.

This article is categorized under:
Therapeutic Approaches and Drug Discovery > Emerging Technologies
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drug delivery, drug discovery, target discovery

1 | INTRODUCTION

The emergence of new computational technologies has had a significant impact on modern science, serving as a substantial contribution to scientific research. The primary goal of these new tools is to accelerate the process of research, using available computational resources, in different areas such as chemistry and biological biotechnology, among others, on such magnitude that they surpass human discernment. In this context, beginning in 1960, several scientists began to develop theories relating to molecular evolution, laying the basis for bioinformatics by publishing the first Atlas of Protein Sequences; this work is the

pulled to the external face of the membrane, adopting a pancake conformation instead of spherical, which makes its lateral diffusion slower.

Regarding dendrimers, several other approaches have been employed to explore their permeability across membranes. One of them addressed the mechanism of the escape of non-viral vectors from endosomes, which is critical for the development of gene delivery systems. De Tian et al. employed coarse-grained molecular dynamics simulations to give insight into the gene escape mechanism and in the effect of the tension of the membranes in the penetration of nanoparticles, suggesting a cooperative effect between the osmotic pressure and the increase of dendrimer size in the endosome, besides local effects caused by the electrostatic adsorption of dendrimers (Tian & Ma, 2012). Early in 2006, Lee and Larson provided one of the first insights into PAMAM dendrimer, having different levels of acetylation, in the interaction with model membranes. They choose coarse grained method to evaluate effect of temperature, salt concentration and acetylation degree of the dendrimer on the formation of pores in the membranes, which can be relevant when studying drug delivery mechanisms (Lee & Larson, 2006).

Nanoparticles surface can be also engineered to promote an increased interaction with surfaces or cell membranes, increasing their abilities as drug carriers.

Some studies have been devoted to address the interaction among charged-gold nanoparticles (AuNPs) covered by an amphiphilic organic monolayer that can penetrate lipid bilayers. The pathway by which AuNPs insert into bilayers was analyzed through molecular dynamics, identifying that this process is hydrophobic–hydrophobic, requiring the protrusion of an aliphatic lipid tail into solution (Van Lehn et al., 2014). Studies were conducted through full atom molecular dynamics simulations. Committor analysis was performed to identify the transition state associated with the insertion of an AuNP into the bilayer from an initial surface -adsorbed position. The committor, p, is defined as the probability that a trajectory started from a particular system configuration will reach basin B before basin A , where A and B correspond to free energy minima of interest in some multidimensional free energy landscape.

The committor can yield information about transitions in the system without defining a particular reaction coordinate for the dynamic trajectory, differently from other methods of free energy calculation.

5 | CONCLUSION

Traditionally, the drug discovery process is related to a ligand and a high binding affinity to a target. Similarly to the experimental process of drug discovery, the current computational drug design methods focus on maximizing ligand efficiency, increasing their potency towards one target. Significant advances are required to optimize computational tools so that they can better accomplish this objective. In this sense, a key challenge for the computational field starts with the integration and automation in all databases of targets and molecules or drugs and the inclusion of delivery systems databases which has not been done up until now. One of the biggest problems that the processes of target discovery and drug discovery have faced is in searching the vast wealth of sequence data and new molecules through databases using computer-based methods, which result in challenging and unfriendly situations. The selection and processing of data from several systems and their following analyses require computational tools appropriate for each purpose. In this sense, a lot of molecule databases, even the sequence that is an important data source for many researchers, are privately held (Chandra & Padiadpu, 2013) although public-access databases are continually integrating with the informatics network. This is necessary for recognizing the growing availability of basic database analysis tools, which are turning to bioinformatics solutions to address research challenges.

Although computational searches will never replace laboratory experiments, computational tools have an important role to play in accelerating and enhancing the process of target and drug discovery as well as the discovery of novel computational efficient tools for simulating drug delivery systems, which are under increasing development.

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CONFLICT OF INTEREST

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Inhibition of miR-378a-3p by Inflammation Enhances IL-33 Levels: A Novel Mechanism of Alarmin Modulation in Ulcerative Colitis

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Ulcerative colitis (UC) is an inflammatory bowel disease (IBD) characterized by mucosa damage associated with an uncontrolled inflammatory response. This immunological impairment leads to altered inflammatory mediators such as IL-33, which is shown to increase in the mucosa of active UC (aUC) patients. MicroRNAs present a distorted feature in inflamed colonic mucosa and are potential IL-33 regulating candidates in UC. Therefore, we studied the microRNA and mRNA profiles in inflamed colonic samples of UC patients, evaluating the effect of a microRNA (selected by *in silico* analysis and its expression in UC patients), on IL-33 under inflammatory conditions. We found that inflamed mucosa ($n = 8$) showed increased expression of 40 microRNAs and 2,120 mRNAs, while 49 microRNAs and 1,734 mRNAs were decreased, as determined by microarrays. In particular, IL-33 mRNA showed a 3.8-fold increase and eight members of a microRNA family (miR-378), which targets IL-33 mRNA in the 3′UTR, were decreased (−3.9 to −3.0 times). We selected three members of the miR-378 family (miR-378a-3p, miR-422a, and miR-378c) according to background information and interaction energy analysis, for further correlation analyses with IL-33 expression through qPCR and ELISA, respectively. We determined that aUC ($n = 24$) showed high IL-33 levels, and decreased expression of miR-378a-3p and miR-422a compared to inactive UC ($n = 10$) and controls ($n = 6$). Moreover, both microRNAs were inversely correlated with IL-33 expression, while miR-378c does not show a significant difference. To evaluate the effect of TNFα on the studied microRNAs, aUC patients with anti-TNF therapy were compared to aUC receiving other treatments. The levels of miR-378a-3p and miR-378c were higher in aUC

on soluble inflammatory factors (**Figure 8**). This research contributed to an in-depth analysis of a microRNA controlling IL-33 levels in an inflammatory environment and hopefully represents the basis of new biomarkers or a therapeutic target grounded on microRNA regulation for UC.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GSE133061, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE133061.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Sub-direction of Research, Local Ethics Committee from Clínica Las Condes, Santiago, Chile. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KD-C performed most of the experiments and analysis, designing, and drafting of the manuscript. DD-J, MD, and GL contributed to design and achieve IL-33 detection in biopsies samples and HT-29 experiments. MO-M accomplished IPA and microarray analysis. RQ contributed to study design and sample acquisition. DS and MM enrolled UC and healthy individuals. JAC and XX performed microarray bioinformatics analysis. GG and JX designed and developed the microRNA sensor vector tools. JC developed the pri-miR lentiviral tool. RS-R supported the designing and results analysis of sensor vector experiments. M-JG prarticipated in the analysis and discussion of results. AC contributed with RNA: microRNA interaction analysis. MH contributed to study design, supervised work. All the authors contributed to the drafting and discussion of the manuscript.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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The Neuronal Glutamate Transporter EAAT3 in Obsessive-Compulsive Disorder

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Obsessive compulsive disorder (OCD) is a heterogeneous psychiatric disorder affecting 1%–3% of the population worldwide. About half of OCD afflicted individuals do not respond to currently available pharmacotherapy, which is mainly based on serotonin reuptake inhibition. Therefore, there is a critical need to search novel and improved therapeutic targets to treat this devastating disorder. In recent years, accumulating evidence has supported the glutamatergic hypothesis of OCD, and particularly pointing a potential role for the neuronal glutamate transporter EAAT3. This mini-review summarizes recent findings regarding the neurobiological basis of OCD, with an emphasis on the glutamatergic neurotransmission and EAAT3 as a key player in OCD etiology.

Keywords: EAAT3, OCD, obsessive-compulsive disorder, glutamate transporter, synaptic function, NMDAR, animal model, SLC1A1

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OBSESSIVE COMPULSIVE DISORDER

Obsessive compulsive disorder (OCD) is a psychiatric disorder affecting 1%–3% of the population worldwide (Grabe et al., 2000; Angst et al., 2004). It is characterized by recurrent, intrusive worries, feelings or unwanted thoughts (obsessions), and repetitive, structured, ritualistic mental acts and/or behaviors (compulsions). OCD is clinically heterogeneous, displaying a wide range of symptomatic expression (Murphy et al., 2013). Most OCD patients also have high levels of anxiety, likely due to the inability to control or stop the appearance of obsessions (Pauls et al., 2014). Both obsessions and compulsions are time consuming, causing a high impairment in social and occupational areas (American Psychiatric Association, 2013).

Current OCD treatment involves cognitive-behavioral therapy alone or combined with pharmacotherapy mainly based on serotonin reuptake inhibitors (SRI), such as fluoxetine, clomipramine or paroxetine (Bouvard et al., 2004; Cottraux et al., 2005; Skapinakis et al., 2016). However, only about half of patients achieve an adequate decrease in the severity of symptoms (Hirschtritt et al., 2017), highlighting the need for new therapeutic options to treat this devastating disorder.

CORTICO-STRIATO-THALAMO-CORTICAL LOOP MODIFICATIONS IN OCD

Since original reports, neuroimaging studies have consistently indicated that a dysfunction in the cortico-striato-thalamo-cortical (CSTC) loop is involved in OCD (Milad and Rauch, 2012; Nakao et al., 2014; Burguiere et al., 2015). In a brief and oversimplified manner, the CSTC loop

the interplay between of NMDAR and redox balance in the etiology of OCD.

AUTHOR CONTRIBUTIONS

PM conceived the work. AE, JW, AC, PM drafted the work, critically revised the work and provided important intellectual content. AE, JW, AC, PM provided approval for publication of the content and agreed to be accountable for all aspects of the work.

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Chapter 1
Rapid Prototyping for Bio–Inspired Robots

María-José Escobar, Frédéric Alexandre, Thierry Viéville and Adrian G. Palacios

1.1 Introduction

Robots are powerful and reliable systems and perform better than humans in several domains, for example considering very precise manipulations in stereotyped tasks. In other domains, their performances remain very poor and disappointing. It is often said that they lack intelligence that a biological system would display for such cases and this has opened a multifarious activity of research to associate robotics with theoretical biology and artificial intelligence.

At the birth of computer science, defining intelligence as a Physical Symbol System opened the field of Artificial Intelligence (AI) [83]. Indeed, computers have all the characteristics of a Physical Symbol System: they encode symbols (variables), they combine them into structures (expressions) and manipulate them with processes (programs) to produce new expressions. Consequently the fathers of AI thought that, if intelligence has also these characteristics, a computer will implement them and become an intelligent machine. In spite of many successes of traditional AI, it is known today that this view is too narrow. One weakness is the Symbol Grounding Problem defined by S. Harnard [43]. For example, performing scene in-

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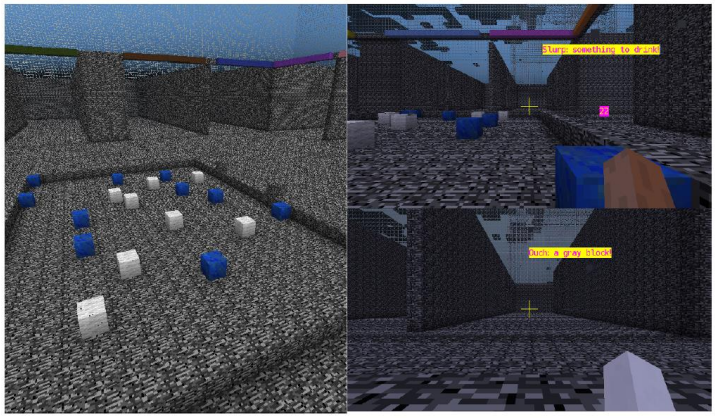

Fig. 1.17 A Platform for Systemic Neuroscience Simulation [27]. Using the MineCraft survival game environment, a simple survival behavior is simulated in which the human player has been replaced by a bot, that implements Pavlovian mechanism related to the role of the amygdala. This simulation is an unpublished ongoing work following the modeling proposed by [18] and considering mechanism of reinforcement learning [10].

nities. This capablity has been transposed to robots [70] showing that it is a crucial step towards machines capable of acting in open-ended [49].

Yet another step further, a great challenge would be to be able to explain the whole complex ground behavior of "survival" of a biological or robotic system by a unifying general "fundamental law". This huge challenge has been taken up by [50], including for the modeling of goal-oriented behavior [35] involving the basal-ganglia [36]. The key idea is to state that a system survives if it is able to maintain its vital variables within certain bounds, which in a non-deterministic environment means to minimize the probabilistic surprise with respect to the system observable. What makes this idea quite fruitful is the fact that such "surprise" is bounded by the system information free-energy (as stated by Feynman and revisited by Friston in neuroscience), which is an observable quantity. Up to now, in neuroscience, this principle has been set as compatible with the main notions related to cognition and active inference, whereas its application to robotics is still to be done.

Though non exhaustive, these glimpses of ongoing neuroscience researches yielding effective systemic modeling of cognitive behaviors really indicate that bio-inspired robots still have a lot to learn from neuroscience.

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ORIGINAL ARTICLE

Gap junctions coordinate the propagation of *glycogenolysis* induced by norepinephrine in the pineal gland

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Abstract

Chemical and electrical synapses are the two major communication systems that permit cell-to-cell communication within the nervous system. Although most studies are focused on chemical synapses (glutamate, γ-aminobutyric acid, and other neurotransmitters), clearly both types of synapses interact and cooperate to allow the coordination of several cell functions within the nervous system. The pineal gland has limited independent axonal innervation and not every cell has access to nerve terminals. Thus, additional communication systems, such as gap junctions, have been postulated to coordinate metabolism and signaling. Using acutely isolated glands and dissociated cells, we found that gap junctions spread glycogenolytic signals from cells containing adrenoreceptors to the entire gland lacking these receptors. Our data using glycogen and lactate quantification, electrical stimulation, and high-performance liquid chromatography with electrochemical detection, demonstrate that gap junctional communication between cells of the rat pineal gland allows cell-to-cell propagation of norepinephrine-induced signal that promotes glycogenolysis throughout the entire gland. Thus, the interplay of both synapses is essential for coordinating glycogen metabolism and lactate production in the pineal gland.

Keywords: connexin, electrical and chemical synapses, glycogen, hemichannels.

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The rat pineal gland is a neuroendocrine organ innervated by sympathetic neurons that end in the center of the gland, coordinating the light/dark cycle for the production of melatonin. The activity of the pineal gland is driven by the suprachiasmatic nucleus, which regulates the phasic release of norepinephrine (NE) from sympathetic neurons (Colwell 2000a; Colwell 2000b). NE action is transduced post-synaptically by β- and α_1- adrenoreceptors (Axelrod 1974; Klein *et al.* 1981; Eugenin *et al.* 1997), through which it induces synthesis and releases of melatonin, a hormone that is involved in the photoperiodic regulation of reproduction and several other physiologic functions (Reiter 1980).

The rat pineal gland is made up mainly by pinealocytes, which are responsible for melatonin synthesis, and astrocytes (Freund *et al.* 1977), which constitute around 5–10% of the total cell content in the gland (Moller 1978; Moller *et al.* 1978; Schachner *et al.* 1984). A key source of energy within the central nervous system (CNS) is glycogen, which is

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Abbreviations used: AGA, 18α-glycyrrhetinic acid; BAPTA, 1,2-bis (2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; $CaCl_2$, calcium chloride; Ca^{2+}, calcium ion; CNS, central nervous system; Cx, connexin; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; eNE, endogenous noradrenalin; FBS, fetal bovine serum; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; GJ, gap junction; HPLC-EC, high-performance liquid chromatography with electrochemical detection; IP_3, inositol trisphosphate; KCl, potassium chloride; $MgSO_4$, magnesium sulfate; NaCl, sodium chloride; Na_2EDTA, ethylenediaminetetraacetic acid disodium salt; NaH_2PO_4, monosodium phosphate; $Na_2S_2O_3$, sodium thiosulfate; NE, norepinephrine; O, octanol; OCT, embedding medium for frozen tissue specimens to ensure optimal cutting temperature; PAS, periodic acid-Schiff staining; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RRID, Research Resource Identifier.

ORIGINAL ARTICLE

mostly dependent on Cx43 instead of Cx36, present on pinealocytes (the most abundant cell type in the pineal gland). In agreement, with the importance of Cx43 containing channels, but not Cx36 containing channels, for glycogen degradation (Fig. 2b), dye coupling (Fig. 4f) and signaling (Fig. 5) was fully dependent on Cx43 channels, but not Cx36. Our data indicate that most glycogen stores are in astrocytes (Eugenin *et al.* 1997), supporting the importance of Cx43 containing channels in spreading glycogenolytic signals across the entire pineal gland.

A similar mechanism of localized receptor expression and GJ amplification was described in the liver (Eugenin *et al.* 1998). Our publications indicated that the glycogenolytic response of the liver to vasopressin was mostly transmitted from the peri-central into the peri-portal acinus area. Several laboratories demonstrated that this metabolic cooperation was mostly mediated by a calcium ions and IP_3 auto-regeneration mechanism. also termed calcium waves (Rooney *et al.* 1990; Thomas *et al.* 1991; Nathanson *et al.* 1994; Nathanson *et al.* 1995; Tordjmann *et al.* 1997; Nathanson *et al.* 1999a; Dupont *et al.* 2000a). Calcium waves can reach long distances and mostly present in cells expressing GJs. Indeed, GJs create an efficient syncytium enabling small second messengers such as calcium, IP_3, and cyclic nucleotides to diffuse between connected non-excitable cells. In excitable cells (e.g., neurons), the major function of GJs is the fast relay of current generated at the plasma membrane by the exchange and intercellular diffusion of K^+, Na^+, and Cl^- ions. We propose that in the pineal gland both systems are active and cooperative. In agreement, it has been described that Cx36 and Cx43 have particular and well-defined permeabilities that promote the spread of particular second messengers (Bukauskas 2012). We believe that these differences in GJ permeability could account for the dependency of glycogen degradation on Cx43 (only present in 10% of the cells, astrocytes), instead of Cx36 present in 90% of all cells, pinealocytes. Thus, the main framework of the metabolic changes in the pineal gland is driven by astrocytes communicated by Cx43.

The finding that small dyes that can only diffuse via GJ channels through astrocytes and pinealocytes in the whole pineal gland confirms that both cell types form extensive functional gap junctional networks, as it has been shown in isolated astrocytes and pinealocytes maintained in culture (Saez *et al.* 1991). Furthermore, the dye coupling found in the whole pineal gland was more extended than was found in cultured pineal cells (Saez *et al.* 1991; Saez *et al.* 1994), suggesting that GJs play a more relevant role than expected from results obtained in dissociated cells. In the pineal gland, pinealocytes and astrocytes may form independent networks, but under specific conditions they might interact, forming a unique network via heterotypic GJs. Our data support a physiological role for gap junctional communication in the CNS, which is to coordinate metabolic response and glycogen degradation in response to the exogenous and endogenous neurotransmitter, NE.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

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Worm corpses affect quantification of dauer recovery

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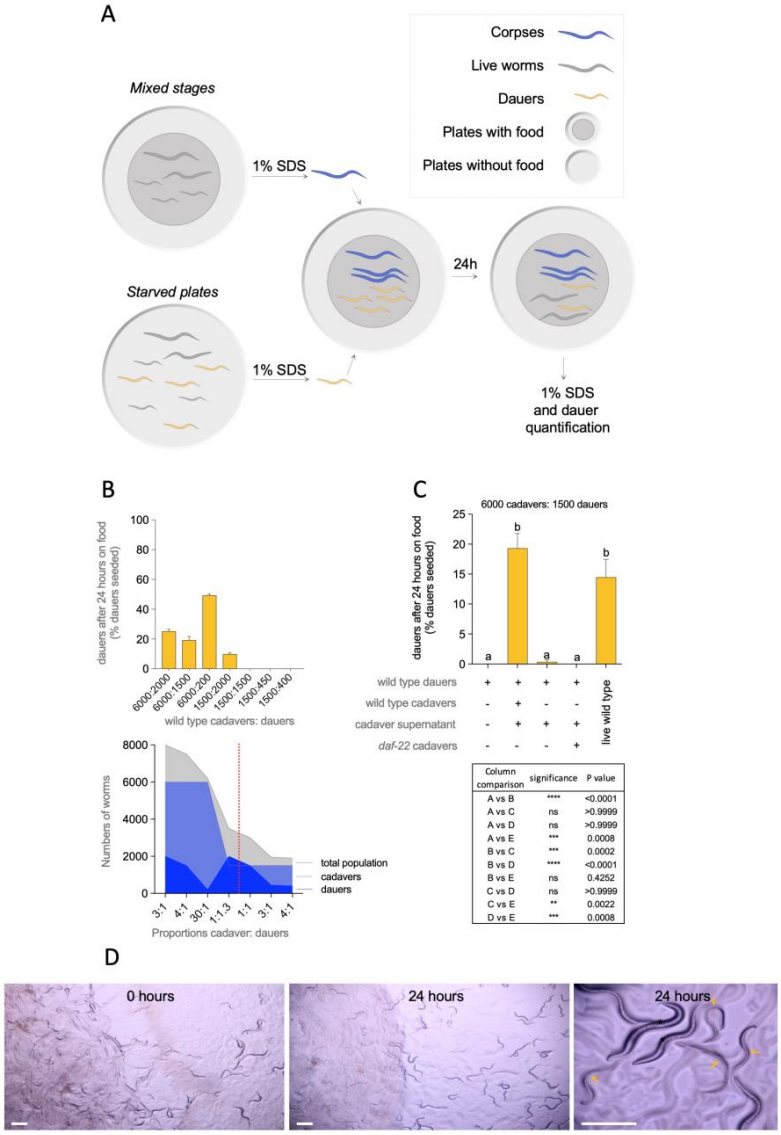

Figure 1 Dauer recovery on *E. coli* OP50 under different conditions of population size and genotype. (A) Methodological outline used for recovery of dauers in the presence of corpses or live animals in plates with *E. coli* OP50 food. (B) Percent of dauer recovery after 24 hours of exposure to food on different proportions of cadavers and dauers (upper panel) and representation of numbers of dauers, cadavers and total population on each proportion (lower panel). (C) Percent of dauer recovery after 24 hours of exposure to food in the proportion 1:4 (6000 cadavers

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Preparation of corpses: Corpses were obtained from mixed plates with high density of worms with an approximate count of 5000 total worms. As a general rule after examining many plates, the proportion of animals in L1-2, L3, L4 and adults was relatively constant: 42% of animals treated were L1-L2, 24% were L3, 16% were L4 and 17% were adults. Animals from a mixed stage plate were washed once with M9 and treated with 1% SDS for 20 min. After SDS treatment tubes were centrifuged for 2 minutes at 2500 rpm. Supernatant was discarded, leaving 45 ml of liquid remaining. To count the number of corpses samples treated with 1% SDS were diluted 1:10 in M9. 10 μl of each dilution was used to count the number of corpses under a Nikon SMZ745 stereoscope.
The mixture of cadavers and dauers was made in a tube (after having the quantification of each one), mixed by pipetting and seeded on a 60 mm plate. Mixed cadavers and dauers were always seeded together in one spot over the *E. coli* OP50 food lawn.

Experimental replicas and statistics: All experiments were done at least 3 times (three biological replicas, started in different days and from different starting plates). Each biological replica contained a triplicate (three technical replicas). Statistical analysis was done by a one-way ANOVA with post-hoc analysis (Tukey´s test).

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Orientational and Folding Thermodynamics via Electric Dipole Moment Restraining.

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Abstract

The projection of molecular processes onto a small set of relevant descriptors, the so called reaction coordinates or collective variables, is a technique nowadays routinely employed by the biomolecular simulation community. In this work, we implemented two collective variables to manipulate the orientation (i.e. angle) ($\vec{\mu}_a$) and the magnitude ($|\vec{\mu}|$) of the electric dipole moment. In doing so, we studied the thermodynamics of water orientation under the application of external voltages and the folding of two polypeptides at zero-field conditions. The projection of the free-energy (PMF) along water orientation defined an upper limit of around 0.3 volts for irrelevant thermodynamic effects. On the other hand, sufficiently strong $\vec{\mu}_a$ restraints applied on 12-alanine

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Supporting Information Available

The following files are available free of charge.

- Sup.Mat.pdf: Supplementary material including details of mathematical derivations, angle based PMF's (Fig. S1) $\vec{\mu}_a$ restraints on small flexible species (Fig.S2), $\vec{\mu}_a$ restraints (Fig.S3 and Tables S1, S2, S3) and $|\vec{\mu}|$ restraints on small flexible species (Figs. S5, S6).

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Role of a RhoA/ROCK-Dependent Pathway on Renal Connexin43 Regulation in the Angiotensin II-Induced Renal Damage

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Abstract: In various models of chronic kidney disease, the amount and localization of Cx43 in the nephron is known to increase, but the intracellular pathways that regulate these changes have not been identified. Therefore, we proposed that: "In the model of renal damage induced by infusion of angiotensin II (AngII), a RhoA/ROCK-dependent pathway, is activated and regulates the abundance of renal Cx43". In rats, we evaluated: 1) the time-point where the renal damage induced by AngII is no longer reversible; and 2) the involvement of a RhoA/ROCK-dependent pathway and its relationship with the amount of Cx43 in this irreversible stage. Systolic blood pressure (SBP) and renal function (urinary protein/urinary creatinine: Uprot/UCrea) were evaluated as systemic and organ outcomes, respectively. In kidney tissue, we also evaluated: 1) oxidative stress (amount of thiobarbituric acid reactive species), 2) inflammation (immunoperoxidase detection of the inflammatory markers ED-1 and IL-1β), 3) fibrosis (immune detection of type III collagen; Col III) and 4) activity of RhoA/ROCK (amount of phosphorylated MYPT1; p-MYPT1). The ratio Uprot/UCrea, SBP, oxidative stress, inflammation, amount of Cx43 and p-MYPT1 remained high 2 weeks after suspending AngII treatment in rats treated for 4 weeks with AngII. These responses were not observed in rats treated with AngII for less than 4 weeks, in which all measurements returned spontaneously close to the control values after suspending AngII treatment. Rats treated with AngII for 6 weeks and co-treated for the last 4 weeks with Fasudil, an inhibitor of ROCK, showed high SBP but did not present renal damage or increased amount of renal Cx43. Therefore, renal damage induced by AngII correlates with the activation of RhoA/ROCK and the increase in Cx43 amounts and can be prevented by inhibitors of this pathway.

Keywords: Hypertensive nephropathy; oxidative stress; fibrosis; inflammation; Cx43; Fasudil

1. Introduction

In the last years Chronic kidney disease (CKD) has been increasing steadily worldwide turning into a public health problem. In fact, about 10% of adults in developed countries suffer some degree of kidney damage [1]. Patients with this disease, usually develop a progressive kidney damage characterized by tubulointerstitial fibrosis and/or glomerular sclerosis, eventually leading to the last stage of this condition, end-stage renal disease [2]. In this process there is an increase in damaged nephrons which leads to a progressive reduction of glomerular filtration rate (GFR), and eventually to

lysis buffer) containing 100 mM Tris-HCl pH 7.4, 5 mM EDTA, 1% SDS, 1 μM PMSF and a protease inhibitor cocktail (Halt™ Protease Inhibitor Cocktail, Pierce, Rockford, IL, USA). Then, samples were centrifuged at 14,000× *g* for 10 min (Eppendorf 5415C, Eppendorf, Hamburg, Germany). Supernatants were collected and protein content assayed by Bicinchoninic Acid (BCA) Protein Assay. Cytokine levels were determined by sandwich ELISA, according to the manufacturer's protocol (IL-1β EIA kit, Enzo Life Science, USA). For the assay, 100 μL of samples were added per well and incubated at 4 °C overnight. A calibration curve with recombinant cytokine was included. Samples were incubated with secondary antibody at room temperature for 2 h and the reaction was developed with avidin–HRP and substrate solution. Absorbance was measured at 450 nm with reference to 570 nm with the microplate reader Synergy HT (Biotek Instruments). The results were normalized by protein amount in pg/g protein.

4.10. Western Blot Assays

Proteins from renal tissue samples (50 μg of proteins) were resolved by electrophoresis in 10% SDS-polyacrylamide gel, using one lane for the pre-stained molecular weight markers. Proteins were transferred to a PVDF membrane (pore size: 0.45 μm), blocked at room temperature with Tris pH 7.4, 5% skim milk (w/v) and 1% BSA (w/v). Then, the PVDF membrane was incubated overnight at 4 °C with anti-Cx43 (1:1,000), anti-p-MYPT1 (1:500), or anti-MYPT1 (1:1,000) antibody, followed by incubation with secondary antibody conjugated to peroxidase (1:2,000) for 1 h at room temperature. Then, the PVDF membrane was stripped and reblotted with the anti-α-tubulin antibody (1:5,000) used as loading control, following the same procedure described above. After repeated rinses, immunoreactive proteins were detected with ECL reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. The bands detected were digitized and subjected to densitometry analysis using the software Image J (Version 1.50i, NIH, Washington, DC, USA).

4.11. Statistical Analysis

Results were expressed as mean ± standard error (SE); *n* is the number of independent experiments. Figure legends included detailed statistical results. GraphPad Prism (version 7, GraphPad Software, La Jolla, CA) was used to perform statistical analyses. One or two-way analysis of variance (ANOVA) was performed for multiple comparisons When significance was obtained, a Tukey's post-hoc test was performed. A probability of $p < 0.05$ was considered statistically significant. Results are expressed as the average of values from each independent experiment ± SE.

Author Contributions: G.I.G., V.V. and J.C.S. conceived and designed the experiments; G.I.G. performed the experiments; G.I.G. and J.C.S. analyzed the data; J.C.S. and V.V. contributed reagents/materials/analysis tools; G.I.G., V.V. and J.C.S. wrote the paper.

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The molecular nature of the 17β-Estradiol binding site in the voltage- and Ca^{2+}-activated K^+ (BK) channel β1 subunit

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The accessory β1 subunit modulates the Ca^{2+}- and voltage-activated K^+ (BK) channel gating properties mainly by increasing its apparent Ca^{2+} sensitivity. β1 plays an important role in the modulation of arterial tone and blood pressure by vascular smooth muscle cells (SMCs). 17β-estradiol (E2) increases the BK channel open probability (P_o) in SMCs, through a β1 subunit-dependent modulatory effect. Here, using molecular modeling, bioinformatics, mutagenesis, and electrophysiology, we identify a cluster of hydrophobic residues in the second transmembrane domain of the β1 subunit, including the residues W163 and F166, as the binding site for E2. We further show that the increase in P_o induced by E2 is associated with a stabilization of the voltage sensor in its active configuration and an increase in the coupling between the voltage sensor activation and pore opening. Since β1 is a key molecular player in vasoregulation, the findings reported here are of importance in the design of novel drugs able to modulate BK channels.

The large conductance, Ca^{2+}- and voltage-activated K^+ (BK) channel is a tetrameric transmembrane protein composed of α subunits that form the K^+ selective pore[1,2]. The α subunit is broadly expressed in mammalian tissues[3], where it is co-expressed with an accessory β subunits in a tissue-specific manner[4,5]. The α subunit comprises seven transmembrane segments (S0-S6), leaving the amino terminus exposed to the external medium. The large intracellular carboxyl terminus domain consisting of two RCK domains contains the Ca^{2+} binding sites. The different β subunits (β1–β4) are formed by two transmembrane segments linked by a large extracellular loop and are responsible for the modulation of the biophysical and pharmacological characteristics of the α subunit[4,6]. The β1 subunit is abundantly expressed in smooth muscle cells and acts to increase the apparent sensitivity to Ca^{2+} and dramatically slows down the activation and deactivation channel-gating kinetics[7–12]. The presence of the BK α/β1 channel lowers the risk of pathologies associated with the vascular tone by causing a membrane hyperpolarization, decreasing vasoconstriction, and inducing a faster relaxation of the blood vessels[13,14]. β subunits appear to regulate the BK channel activity by targeting specific gating mechanisms[11,14,15]. In particular, by biasing the equilibrium resting-active of the voltage sensor towards its active configuration, β1 increases the apparent BK Ca^{2+} sensitivity[14,16] Co-expression of BK α and β subunits can dramatically modify pharmacological responses of the channel[17–19] as potential targets for channel modulators such as alcohol[20], estrogens[19], hormones[21] and fatty acids[22,23].

17β-Estradiol (E2) is the main circulating oestrogen in women and reaches a plasma concentration of 30–400 pg/mL before menopause. E2 regulates growth and the development of the reproductive system, also, helps to maintain the osseous tissue, the central nervous system and the vasodilatation in the vascular tissue[24]. The protective effect of E2 in the vasculature and against cardiovascular disease (CVD) has been demonstrated in several hormone replacement studies[25,26]. E2 activates BK channels[19,27–30] via a process that requires the presence

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Author Contributions

S.T.G. carried out confocal microscopy, flow cytometry assays and ionic current recordings; K.C. and Y.L.-C. performed gating current recordings; F.B.-M., R.S. and M.R. carried out the molecular modelling; E.C. performed ionic current recordings; S.T.G., K.C., Y.L.-C. and R.L. analysed the data; S.T.G., R.L., K.C., W.C.-U. and Y.P.T. wrote the manuscript; R.L., F.G.-N., C.G. and Y.P.T. designed the experiments and directed research activities.

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ORIGINAL ARTICLE

The Ca^{2+} channel subunit $Ca_V\beta2a$-subunit down-regulates voltage-activated ion current densities by disrupting actin-dependent traffic in chromaffin cells

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Abstract

β-Subunits of the Ca^{2+} channel have been conventionally regarded as auxiliary subunits that regulate the expression and activity of the pore-forming α_1 subunit. However, they comprise protein–protein interaction domains, such as a SRC homology 3 domain (SH3) domain, which make them potential signaling molecules. Here we evaluated the role of the β2a subunit of the Ca^{2+} channels ($Ca_V\beta2a$) and its SH3 domain (β2a-SH3) in late stages of channel trafficking in bovine adrenal chromaffin cells. Cultured bovine adrenal chromaffin cells were injected with $Ca_V\beta2a$ or β2a-SH3 under different conditions, in order to acutely interfere with endogenous associations of these proteins. As assayed by whole-cell patch clamp recordings, Ca^{2+} currents were reduced by $Ca_V\beta2a$ in the presence of exogenous α1-interaction domain. β2a-SH3, but not its dimerization-deficient mutant, also reduced Ca^{2+} currents. Na^+ currents were also diminished following β2a-SH3 injection. Furthermore, β2a-SH3 was still able to reduce Ca^{2+} currents when dynamin-2 function was disrupted, but not when SNARE-dependent exocytosis or actin polymerization was inhibited. Together with the additional finding that both $Ca_V\beta2a$ and β2a-SH3 diminished the incorporation of new actin monomers to cortical actin filaments, β2a-SH3 emerges as a signaling module that might down-regulate forward trafficking of ion channels by modulating actin dynamics.

Keywords: β2a subunit, actin dynamics, Ca^{2+} channels, Ca^{2+} currents, protein trafficking.

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Many cellular processes rely on precise and localized elevations of the cytosolic Ca^{2+} level. In electrically excitable cells, the major mechanism that drives cytosolic Ca^{2+} increments is the Ca^{2+} entry through high-voltage-activated (HVA) Ca^{2+} channels (Ca_V) (Neely and Hidalgo, 2014). HVA Ca_V are multiprotein complexes containing a pore-forming α1 subunit and auxiliary subunits (β, $\alpha_2\delta$, and γ) that control the biophysical properties and membrane expression of the pore-forming $Ca_V\alpha1$ (Neely and Hidalgo, 2014; Dolphin, 2016), and thus, these subunits play a critical role in adjusting the amount of Ca^{2+} entry and Ca^{2+}-mediated signaling. Among the HVA Ca_V auxiliary subunits the β subunits stand out as potent regulators of HVA Ca_V function (Buraei and Yang, 2010). $Ca_V\beta$ subunits are a group of signaling proteins that share a common primary structure with two highly conserved domains, a guanylate kinase-like (GK) and a SRC homology 3 domains (SH3). The GK

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Abbreviations used: I–II loop, I–II cytoplasmic loop of the $Ca_V1.2$ subunit fused to GST; I–V, current-voltage; β2a-SH3, SH3 domain of the $Ca_v\beta2a$; amphiphysin-SH3, amphiphysin SH3 domain; AID, α1-interaction domain; ATP, adenosine triphosphate; BACC, bovine adrenal chromaffin cells; BoNT/E, light chain of botulinum neurotoxin E; $Ca_V\alpha1$, α1 subunit of the Ca^{2+} channels; $Ca_V\beta2a$, β2a-subunit of the Ca^{2+} channels; Ca_V, Ca^{2+} channels; cortactin-SH3, cortactin SH3 domain; DAPI, 4′,6-diamidino-2-phenylindole; Dyn2 K44A, dynamin-2 mutant K44A; Dyn2 WT, dynamin-2 wild-type; EGFP, enhanced green fluorescent protein; GK, guanylate kinase-like; GST, glutathione-S-transferase; HVA, high-voltage-activated; LatA, latrunculin A; PRD, proline-rich-domain; SH3, SRC homology 3 domain; WASP, Wiskott–Aldrich-syndrome protein; WAVE, Wiskott–Aldrich syndrome verprolin-homologous protein.

ORIGINAL ARTICLE

formation of $Ca_V1.2$ clustering, and consequently channel activity (Ghosh *et al.,* 2018). In this regard, trafficking and Ca^{2+} channel activity seem to be linked.

Another key regulator of $Ca_V\alpha1$ surface expression is $Ca_V\beta2a$ that via its SH3 domain appears to regulate the surface expression of the α1 subunit by different routes whose relevance varies according to the cell context and experimental conditions: (i) β2a-SH3 may promote dynamin-dependent endocytosis with the consequent reduction in the number of channels as reported in *Xenopus* oocytes (Gonzalez-Gutierrez *et al.,* 2007), and (ii) it may facilitate the actin-dependent forward traffic as observed in HL-1 cardiomyocytes (Stölting *et al.,* 2015). Here we proposed a third route that operates in BACC in which β2a-SH3 down-regulates the expression of Ca^{2+} and Na^{+} currents (Figs 2 and 6) that as discussed below it correlated with a reduction in actin-dependent forward traffic (Figs 3 and 4). Such ample repertoire of regulatory mechanisms should not be surprising considering that SH3 domains are protein modules that bind a variety of PRD-containing effector proteins such as dynamin, myosin, and actin nucleation promoting factors, including Wiskott–Aldrich-syndrome protein and Wiskott–Aldrich syndrome verprolin-homologous protein family members (Kurochkina and Guha, 2013). Different factors may influence the interaction of SH3 domains with PRDs, including the oligomeric state of the SH3-containing protein. In *Xenopus* oocytes for example, β2a-SH3 promotes dynamin-dependent endocytosis only when able to form homodimers (Miranda-Laferte *et al.,* 2011). In chromaffin cells, we proposed that β2a-SH3 dimerization seems also to be critical to the regulation of actin dynamics (Fig. 4), as well as for Ca^{2+} current expression (Fig. 2).

Our results show that both $Ca_V\beta2a$ and β2a-SH3 inhibited the incorporation of G-actin into filaments (Fig. 5). However, β2a-SH3 reduced Ca^{2+} current densities by itself, whereas, $Ca_V\beta2a$ reduced Ca^{2+} currents only when is co-injected with $Ca_V\alpha1$ I–II loop. These differences cannot be attributed to their ability to alter actin dynamics since the effects of $Ca_V\beta2a$ and β2a-SH3 on G-actin incorporation are not additive (Fig. 5). We should take into consideration that reduction in ionic currents occurs in relatively intact cells whereas assays on actin dynamics are carried out in digitonin-permeabilized cells. It is possible then that the intracellularly injected $Ca_V\beta2a$ undergoes post-translational modifications that hamper its ability to down-regulate actin dynamics. This modification may be lost in digitonin-permeabilized cells as a consequence of protein leakage (Sarafian *et al.,* 1987).

In previous studies, the role of the $Ca_V\beta$ subunits in chromaffin cells was investigated under their chronic deletion or sequestration, and therefore all stages of the Ca^{2+} channel biogenesis may have been compromised. This is the case of chronic ablation of the $Ca_V\beta3$ in knockout mice that reduced protein plasma membrane expression and current densities of both L and N Ca_V subtypes (Ohta *et al.,* 2010). Chronic sequestration of $Ca_V\beta$ subunits in BACC, by expressing a chimera that coupled the I–II loop of $Ca_V2.1$ to membranes was also shown to reduce over 50% the overall Ca^{2+} current density (Cuchillo-Ibañez *et al.,* 2003). Here, by injecting recombinant proteins and measuring current densities within 30–45 min, late stages of the channel life cycle are being intervened and the forward trafficking emerges as a major target for the regulation exerted by $Ca_V\beta2a$. At this moment, we have not yet studied whether this mechanism is extended to other Cavβ subunits. However, considering that the SH3 domain is a highly conserved region (Buraei and Yang, 2010), it is likely that other $Ca_V\beta$ subunits also regulate actin dynamics. Furthermore, this mechanism seems not to be extended to other types of SH3-containing proteins, as SH3 domains of cortactin and amphiphysin influenced Ca^{2+} current densities dissimilarly (Fig. S2). In contrast to β2a-SH3, cortactin-SH3 increases actin polymerization (González-Jamett *et al.,* 2017) and does not interfere with Ca^{2+} current expression (Fig. 2S). On the other hand, amphiphysin-SH3 by itself does not influence actin polymerization (Yamada *et al.,* 2009), but increases Ca^{2+} current densities (Fig. 2S). Then, the effects of the β2a-SH3 on actin dynamics and Ca^{2+} current densities seem to be unique and different to other SH3 domains.

To unveil the mechanism by which the β2a subunit remodels actin filaments it would be necessary to identify the motif in the β2a-SH3 domain that binds to actin or perhaps an actin-binding protein. Since we have no clue on the potential determinant of this interaction, an extensive mutagenesis scanning would have to be undertaken. Moreover, to assess the impact of these mutations on actin binding and channels' trafficking would require sophisticated imaging techniques such as FRET/BRET (Guhathakurta *et al.,* 2018). Nevertheless, our data support the idea that $Ca_V\beta2a$, via its SH3 domain, might constitute a regulatory molecule that controls the plasma membrane expression of ion channels by modulating actin remodeling. This process could be especially relevant for the function of excitable cells such as chromaffin cells.

Acknowledgments and conflict of interest disclosure

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ORIGINAL RESEARCH
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Pannexin-1 Channels Are Essential for Mast Cell Degranulation Triggered During Type I Hypersensitivity Reactions

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Mast cells (MCs) release pro-inflammatory mediators through a process called degranulation response. The latter may be induced by several conditions, including antigen recognition through immunoglobulin E (IgE) or "cross-linking," classically associated with Type I hypersensitivity reactions. Early in this reaction, Ca^{2+} influx and subsequent increase of intracellular free Ca^{2+} concentration are essential for MC degranulation. Several membrane channels that mediate Ca^{2+} influx have been proposed, but their role remains elusive. Here, we evaluated the possible contribution of pannexin-1 channels (Panx1 Chs), well-known as ATP-releasing channels, in the increase of intracellular Ca^{2+} triggered during cross-linking reaction of MCs. The contribution of Panx1 Chs in the degranulation response was evaluated in MCs from wild type (WT) and Panx1 knock out (Panx1$^{-/-}$) mice after anti-ovalbumin (OVA) IgE sensitization. Notably, the degranulation response (toluidine blue and histamine release) was absent in Panx1$^{-/-}$ MCs. Moreover, WT MCs showed a rapid and transient increase in Ca^{2+} signal followed by a sustained increase after antigen stimulation. However, the sustained increase in Ca^{2+} signal triggered by OVA was absent in Panx1$^{-/-}$ MCs. Furthermore, OVA stimulation increased the membrane permeability assessed by dye uptake, a prevented response by Panx1 Ch but not by connexin hemichannel blockers and without effect on Panx1$^{-/-}$ MCs. Interestingly, the increase in membrane permeability of WT MCs was also prevented by suramin, a P2 purinergic inhibitor, suggesting that Panx1 Chs act as ATP-releasing channels impermeable to Ca^{2+}. Accordingly, stimulation with exogenous ATP restored the degranulation response and sustained increase in Ca^{2+} signal of OVA stimulated Panx1$^{-/-}$ MCs. Moreover, opening of Panx1 Chs in Panx1 transfected HeLa cells increased dye uptake and ATP release but did not promote Ca^{2+} influx, confirming that Panx1 Chs permeable to ATP are not permeable to Ca^{2+}. These data strongly suggest that during antigen recognition, Panx1 Chs contribute to the sustained Ca^{2+} signal increase via release of ATP that activates P2 receptors, playing a critical role in the sequential events that leads to degranulation response during Type I hypersensitivity reactions.

Keywords: inflammation, immune cells, ovalbumin, pannexon, degranulation, histamine, ATP

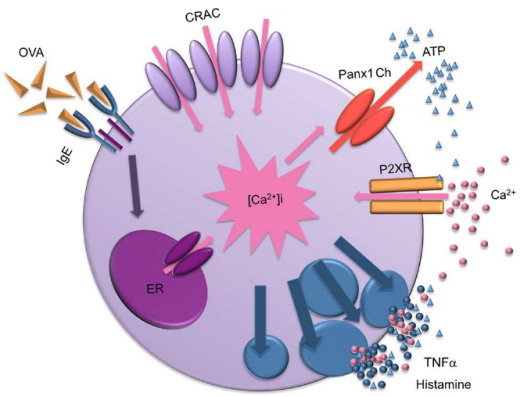

FIGURE 6 | Scheme of the mast cell degranulation process induced by IgE cross-linking and the involvement of Panx1 channels. After antigen recognition, biochemical events lead to Ca^{2+} release from intracellular stores likely to be the endoplasmic reticulum (ER), causing a transient increase in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]$). Then, Ca^{2+} released from the ER activates the Ca^{2+} influx through membrane channels (calcium released activates CRAC channels) slowly replenishing intracellular stores. Sustained increase in Ca^{2+} signal also favors the transient opening of Panx1 Chs, allowing the release of ATP to the extracellular media and activation of purinergic receptors (P2XRs). Active P2XRs contribute to the sustained increase of $[Ca^{2+}]_i$, promoting activation of Panx1 Chs and mast cell degranulation, leading to release of pro-inflammatory compound such as TNF-α and histamine. Finally, high extracellular ATP concentration could block Panx1 Chs.

Ca^{2+}-dependent protein phosphorylation (38). Accordingly, Panx1 has two threonine residues (Thr302 and Thr384) in consensus sites for calmodulin-dependent protein kinase II (38). Nevertheless, further studies are required to determine their possible participation in activation of Panx1 Chs. Interestingly, we found that the increase in membrane permeability to Etd via Panx1 Chs was transient (~2.5 min), which might be explained by inhibition of Panx1 Chs by a high extracellular ATP concentration due to opening of the same Panx1 Chs (19).

Consistent with the notion that Panx1 Chs are non-selective membrane channels, we found that exposure to extracellular alkaline solution, known to promote opening of fish Panx1 isoforms (32, 33), also induces opening of mammalian Panx1 Chs. The latter was revealed by the increase of ATP release and DAPI uptake inhibited by low (5 μM) carbenoxolone concentration that acts as Panx1 Ch blocker (34). However, in HeLa Panx1 cells, pH 8.5 did not affect the Ca^{2+} signal, suggesting that these channels did not allow Ca^{2+} influx in MCs.

Considering the fact that the alkaline solution could activates other Ca^{2+} permeable membrane channels (e.g., through ACIS, Piezol or TRP channels), we cannot rule out the possible involvement of other membrane channels activated by alkaline pH that could indirectly leads to activation of Panx1 Chs. However, in HeLa-Parental cells, we could not detect an increase in membrane permeability to DAPI or ATP release in response to alkaline pH, suggesting that these responses of HeLa transfectants are in fact mediated by Panx1 Chs and therefore indicate that Panx1 Chs are directly activated by alkaline pH.

In support to the above proposal, it is conceivable that P2X receptors activated by extracellular ATP released via Panx1 Chs participates in the plateau phase of the Ca^{2+} signal of sensitized MCs exposed to OVA. Accordingly, several P2X receptors are permeable to Ca^{2+} (38) and their involvement in the plateau phase of the Ca^{2+} signal is supported by the following findings: (1) the dye uptake response was promoted by 500 μM ATP, which is a concentration that activates P2X receptors (38). (2) The dye uptake induced by OVA was prevented by suramin, which blocks P2 receptors (38). (3) In Panx1$^{-/-}$ MCs, exogenous ATP bypassed the absence of Panx1, promoting histamine secretion and Ca^{2+} signal similar to that induced by OVA in WT MCs. As additional support, it is relevant to notice that P2X receptors do not desensitize upon activation by high extracellular ATP concentration (38), and in this way, their persistent open state can explain the long-lasting duration (>80 s) of the plateau phase of the Ca^{2+} signal. Accordingly, it has been shown that MCs express a wide variety of damage-associated receptors involved in Type I hypersensitivity reactions, like purinergic P2X receptors (39).

In summary, after OVA cross-linking recognition, characterized by (1) a rapid and transient Ca^{2+} signal, (2) followed by a sustained Ca^{2+} influx, we propose that Panx1 Chs indirectly contribute to the latter event, since they are not permeable to Ca^{2+}. In this context, Panx1 Chs opening leads to activation of a purinergic signal pathway through an autocrine process, as ATP released through Panx1 Chs could activate P2X receptors. These key events lead to degranulation and consequent histamine release to the extracellular milieu, a characteristic feature of Type I hypersensitivity reactions (**Figure 6**).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Comisión de Bioética y Bioseguridad de la Pontificia Universidad Católica de Chile.

AUTHOR CONTRIBUTIONS

PH and JS designed the experiments and wrote the paper. PH, XL, PS, PF, IB, and AM performed the experiments. PH analyzed the data.

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Clinical Research

Neutrophil Extracellular Traps Exert Potential Cytotoxic and Proinflammatory Effects in the Dental Pulp

Michelle J. Holder, BSc, Helen J. Wright, BSc, PhD,* Eduardo Couve, DDS,†‡ Michael R. Milward, BDS, PhD,* and Paul R. Cooper, BSc, PhD**

Abstract

Introduction: Neutrophil extracellular traps (NETs) are an important innate immune mechanism aimed at limiting the dissemination of bacteria within tissues and localizing antibacterial killing mechanisms. There is significant interest in the role of NETs in a range of infectious and inflammatory diseases; however, their role in diseased pulp has yet to be explored. Our aim was to determine their relevance to infected pulp and how their components affect human dental pulp cell (HDPC) responses. **Methods:** Diseased pulp tissue was stained for the presence of extracellular DNA and elastase to detect the presence of NETs. Bacteria known to infect pulp were also assayed to determine their ability to stimulate NETs. Coculture studies and NET component challenge were used to determine the effect of extracellular NET release on HDPC viability and inflammatory response. NET-stimulated HDPC secretomes were assessed for their chemotactic activity for lymphocytes and macrophages. **Results:** Data indicate that NETs are present in infected pulp tissue and whole NETs, and their histone components, particularly H2A, decreased HDPC viability and stimulated chemokine release, resulting in an attraction of lymphocyte populations. **Conclusions:** NETs are likely important in pulpal pathogenesis with injurious and chronic inflammatory effects on HDPCs, which may contribute to disease progression. Macrophages are chemoattracted to NET-induced apoptotic HDPCs, facilitating cellular debris removal. NETs and histones may provide novel prognostic markers and/or therapeutic targets for pulpal diseases. *(J Endod 2019;45:513–520)*

Key Words

Damage-associated molecular patterns, dentin-pulp complex, inflammation, polymorphonuclear leukocytes, pulp biology

Significance

Neutrophil extracellular traps are present in diseased pulp. Although their release is aimed at combatting bacteria, the associated histones may exert cytotoxic and proinflammatory effects. Levels of histones may serve as prognostic and therapeutic targets in pulpal diseases.

If unchecked, caries can progress to a deeper endodontic polymicrobial infection dominated by anaerobic bacteria that colonize the necrotic pulp (1, 2). The pulp's innate immune responses aim to combat this infection and are orchestrated by a plethora of cytokines. Neutrophils are subsequently chemoattracted in large numbers to the diseased pulp where they provide a first line of defense (3–5). They can be initially primed by cytokines or complement or bacterial components in the bloodstream during infection and subsequently exhibit increased longevity at the diseased site (6). Once in the pulp, neutrophils can phagocytose bacteria for intracellular killing, or they can degranulate, releasing reactive oxygen species (ROS) and antimicrobial proteins (AMPs), such as cathepsins, defensins, lactoferrin and lysozyme, for extracellular killing. Notably, the neutrophil trafficking process and extracellular killing mechanisms can cause significant host collateral tissue damage (7, 8).

In 2004, a novel mode of neutrophil-mediated pathogen containment and killing was identified and termed *neutrophil extracellular traps* (NETs). NETs are extracellular weblike structures containing decondensed nuclear chromatin adorned with antimicrobial molecules, including histones and AMPs (9). Electrostatic charge interactions between core DNA and the bacterial outer membrane are understood to enable bacterial entrapment, and killing is enabled via the colocalization of high concentrations of antimicrobial molecules (10). The induction of NETs requires complex signaling with stimuli including nitric oxide, cytokines, and Gram-positive/-negative bacterial components (11). NET release is regarded as a "last resort" killing mechanism because it represents a form of programmed cell death termed *NETosis*, which is distinct from apoptosis and necrosis (10). Neutrophil ROS generation underpins the signaling for NET production, and the activity of the calcium-dependent enzyme peptidyl arginine deiminase 4 (PAD4) is also essential. PAD4 citrullinates positively charged arginine residues in histones to neutrally charged citrulline, enabling DNA unpacking. Additionally, granule-derived neutrophil elastase can enter the nucleus and partially degrade histones, enabling binding of myeloperoxidase, which facilitates chromatin decondensation (12–14). The demonstration of neutrophil elastase colocalized with

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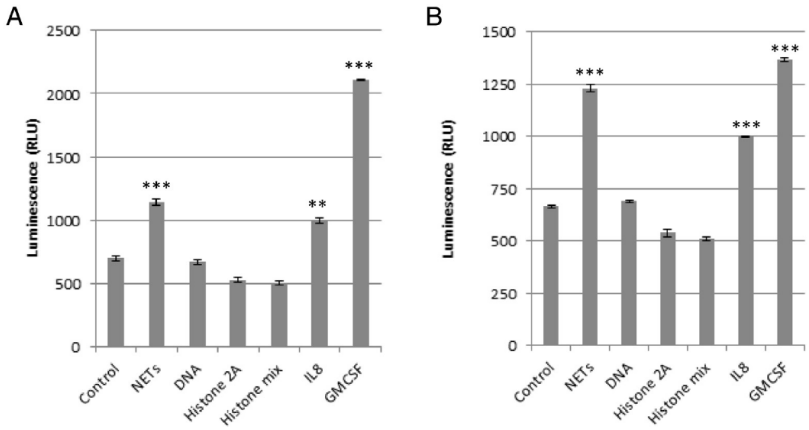

Figure 4. Chemotaxis assay for (*A*) whole lymphocyte preparations and (*B*) macrophages in response to HDPC-stimulated secretomes. Control supernatants from HDPC cultures collected at 24 hours were unexposed, whereas test secretomes were generated from NETs (10 μg/mL), histones (2A [100 μg/mL] and mix [100 μg/mL]), and calf thymus DNA (10 μg/mL). Positive controls for lymphocyte and macrophage chemoattraction were IL-8 (25 μg/mL) and granulocyte-macrophage colony-stimulating factor (25 μg/mL). The results are mean ± standard error (n = 3). Statistical significant increases versus unstimulated HDPC control secretomes are shown: $^{**}P < .01$ and $^{***}P < .001$.

(15). Several cell stress–associated events have now been shown to result in their extracellular discharge including apoptosis, necrosis, and, more recently, NETosis. When histones are released extracellularly, they are detected by Toll-like receptor (TLR) family members, including TLR2, TLR4, and TLR9, present on the local cell surfaces. Indeed, several TLR family members are already known to be expressed on HDPC populations (26). Receptor binding results in proinflammatory cytokine/chemokine release via MyD88, nuclear factor kappa B, and NLRP3 inflammasome-dependent and caspase-1 pathways (15). The data presented here now indicate that the histones released during NETosis exert cytotoxic and proinflammatory effects on HDPCs, findings that are consistent with previous studies in other cell systems. Notably, relatively high concentrations of histones have been detected in tissues from animals and patients with a range of diseases, and inadequate clearance of this form of molecular debris by macrophages reportedly leads to their accumulation (35). It is conceivable that similar processes could also occur here, which would lead to chronic inflammation within the pulp. Therefore, it is important for disease resolution that macrophage chemoattraction is signaled in the pulp, as has been identified here, which will lead to the phagocytotic removal of histones and other cellular and molecular debris, enabling a return to tissue homeostasis.

In conclusion, the data presented here support a role for NETosis in diseased and infected pulp. The release of NET DNA will also lead to the presence of DAMPs within the tissue, in the form of histones, which may also exert deleterious effects on HDPCs in the form of cytotoxicity and the stimulation of inflammatory responses. The signaling of macrophage recruitment by HDPCs may enable the removal of cell debris, which ultimately will facilitate resolution of inflammation. Extracellular histones are now being considered as prognostic and therapeutic targets for many infectious and inflammatory disorders (15); subsequently, further studies are now warranted to determine whether these molecules could be used for these purposes in the future for patients with deep caries or endodontic infections.

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Supplementary Material

Supplementary material associated with this article can be found in the online version at www.jendodon.com (https://dx.doi.org/10.1016/j.joen.2019.02.014).

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PROTOCOL

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Nongenetic optical neuromodulation with silicon-based materials

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Optically controlled nongenetic neuromodulation represents a promising approach for the fundamental study of neural circuits and the clinical treatment of neurological disorders. Among the existing material candidates that can transduce light energy into biologically relevant cues, silicon (Si) is particularly advantageous due to its highly tunable electrical and optical properties, ease of fabrication into multiple forms, ability to absorb a broad spectrum of light, and biocompatibility. This protocol describes a rational design principle for Si-based structures, general procedures for material synthesis and device fabrication, a universal method for evaluating material photoresponses, detailed illustrations of all instrumentation used, and demonstrations of optically controlled nongenetic modulation of cellular calcium dynamics, neuronal excitability, neurotransmitter release from mouse brain slices, and brain activity in the mouse brain in vivo using the aforementioned Si materials. The entire procedure takes ~4–8 d in the hands of an experienced graduate student, depending on the specific biological targets. We anticipate that our approach can also be adapted in the future to study other systems, such as cardiovascular tissues and microbial communities.

Introduction

Electrical neuromodulation constitutes the basis of many implantable medical devices that are valuable in treating debilitating medical conditions such as Parkinson's disease, clinical depression, and epilepsy[1–8]. Although traditional stimulation electrodes have substantially improved patients' quality of life, they are often limited by their need to be tethered to electrical wiring and their inability to target single cells. Moreover, the bulky and rigid nature of these electrodes can induce severe inflammation in target tissue[9–13]. Alternative neuromodulation methods using optical stimuli can significantly alleviate tissue inflammatory responses and offer greater flexibility with even single-cell resolution. Over the past decade, optogenetics, or genetic engineering that allows optical control of cellular activity, has been able to address many of the aforementioned issues with physical electrodes[14–17]. However, neuromodulation methods that rely on genetic modifications are technically challenging in larger-brained mammals, as they may produce unpredictable or even permanent side effects and are controversial if applied to human subjects. On the other hand, nongenetic photostimulation approaches could revolutionize the field of neuromodulation, especially when they are minimally invasive, tightly integrated with the target biological system, and able to achieve a high spatiotemporal resolution. Unlike optogenetics, which expresses light-gated ion channels or pumps on the cell membrane, nongenetic photostimulation does not alter the biological targets, but instead uses the transient physicochemical outputs from synthetic materials that are attached to the cells or tissues. When the material is illuminated, its light-induced electrical or thermal output yields temporary biophysical responses (e.g., membrane electrical capacitance increase) in neurons, producing a neuromodulation effect in close proximity to the material.

So far, several light-responsive materials have been utilized for optical neuromodulation, including quantum dots, gold (Au) nanoparticles, and semiconducting polymers, and these studies have already

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Author contributions
Y.J., R.P., X.L., J.L.C.-d.-S., F.B., G.M.G.S., and B.T. developed the protocol. Y.J., R.P., X.L., and J.L.C.-d.-S. performed the experiments. Y.J., R.P., X.L., and B.T. wrote the manuscript with input from J.L.C.-d.-S., X.G., L.M., F.B., and G.M.G.S.

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METHODS AND APPROACHES

Methodological improvements for fluorescence recordings in *Xenopus laevis* oocytes

Elizabeth E.L. Lee[1] and Francisco Bezanilla[1,2]

Xenopus laevis **oocytes are a widely used model system because of their capacity to translate exogenous mRNA, but their high intrinsic background fluorescence is a disadvantage for fluorescence recordings. Here, we developed two distinct methods for improving fluorescence recordings from oocytes. One was a pharmacological method in which a small-molecule salt-inducible kinase inhibitor was co-injected with the mRNA of interest to stimulate melanin production. We interrogated the oocytes using cut-open voltage clamp with simultaneous fluorescence recording and found that by increasing the amount of light-absorbing melanin in these oocytes, we decreased their intrinsic background fluorescence. The treated oocytes produced fluorescence signals that were approximately four times larger. The second method consisted of direct injection of synthetic melanin. This method also significantly improved (doubled) fluorescence signals and allowed any oocyte to be used for fluorescence recording. These two methods provide significant improvements of the signal quality for fluorescent oocyte recordings and allow all healthy oocytes to be used for high-sensitivity recordings.**

Introduction

Xenopus laevis oocytes are a model in vitro system for development, pharmacology, and electrophysiology (Gurdon et al., 1971). In particular, oocytes have been established as an important system to study membrane proteins and ion channels (Kusano et al., 1977). When voltage-clamp fluorimetry for oocytes was first introduced 20 yr ago (Mannuzzu et al., 1996; Cha and Bezanilla, 1997), it paved the way for novel optical interrogations of membrane proteins with simultaneous electrophysiology. However, optical measurements are currently limited in sensitivity by the oocyte's high intrinsic background fluorescence in the 300–500 nm wavelength range, thus decreasing the signal-to-noise ratio of recordings in this range.

Recent developments have allowed us to decrease the intrinsic fluorescence of the oocyte through melanin. Melanin is a general name for a group of small light-absorbing polymers. This dark-pigmented molecule plays a role in protection from UV radiation (Costin and Hearing, 2007). Melanin is a useful molecule for suppressing background fluorescence due to its wide range of absorbance (Fig. 4 A). It is well known that the WT expression of melanin in oocytes causes the animal pole to be darker visually and more light absorbent and thus have less fluorescent background compared with the lighter-colored vegetal pole. Thus, we sought to artificially increase the melanin content of the oocyte to further decrease the endogenous fluorescence signal in our recordings.

We demonstrate a novel drug-based method to decrease the endogenous background fluorescence of injected oocytes. A recent study found that the addition of a salt-inducible kinase (SIK) inhibitor increases melanin production in human melanocytes and in vivo in mice (Mujahid et al., 2017). This drug, HG 9-91-01, inhibits SIK2, which in turn stimulates melanogenesis-associated transcription factor, resulting in melanin production by melanosomes. *X. laevis* have homologues for both enzymes and transcription factors, and melanin production can be similarly regulated with HG 9-91-01.

We next demonstrate a second method to decrease endogenous background fluorescence through a direct injection of melanin. For this specific study, we have chosen to use neuromelanin, which is melanin created from the oxidation of dopamine (Zeise et al., 1992). A recent study using synthetic dopamine generated melanin as a photothermal method for treating cancer in vivo (Liu et al., 2013). We used this method of synthesizing melanin, but with the aim of reducing the fluorescence background to improve oocyte recordings. These methodological improvements offer many opportunities for better fluorescence recordings in oocytes.

Materials and methods

Oocyte preparation

Oocytes were harvested from *X. laevis* in accordance with experimental protocols approved by the University of Chicago Animal Care and Use Committee. Following collagenase digestion of the follicular membrane, oocytes were stored at 18°C in standard

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sible for this method to be applied to other experimental systems where endogenous fluorescence is a systematic problem, such as total internal reflection microscopy, single-molecule tracking, and fluorescence resonance energy transfer studies.

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Author contributions: E.E.L. Lee and F. Bezanilla designed experiments, E.E.L. Lee carried out experiments and analysis, and both authors contributed to data interpretation and writing of the paper.

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Current Opinion in Systems Biology

RNA language in *Caenorhabditis elegans* and bacteria interspecies communication and memory

Marcela Legüe[1,2] and Andrea Calixto[1,2]

Abstract

Bacteria were the first inhabitants on earth, and worms one of the earliest animals. Their mutual adaptation points to a remarkable genetic plasticity, underlying rapidly evolving molecular and behavioral strategies for long-term survival. Behavioral adaptive responses require the active communication between bacteria and the animal's intestine. After their encounter the *holobiont* changes at many levels, including global transcriptomic and epigenetic adaptations. RNA is a likely mediator of these changes because it is a mobile molecule capable of messaging complex and precise information to distant targets and across generations. *Caenorhabditis elegans* and their commensal and pathogenic bacteria are a good model to study the components and dynamics of interspecies RNA communication *in vivo* and across generations. We focus on current research on extracellular RNA transport and uptake, and in putative ways in which exogenous RNA can affect global transcriptomic and epigenomic profiles in other species, highlighting its role in interspecies communication and memory. Some epigenetic effectors are sRNA-mediated which opens a complex landscape of possible pathways. We advocate the idea that the export, travel and import of small RNAs between bacteria and host is an active and targeted process, and there is increasing evidence that points in that direction. Some mechanistic proposals and future directions are outlined.

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This review comes from a themed issue on **Systems biology of model organisms**

Edited by **Denis Dupuy** and **Baris Tursun**

For a complete overview see the Issue and the Editorial

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Keywords

RNA, Transgenerational, *C. elegans*, Bacteria, Epigenetic.

Introduction: bacteria worm holobiont

In his book *Nematodes and Their Relationships*, Nathan Cobb wrote, "if all the matter in the universe except the nematodes were swept away, our world would still be dimly recognizable" [1]. This phrase alludes to the omnipresence of worms; capable of inhabiting hot springs, polar ice, soil, fresh and salty water, and as deep as 1.4 km under Earth surface in the gold mine of Kopanang in South Africa [2]. The vast majority is free living, feeding on a variety of microbes, mainly bacteria. Bacteria were the first inhabitants on earth, since at least 3.5 billion years and were likely to contribute to the origin and evolution of animals [3]. Even though the origin of metazoan is uncertain, worm-like fossil records suggest that nematodes existed as early as in the Precambrian supereon [4]. In this context, nematodes and bacteria have had time to rehearse, improve, adapt and learn strategies to live with one another and are an excellent "*holobiont*" model [5] to study interspecies relationships.

Here we discuss the relationships maintained by the bacterivore nematode *Caenorhabditis elegans* and bacteria it feeds on and defends from, the molecular mechanisms underlying this interaction and the reciprocal influences in transcriptional programs with focus on small RNAs. We review possible ways by which this complex interaction leaves a long-lasting memory in the worm and is inherited by their progeny.

Symbiosis and survival strategies

The success of Nematoda in populating virtually every niche resides in part in their ability to establish productive relationships with bacteria populating those environments. Their mutual adaptation points to a remarkable genetic plasticity, underlying rapidly evolving molecular and behavioral strategies for long-term survival of both communities.

In nature *C. elegans* lives on the soil and feeds on a great diversity of commensal and pathogenic bacteria, which provide nutrients and can also cause disease. Bacterial particles enter the buccal cavity of the worm and move toward the intestine pushed by a cycle of contraction relaxation of the pharyngeal muscle. The grinder located in the posterior part of the pharynx breaks standard laboratory bacteria, allowing the debris to pass to the intestine [6,7]. It has however been demonstrated that other bacteria are capable of passing intact through the grinder and reproduce in the worm's intestine [8], especially pathogens [9–11]. Then, the form in which bacterial contents are made accessible to the cells of the intestinal lumen can be either passive by the grinding of

faded. Other transcriptional changes persist shortly, likely due to passive mechanisms such as gene loop formation [73]. Epigenetic regulation is that which remains stable through mitosis, hence persists after the disappearance of the initiating signal [74,75]. Even more, upon a certain trigger threshold (duration, magnitude or biological relevance) the memory can last in a multigenerational or even transgenerational mode.

Transgenerationality requires germ line transmission of epigenetic information in the absence of direct environmental exposure [76]. This inheritance requires actively passing the Weismann soma-germline barrier [77], which has been shown to be achieved by vesicles containing RNA, retrotransposons, sRNAs and viruses [34,78–81]. Small RNAs are epigenetic effectors able to sustain transgenerationality [82] mainly secondary and tertiary siRNAs that interact with Argonaute proteins, and Piwi-interacting RNAs. sRNAs influence the formation of boundaries blocking the spread of heterochromatin, one of the most stable epigenetic marks [25]. Histone modifications affect sRNA dynamics: while H3K9me3 does not mediate gene silencing [83], MET-2 mediated methylation of histones restricts the perpetuation of the RNAi response [84]. An example of transgenerationally-inherited behavior is the *C. elegans* entry into diapause (dauers) to avoid pathogenesis [9]. Dauers form in the second generation of animals exposed to pathogens revealing the need for the accumulation of information about the pathogenic nature of bacteria before the behavioral change takes place. Dauer formation is tightly regulated, for it delays reproduction. This defensive strategy depends on RNAi effectors at diverse levels from environmental to cell autonomous and is transmitted transgenerationally through the maternal germline. This mechanism is adaptive for survival in conditions were pathogens are the only food source. Importantly for adaptation, in absence of pathogens this behavior is maintained for a number of generations and later forgotten [9].

Conclusions

RNA is the most ancient of macromolecules existing in all forms of life. Since the primordial RNA world, it has perpetuating information being both the carrier and the message. Sophisticated mechanisms that selectively filter RNA-coded information about environmental cues, provide a finely tuned and complex language that allows fitness in a multilevel and time-dependent manner.

One of the main challenges of an organism is to correctly interpret this language to preserve homeostasis and perpetuate and increase descendant's fitness in the encounter with other species. From the vast variety of molecules implicated in interspecies crosstalk, small RNAs offer an exquisitely precise communication means.

The interaction between *C. elegans* and the bacteria it eats provide a simple pair to thoroughly dissect the components of this crosstalk, which can be escalated to multi-species studies. Future directions include *i)* evidencing the mechanisms of sRNA transport *in vivo* and *ii)* discovering sRNA novel functions in target cells and unveil the sRNA-triggered epigenetic landscape modification.

Deciphering the RNA language in interspecies and transgenerational memory is a fascinating field that compels us to integrate diverse disciplines such as evolutionary biology, microbiology, bioinformatics, epigenetics and others with a systemic biology perspective.

Conflict of interest statement

Nothing declared.

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ORIGINAL RESEARCH ARTICLE

Connexin 43 deletion in astrocytes promotes CNS remyelination by modulating local inflammation

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Abstract

As the most abundant gap junction protein in the central nervous system (CNS), astrocytic connexin 43 (Cx43) maintains astrocyte network homeostasis, affects oligodendroglial development and participates in CNS pathologies as well as injury progression. However, its role in remyelination is not yet fully understood. To address this issue, we used astrocyte-specific Cx43 conditional knockout (Cx43 cKO) mice generated through the use of a hGFAP-cre promoter, in combination with mice carrying a floxed Cx43 allele that were subjected to lysolecithin so as to induce demyelination. We found no significant difference in the demyelination of the corpus callosum between Cx43 cKO mice and their non-cre littermate controls, while the remyelination process in Cx43 cKO mice was accelerated. Moreover, an increased number of mature oligodendrocytes and an unaltered number of oligodendroglial lineage cells were found in Cx43 cKO mouse lesions. This indicates that oligodendrocyte precursor cell (OPC) differentiation was facilitated by astroglial Cx43 depletion as remyelination progressed. Underlying the latter, there was a down-regulated glial activation and modulated local inflammation as well as a reduction of myelin debris in Cx43 cKO mice. Importantly, 2 weeks of orally administrating boldine, a natural alkaloid that blocks Cx hemichannel activity in astrocytes without affecting gap junctional communication, obviously modulated local inflammation and promoted remyelination. Together, the data suggest that the astrocytic Cx43 hemichannel is negatively involved in the remyelination process by favoring local inflammation. Consequently, inhibiting Cx43 hemichannel functionality may be a potential therapeutic approach for demyelinating diseases in the CNS.

KEYWORDS
boldine, connexin, glial cells, hemichannel, lysolecithin, oligodendrocyte

1 | INTRODUCTION

Remyelination by newly generated oligodendrocytes (OLs), a process which includes the recruitment and differentiation of oligodendrocyte precursor cells (OPCs), is vital for rebuilding myelin structure when the myelin sheath is destroyed (Franklin & Ffrench-Constant, 2008). Based on current clinical knowledge, OPC differentiation in a demyelinated environment is frequently disturbed, and because of this the remyelination process fails (Chang, Tourtellotte, Rudick, & Trapp, 2002; Kuhlmann et al., 2008). Therefore, identifying the

compared to WT mice. These results indicate that astrocytic Cx43 hemichannels are negatively related to myelin regeneration in terms of contributing to the inflammatory process during myelin injury. Finally, this finding was strengthened by chronic administration of boldine, a natural alkaloid, which prevented Cx hemichannel activity from increasing without impacting gap junction communication in astrocytes. This compound mimics the effects of Cx43 cKO, although only hemichannel activity is inhibited in glia (Yi et al., 2017).

In the present study, a "friendlier" local inflammation level favors OPC differentiation into mature OLs, and the remyelination process in the lesion area. Previous studies have shown that, after myelin damage, microglia and astrocyte activation has both detrimental and beneficial effects on myelin repair (Domingues, Portugal, Socodato, & Relvas, 2016). The classic role of reactive astrocytes and/or activated microglia is favoring OPC recruitment and the clearance of myelin debris (Kotter et al., 2006). However, a number of works have indicated that excessive inflammation exacerbates damage to surrounding cells (Bar & Barak, 2019; Domingues et al., 2016; Kiray et al., 2016). Reactive astrocytes are capable of modifying the extracellular matrix in MS by secreting different components, such as FGF-2, hyaluronan and endothelin-1, which can directly affect remyelination in MS lesions (Chang et al., 2012; Hammond et al., 2014; Holley, Gveric, Newcombe, Cuzner, & Gutowski, 2003). Moreover, activated microglia hinder OPC differentiation by inducing nitric oxide-dependent oxidative damage and TNF-α-induced toxicity (Pang et al., 2010). In accordance with these findings, studies analyzing the brain tissue of postmortem MS patients have found that cortical demyelinated lesions with fewer reactive astrocytes always show greater remyelination (Chang et al., 2012). A recent study of in vitro hypoxia revealed that the application of astrocytic Cx43 blockers suppresses the release of glutamate associated with hemichannel activity, attenuates astrocyte activation and results in improved OPC differentiation (Wang et al., 2018). Cx43 hemichannels are activated under certain conditions, such as Alzheimer's Disease or lysolecithin-induced myelin injury used in our study, leading to the release of ATP and glutamate into the extracellular medium (Yi et al., 2016). The released ATP can activate the purinergic receptor P2RX7, which results in astrocyte and microglia activation (Huang et al., 2012). The above processes are expected to form a vicious cycle, resulting in excessive inflammation after myelin damage. In this context, our work showed that astrocytic Cx43 cKO reduced the activation levels of astrocyte and microglia and down-regulated the expression of proinflammatory cytokines (IL-1β, TNF-α), suggesting that M1 microglial cell/macrophage activation was inhibited. Furthermore, we found that astrocytic Cx43 deletion resulted in more Arg1 expression and likely favor the generation of M2 microglia/macrophages, which has been shown to be positively involved in the phagocytosis of myelin debris and the secretion of factors beneficial for OPC differentiation and remyelination (Miron & Franklin, 2014). In agreement with that, our results revealed that the myelin debris in the lesion areas was reduced after astrocytic Cx43 deletion. It is worth to note that although IL-1β has been shown to be cytotoxic to mature OLs in vitro (Merrill, 1991), there is also evidence that IL-1β deletion induces a delay of OPC differentiation and impaired myelin repair (Mason, Suzuki, Chaplin, & Matsushima, 2001). Accordingly, the remaining lower amount of IL-1β in our model may contribute to the myelin repair process. Meanwhile, we did not observe any increases in relative neurotrophic factors that could be secreted by M2 microglia/macrophages. One explanation of the latter is that such neurotrophic factors could also be generated from astrocytes or neurons (Verkhratsky & Nedergaard, 2018), which were decreased after Cx43 cKO. Taken together, modulating microglia/macrophages to be pro-regenerative (increased M2) and limiting the secretion of toxic molecules (decreased M1) by deletion of Cx43 in astrocytes may enhance the clearance of myelin debris, thus could facilitate OPC differentiation and remyelination.

Regarding the underlying action of astrocytic Cx43 in this model, we demonstrated that the observed effect was probably caused by a lack of hemichannel activity rather than gap junctional communication, and this was observed by applying a selected astrocytic hemichannel blocker (Yi et al., 2017). Particularly, our previous study showed that astrocytic Cx43, under physiological conditions, is responsible for the maintenance of extracellular glucose levels, which are critical for OPC proliferation (Niu et al., 2016). Moreover, myelin maintenance and formation can also be substantially influenced by the expression of Cx43 and Cx30 in astrocytes, as shown in mice in which Cxs produced by astrocytes and OLs are knocked out (Kiray et al., 2016). Although Cx43 may play a role in metabolic support for the recruitment of OPCs, hemichannel-mediated inflammation during remyelination seems to play a dominant role in the inhibition of remyelination.

Accumulating studies of other types of CNS injuries have found that the inhibition of Cx43 expression or its channel functions (the function of both hemichannel and gap junction channel) can protect neurons from cell death and improve functional recovery (Chew et al., 2010). Based on current studies, Cx43 gap junctional coupling may exacerbate injury by allowing for the spread of toxins and death signals. Thus, the functional loss of Cx43 gap junction channels may also contribute to improve the remyelination observed in Cx43 cKO mice. At the same time, it is believed that CNS injury is a dynamic process in which the role of astrocytic Cx43 channels varies over time. Cx43 gap junctions seem to be required for neuroprotection and maintenance of CNS homeostasis in later injury stages (Frantseva, Kokarovtseva, & Perez Velazquez, 2002). Therefore, future studies are needed to clarify the functional time window of Cx43 channels. In addition, the role of another astrocytic hemichannel subtype formed by pannexin1, which also takes part in the inflammatory process and may be blocked by boldine (Yi et al., 2017), should be taken into account in future studies.

In conclusion, the present study identified astrocytic Cx43 hemichannel activity as a crucial factor in modulating the inflammatory process during myelin injury, hence providing favorable conditions for OPC differentiation and remyelination. Consequently, inhibition of Cx43 hemichannel activity may be a potential therapeutic strategy for modulating the pathological environment and promoting myelin repair.

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Short communication

Opposing action of NCoR1 and PGC-1α in mitochondrial redox homeostasis

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ABSTRACT

The ability to respond to fluctuations of reactive oxygen species (ROS) within the cell is a central aspect of mammalian physiology. This dynamic process depends on the coordinated action of transcriptional factors to promote the expression of genes encoding for antioxidant enzymes. Here, we demonstrate that the transcriptional coregulators, PGC-1α and NCoR1, are essential mediators of mitochondrial redox homeostasis in skeletal muscle cells. Our findings reveal an antagonistic role of these coregulators in modulating mitochondrial antioxidant induction through *Sod2* transcriptional control. Importantly, the activation of this mechanism by either PGC-1α overexpression or NCoR1 knockdown attenuates mitochondrial ROS levels and prevents cell death caused by lipid overload in skeletal muscle cells. The opposing actions of coactivators and corepressors, therefore, exert a commanding role over cellular antioxidant capacity.

1. Introduction

Mitochondria are dense double membrane organelles known for their ability to produce ATP from the oxidation of carbon substrates. They also play a crucial role in the maintenance of redox homeostasis by controlling reactive oxygen species (ROS) levels produced from the oxidation of NADH and $FADH_2$ at the electron transport chain [1]. Therefore, mitochondrial redox and oxidative signaling pathways must be tightly synchronized to adapt mitochondrial function to homeostatic fluctuations within the cell. Recent evidence demonstrated that the signaling axis that controls oxidative metabolism in skeletal muscle involves an antagonistic action between the coactivator PGC-1α and the corepressor NCoR1 on PPARβ/δ and ERRα transcriptional activity [2,3]. However, the mechanism by which these coregulators act on mitochondrial redox homeostasis is poorly understood.

The shift from transcriptional repression to activation is fine-tuned by the association with coactivators and the dismissal of corepressors on nuclear receptors (NRs). The corepressor NCoR1 represses gene expression when the complex is bound to NRs [4]. Although NCoR1 have been shown to control mitochondrial biogenesis through its action on PPARs and ERRs [5,6], it is unknown whether NCoR1 regulates mitochondrial redox homeostasis. Here we show that both PGC-1α and NCoR1 govern the antioxidant status in skeletal muscle cells acting as activator and repressor of *Sod2* expression, respectively. This mechanism is essential for the cellular antioxidant response and protects skeletal muscle cells against lipid-induced ROS generation and cell death.

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mitochondrial redox regulation in skeletal muscle cells. The first evidence of this mechanism came from the analysis of *Sod2* promoter where we identified a strong enrichment of NCoR1. When cells are exposed to high ROS levels, NCoR1 dissociates, and PGC-1α binds at *Sod2* promoter. The functional consequence of this mechanism converges in Sod2 gene expression and reduction of ROS levels. Interestingly, this mechanism revealed to be conserved through evolution as silencing the NCoR1 homolog *gei-8* in *C. elegans* also activates mitochondrial *Sod* gene.

The PPAR family of nuclear receptors has been shown to act as key transcriptional regulators of antioxidant defense genes [17]. Our study suggests a mechanism where mitochondrial ROS detoxification system is regulated by a molecular switch that depends on the exclusive exchange of coregulators on PPREs located at the *Sod2* core promoter. This hypothesis is supported by the opposing effect of PGC-1α and NCoR1 on PPRE transactivation and *Sod2* promoter induction. Also, silencing PPARβ, the main PPAR isoform in skeletal muscle, completely prevents *Sod2* promoter activation by PGC-1α indicating that PPARβ is a critical mediator of the coregulator action on *Sod2* gene. These data are in accordance with previous studies showing that coactivators and corepressors act on the same hydrophobic pocket on nuclear receptors suggesting that coregulators exchange is mutually exclusive [18,19]. Importantly, our results revealed that turning on this switch by either silencing NCoR1 or overexpressing PGC-1α attenuates lipid-induced mitochondrial ROS production and cell death in skeletal muscle cells. Thus, this mechanism offers a potential signaling interface that could be explored in pathological conditions associated with oxidative and metabolic stress in skeletal muscle.

5. Conclusions

In summary, we demonstrated that the transcriptional coregulators PGC-1α and NCoR1 are essential mediators of mitochondrial redox homeostasis in skeletal muscle cells. Our findings revealed that they have antagonistic roles in mediating mitochondrial antioxidant capacity through *Sod2* transcriptional control. Pharmacological inhibition of NCoR1 interaction with selected nuclear receptors may be an interesting approach to boost muscle antioxidant defense. This regulatory axis in which the balance of coactivator and corepressor determine the mitochondrial redox status brings out a novel therapeutic opportunity for the treatment of metabolic diseases such as obesity and type 2 diabetes.

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Appendix A. Supplementary data

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RESEARCH ARTICLE

Calcium-driven regulation of voltage-sensing domains in BK channels

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Abstract Allosteric interactions between the voltage-sensing domain (VSD), the Ca^{2+}-binding sites, and the pore domain govern the mammalian Ca^{2+}- and voltage-activated K^+ (BK) channel opening. However, the functional relevance of the crosstalk between the Ca^{2+}- and voltage-sensing mechanisms on BK channel gating is still debated. We examined the energetic interaction between Ca^{2+} binding and VSD activation by investigating the effects of internal Ca^{2+} on BK channel gating currents. Our results indicate that Ca^{2+} sensor occupancy has a strong impact on VSD activation through a coordinated interaction mechanism in which Ca^{2+} binding to a single α-subunit affects all VSDs equally. Moreover, the two distinct high-affinity Ca^{2+}-binding sites contained in the C-terminus domains, RCK1 and RCK2, contribute equally to decrease the free energy necessary to activate the VSD. We conclude that voltage-dependent gating and pore opening in BK channels is modulated to a great extent by the interaction between Ca^{2+} sensors and VSDs.
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Introduction

Diverse cellular events involve calcium ions as a primary mediator in the signal transduction pathways triggering, among other signaling processes, Ca^{2+}-activated conductances. Since the BK channels are regulated by cytosolic Ca^{2+} and depolarizing voltages (***Marty, 1981***; ***Pallotta et al., 1981***; ***Latorre et al., 1982***), they are integrators of physiological stimuli involving intracellular Ca^{2+} elevation and membrane excitability. BK channels are modular proteins in which each module accomplishes a specific function. Thus, different modules harbor voltage and Ca^{2+} sensors that communicate allosterically with the channel gate (***Cox et al., 1997***; ***Horrigan and Aldrich, 1999***; ***Horrigan and Aldrich, 2002***; ***Horrigan et al., 1999***; ***Rothberg and Magleby, 1999***; ***Rothberg and Magleby, 2000***; ***Cui and Aldrich, 2000***). Functional BK channels are formed by homotetramers of α-subunits (***Shen et al., 1994***), each comprising a transmembrane voltage-sensing domain (VSD) and an intracellular Ca^{2+}-sensing C-terminal domain (CTD) that can independently modulate the ion conduction gate in the pore domain (PD) (***Latorre et al., 2017***). The CTDs consist of two non-identical regulators of the conductance of K^+ domains (RCK1 and RCK2) arranged in a ring-like tetrameric structure dubbed the gating ring (***Wu et al., 2010***; ***Yuan et al., 2010***; ***Yuan et al., 2012***; ***Hite et al., 2017***; ***Tao et al., 2017***). Each RCK domain contains distinct ligand-binding sites capable of detecting Ca^{2+} in the micromolar range (***Schreiber and Salkoff, 1997***; ***Bao et al., 2002***; ***Xia et al., 2002***).

In the absence of Ca^{2+}, the activation of the VSD decreases the free energy necessary to fully open the BK channels in an allosteric fashion (***Horrigan and Aldrich, 1999***; ***Horrigan et al., 1999***). With 0 Ca^{2+}, very positive membrane potentials are required to drive all voltage sensors to their active conformations (***Cui et al., 1997***; ***Stefani et al., 1997***; ***Horrigan et al., 1999***; ***Contreras et al.,***

values (***Akaike, 1974***), calculated as $\mathrm{AIC}_i = 2p_i - 2\ln(L_i)$, where p_i is the number of free parameters and $\ln(L_i)$ is the maximum log-likelihood of the model i. The best fit being the one that achieves the lowest AIC_i value. Minimum AIC_i ($\mathrm{AIC}_{\mathrm{MIN}}$) values were used as model selection criteria. Using the AIC_i weights (w_i), we estimated the probability of model i is the best model given the data and the set of candidate models. w_i are based on the relative likelihood of each tested model i which is a function of the difference in AIC_i score and the best model: $\Delta\mathrm{AIC}_i = \mathrm{AIC}_i - \mathrm{AIC}_{\mathrm{MIN}}$ (***Anderson and Burnham, 2002***). From the $\Delta\mathrm{AIC}_i$ we obtained an estimate of the relative likelihood of model i ($\mathfrak{L}_i$) by the simple transform: $\mathfrak{L}_i = exp\left(-\frac{1}{2}\,\Delta\mathrm{AIC}_i\right)$. w_i is calculated normalizing the $\mathfrak{L}_i$ for each model: $\mathrm{w}_i = \mathfrak{L}_i / \sum_{k=1}^{K} \mathfrak{L}_k$, where K is the number of candidate models.

The models for the Ca^{2+}-VSD interaction schemes were extended including two high-affinity Ca^{2+}-binding sites per α-subunit (***Figure 3—figure supplement 1D-E***). The contribution of each Ca^{2+}-binding site to the free energy of the voltage sensor equilibrium may be split in two, such as $E = E_{S1} * E_{S2} = e^{-\left(\Delta\Delta G_V^{Ca}(S1) + \Delta\Delta G_V^{Ca}(S2)\right)/RT}$, where E_{S1} and E_{S2} are the allosteric factors E for the RCK1 and RCK2 sites. Thus, for the global fit of the $Q_C(V, [\mathrm{Ca}^{2+}])$ curves, we constrained the allosteric parameter E_{S1} and E_{S2} obtained experimentally for the RCK2 Ca^{2+}-sensor mutant (5D5A) and RCK1 Ca^{2+}-sensor mutant (D362A/D367A), respectively, as described above. The rest of the parameters z_J, J_0, K_{D1}, K_{D2}, and G, where K_{D1} and K_{D2} are the dissociation constants of the RCK1 and RCK2 sites and G is a cooperativity factor between the two sites within the same α-subunit of the BK channel, were allowed to vary freely.

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Neurophysiological Muscle Activation Scheme for Controlling Vocal Fold Models

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***Abstract*—A physiologically-based scheme that incorporates inherent neurological fluctuations in the activation of intrinsic laryngeal muscles into a lumped-element vocal fold model is proposed. Herein, muscles are activated through a combination of neural firing rate and recruitment of additional motor units, both of which have stochastic components. The mathematical framework and underlying physiological assumptions are described, and the effects of the fluctuations are tested via a parametric analysis using a body-cover model of the vocal folds for steady-state sustained vowels. The inherent muscle activation fluctuations have a bandwidth that varies with the firing rate, yielding both low and high frequency components. When applying the proposed fluctuation scheme to the voice production model, changes in the dynamics of the system can be observed, ranging from fluctuations in the fundamental frequency to unstable behavior near bifurcation regions. The resulting coefficient of variation of the model parameters is not uniform with muscle activation. The stochastic components of muscle activation influence both the fine structure variability and the ability to achieve a target value for pitch control. These components can have a significant impact on the vocal fold parameters, as well as the outputs of the voice production model. Good agreement was found when contrasting the proposed scheme with prior experimental studies accounting for variability in vocal fold posturing and spectral characteristics of the muscle activation signal. The proposed scheme constitutes a novel and physiologically-based approach for controlling lumped-element models for normal voice production and can be extended to explore neuropathological conditions.**

***Index Terms*—Muscle activation, larynx, vocal folds, voice.**

I. Introduction

Phonation is the primary physiological process of speech production, in which the coordinated activation of breathing and laryngeal muscles controls the interaction of airflow, the vibratory activity of the vocal folds (VF), and sound. Phonation determines distinctive features of speech production, defining the fundamental frequency (f_o), amplitude, quality, and temporal patterns of vocalization.

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A significant amount of data describing voice production from research and clinical perspectives have been collected in the past few decades using imaging and acoustic signal recording techniques. These efforts have resulted in mathematical models able to reproduce different aspects of the phonatory process in normal physiological conditions. Of particular value has been the development of lumped-element models of the VFs, since they can efficiently represent a wide range of gestures and voice qualities, including the self-oscillating modal response of the vibrating VFs [1] [2] [3]. These lumped-element models can be coupled with models of aerodynamic interactions and acoustical features, thus forming a complete framework able to simulate the transmission and propagation of acoustic waves within the vocal tract, the subglottal system, and the VF tissue. Reduced order VF models can also mimic complex pathological phenomena, including incomplete glottal closure [4] and nerve paralysis [5], which opens the possibility of using these models in the diagnosis and treatment of VF pathologies [6], [7]. However, a number of gaps need to be filled before VF modeling can be established as a viable and robust clinical tool. Although efforts to accurately represent an individual patient in a modeling framework have been performed recently [8], [9], [10], a reliable representation of the inter-subject variability inherent in the clinical population has not yet been achieved.

Titze and Story [11] proposed a set of rules to unfold the physiological relationship between laryngeal muscle activation and VF configuration for reduced order models of the VFs [11]. However, there are numerous assumptions in that relevant study that need to be revisited. For instance, the effect of antagonistic muscles is overly simplified and the number of intrinsic laryngeal muscles that the scheme effectively controls is reduced to the thyroarytenoid (TA) and cricothyroid (CT) muscles. Their assumption of simplifying the effect of lateral cricoarytenoid (LCA) and posterior cricoarytenoid (PCA) muscles in a single activation signal reduces the neurological relevance of the adduction process of the VFs. More importantly for the present study, the method by which the muscles are activated does not have a neural basis, which in turn results in fixed, deterministic muscle activation values. These limitations reduce the physiological and clinical relevance of lumped-element VF models, making it difficult to correctly replicate gestures that depend on muscle activation, like phonation onset and offset, among others. Also, disordered speech motor control (e.g., Parkinson's disease, spasmodic dysphonia) cannot be properly represented with fixed muscle activation.

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contrast with studies assessing acoustic perturbations, where resulting jitter and shimmer from the BCM output were in the normal range and had an increasing behavior with frequency, matching prior observations [33], [4]. All these comparisons have limitations that need to be pointed out. The *in vivo* canine experiments from Vahabzadeh *et al.* [26] do not exactly match the anatomical conditions and type of nerve stimulation procedure in our human model, thus affecting mean values and their variability. In terms of the acoustic perturbations, we acknowledge that changes in the neural drive are not the only source that induces variability. Other factors (acoustic, aerodynamic, biomechanical) can result in significant changes in these measures. Furthermore, experiments with excised larynx phonation in neurally dead specimens indicate that vocal perturbation is present without fluctuations in the neural drive [22], [25]. It is interesting to note that the graded artificial stimulation used in these studies does not generate a voice pattern that corresponds to unnatural voice. We could hypothesize that the graded electrical input introduced to the laryngeal nerves in these canine experiments could be described through a deterministic (periodic) MU firing rate contracting the laryngeal muscles using a similar framework as the one proposed in this study, although further research would be needed to relate the electrical nerve activation to the MU constriction.

On the other hand, there is evidence in which twitch variations affect perturbations in the voice [30], particularly in the fundamental frequency. The simulations presented in this study support the idea that small variations in muscular activity can yield perturbations in the voice. One aspect that remains to be explored is how these fluctuations affect phonation stability near bifurcation zones, information that could be useful for modeling voice breaks or tremors. It is important to emphasize that our simulations were obtained for steady state vowels, given that previous studies have illustrated that the neural drive of the muscle activation changes during phonation onset but not in the same way than the resulting acoustic perturbations [52], [19], [47]. Phonation onset has added complexity given the inertial effects in the muscle dynamics [56] that are not captured by the current rules of reduced order models [11], and measures such as the relative fundamental frequency [57] may be more appropriate to assess the variability in pitch than traditional acoustic perturbations.

Future efforts will be devoted to exploring the neural effects of antagonistic muscles and extending the rules for controlling a triangular body-cover model [7]. In addition, a long-term goal of this work is to replicate the neural variability of common muscle-related pathologies like Parkinson's disease. In the case of Parkinson's disease, neurons exhibit an intricate pattern of inhibition and excitation, which leads to altered firing rate patterns [58]. The proposed scheme could potentially replicate this behavior and therefore serve as a starting point to construct a physiologically-relevant model of Parkinson voices, which is currently lacking. There are also other applications of the proposed muscle activation scheme, e.g., a model of the vocal tract that inherent neural fluctuations. Finally, it would be of value to design a comprehensive validation framework of the proposed stochastic muscle activation scheme with intramuscular EMG measurements of intrinsic laryngeal muscle activity of human subjects during phonation, though it could be quite invasive and complex.

V. Conclusion

The present study introduces a neurophysiological muscle activation scheme for intrinsic laryngeal muscles. It is designed to capture the essential characteristics of muscle control, providing an activation signal for use in numerical models of the vocal folds. The resulting muscle activation is controlled by the neural firing rate of the different MUs, therefore establishing a link between the nervous system and laryngeal muscle control. Synaptic stochasticity present in the neuronal input of the MU arises from the temporal and spatial summations that govern superposition of muscle twitches and MU recruitment, respectively. As a result, the muscle response has frequency content centered around both the firing rate and its harmonics, as well as a low-frequency DC component. These components influence both the fine structure variability of the signal, as well as the ability to achieve a target mean activation value for pitch control. The proposed scheme is integrated into a body-cover model of the vocal folds to assess the impact of muscle activation variability on overall laryngeal control. Along with muscle activation rules, neural firing rate becomes a novel control parameter that offers a natural, physiologically-based, framework to govern vocal fold properties. Fluctuations arise in the vocal fold model parameters, which in turn result in measurable changes in the model output. These changes are in agreement with prior experimental studies accounting for changes in vocal fold posturing, spectral characteristics of the muscle activation signal, and perturbations in the fundamental frequency. The variability in the resulting output is not a simple function of one muscle, but exhibits complex interactions between intrinsic laryngeal muscles.

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Ca^{2+}-Independent and Voltage-Dependent Exocytosis in Mouse Chromaffin Cells

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Conflict of interest

The authors have no conflicts of interest to declare.

PeerJ

An update on anatomy and function of the teleost olfactory system

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ABSTRACT

About half of all extant vertebrates are teleost fishes. Although our knowledge about anatomy and function of their olfactory systems still lags behind that of mammals, recent advances in cellular and molecular biology have provided us with a wealth of novel information about the sense of smell in this important animal group. Its paired olfactory organs contain up to five types of olfactory receptor neurons expressing OR, TAAR, VR1- and VR2-class odorant receptors associated with individual transduction machineries. The different types of receptor neurons are preferentially tuned towards particular classes of odorants, that are associated with specific behaviors, such as feeding, mating or migration. We discuss the connections of the receptor neurons in the olfactory bulb, the differences in bulbar circuitry compared to mammals, and the characteristics of second order projections to telencephalic olfactory areas, considering the everted ontogeny of the teleost telencephalon. The review concludes with a brief overview of current theories about odor coding and the prominent neural oscillations observed in the teleost olfactory system.

Subjects Aquaculture, Fisheries and Fish Science, Neuroscience, Freshwater Biology
Keywords Teleost, Fish, Olfaction, Sense of smell, Olfactory bulb, Telencephalon, Olfactory receptor neurons, Odor coding, Olfactory transduction

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INTRODUCTION

The sense of smell plays an important role in fishes, mediating behaviors and physiological responses related to food search and feeding, social interaction, mating, detection of predators and contamination, or migration and search for spawning sites (*Sorensen & Caprio, 1998*; *Bone & Moore, 2008*). As opposed to higher vertebrates (amphibians, reptiles and mammals), which usually have access to a limited degree of light even in moonless nights, fish encounter situations of total darkness easily in turbid or deeper waters and underwater caves, where their olfactory sense may provide a degree of orientation. Olfactory information has long been known to be important for the homing of migrating salmonids and other fishes (*Dittman & Quinn, 1996*; *Bett & Hinch, 2016*). There is evidence for salmonids that some amino acids dissolved in the water of native rivers could mediate migratory conducts (*Yamamoto, Hino & Ueda, 2010*; *Yamamoto, Shibata & Ueda, 2013*). However, in general, the identity of the odors mediating orientation behavior in teleosts remains a topic of active investigation. On the other hand, amino acids are considered the most relevant odorants indicating the presence of a food source to teleosts, given

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The authors declare there are no competing interests.

Author Contributions

- Jesús Olivares performed the experiments, analyzed the data, prepared figures and/or tables, approved the final draft.
- Oliver Schmachtenberg conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Data Availability

The following information was supplied regarding data availability:
This is a review and does not contain any unpublished raw data.

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Odd Loop Regions of XenA and XenB Enzymes Modulate Their Interaction with Nitro-explosives Compounds and Provide Structural Support for Their Regioselectivity

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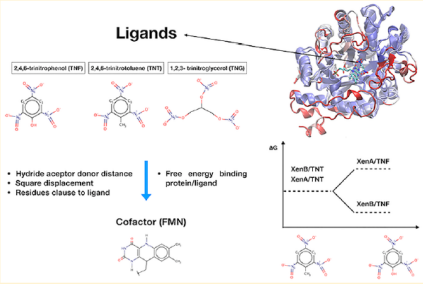
Supporting Information

ABSTRACT: The nitro-explosive compounds 2,4,6-trinitrotoluene, 2,4,6-trinitrophenol, and 1,2,3-trinitroglycerol are persistent environmental contaminants. The presence of different functional groups in these molecules represents a great challenge to enzymatic catalysis. The chemical variety of these three substrates is such that they do not bind and interact with catalytic residues within an enzyme with the same affinity. In this context, two Xenobiotic Reductase enzymes produced by the bacteria *Pseudomonas putida* can catalyze the reduction of these compounds with different affinities and regioselectivity. The structural bases that support this substrate promiscuity and catalytic preferences are unknown. Therefore, through molecular dynamics simulations and free energy calculations, we explored the structural properties driving the specific interactions of these enzymes with their substrates and cofactor. Models of Xenobiotic Reductase A and B enzymes in complex with 2,4,6-trinitrotoluene, 2,4,6-trinitrophenol, or 1,2,3-trinitroglycerol were built, and the ligand enzyme interaction was simulated by molecular dynamics. The structural analysis of the molecular dynamics simulations shows that loops 3, 5, 7, 9, 11, and 13 of Xenobiotic Reductase B, and loops 4, 5, 7, 11, 13, and 15 Xenobiotic Reductase A, are in contact with the ligands during the first stages of the molecular recognition. These loops are the most flexible regions for both enzymes; however, Xenobiotic Reductase B presents a greater range of movement and a higher number of residues interacting with the ligands. Finally, the distance between the cofactor and the different reactive groups in the substrate reflects the regioselectivity of the enzymes, and the free energy calculations are consistent with the substrate specificity of both enzymes studied. The simulation shows a stable interaction between the aromatic ring of the substrates and Xenobiotic Reductase B. In contrast, a less stable interaction with the different nitro groups of the aromatic ligands was observed. In the case of 1,2,3-trinitroglycerol, Xenobiotic Reductase B interacts more closely with the nitro groups of carbon 1 or 3, while Xenobiotic Reductase A is more selective by nitro groups of carbon 2. The obtained results suggest that the flexibility of the loops in Xenobiotic Reductase B and the presence of polar and aromatic residues present in loops 5 and 7 are fundamental to determine the affinity of the enzyme with the different substrates, and they also contribute to the proper orientation of the ligands that directs the catalytic reaction.



INTRODUCTION

The nitro-explosive compounds 2,4,6-trinitrotoluene (TNT), 2,4,6-trinitrophenol (TNF), and 1,2,3-trinitroglycerol (TNG) are toxic for most living organisms and recalcitrant to biodegradation.[1,2] Many nitro compounds are synthesized by organisms as a defense mechanism against microorganisms because of the great cellular toxicity that they exert as a consequence of the low enzymatic activity to degrade them.[3] In this stage, there is a great interest in discovering and studying enzymes that can degrade these compounds to eliminate them from the environment.[4]

The interaction of any enzyme with TNT, TNF, and TNG represents a catalytic challenge, because it requires metabolizing aromatic (TNT and TNT) and aliphatic (TNG) compounds. Principally, TNT or TNF is more rigid than TNG, due to the π bond of the aromatic ring. Regarding the hydroxyl group, TNF is more polar than TNT or TNG, which reduces the chemical complementarity between the catalytic site and the ligand.[5−7] Therefore, the identification of enzymes capable of degrading these three compounds and understanding the phenomena underlying the catalysis would favor their application in bioremediation systems. In addition, understanding the catalytic process and the key residues involved would provide information that could be used to generate more efficient versions of these enzymes and extend their application to other xenobiotic compounds.

Several microorganisms with the capacity to degrade nitro-explosive compounds have been reported to date.[8,9] Among them, *Pseudomonas putida* strain KT2440 possesses remarkable

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restrictions to 300 K in a canonical assembly (NVT); and (5) finally, a production dynamic was carried out with an isothermal isobaric assembly (NPT) without restrictions for 50 ns at 300 K and 1 atm with a temporary passage of 2 fs.[26] In the MD simulation, the temperature was controlled by the Langevin dynamics with a collision frequency of 1 ps^{-1} (NVT) and the pressure with the Berendsen barostat (NPT). In addition, the Particle Mesh Ewald (PME) method with a cutoff value of 10 Å was used to treat nonbonding and long-range electrostatic interactions. To evaluate the reproducibility of the process, two independent MD simulations were carried out starting from the minimization stage (step 1 standard MD protocol), one with 50 ns and another with 300 ns of production for each system (Figures S4 and S5). All MD simulation calculations were performed using the Graphics Processing Unit (GPU) - AMBER Implementations18.[29]

Free Energy Calculation. The binding free energy of each ligand was calculated for the enzymes XenA and XenB by means of MMGBSA.[31] This approximation estimates the free energy difference (ΔG) of binding ($\Delta G_{bind,solvated}$) using a thermodynamic cycle. The $\Delta G_{bind,solvated}$ was obtained of the mean $\Delta G_{bind,solvated} = \Delta G_{bind,vacuum} + \Delta G_{solv,complex} - (\Delta G_{solv,ligand} + \Delta G_{solv,protein})$ equation, where $\Delta G_{bind,vacuum}$ is the ΔG of the complex formation in the vacuum, $\Delta G_{solv,ligand}$ is the ΔG solvation of the ligand, $\Delta G_{solv,protein}$ is the ΔG solved of the protein, $\Delta G_{solv,ligand}$ is the ΔG solved of the ligand, and $\Delta G_{solv,complex}$ is the ΔG solvation of the protein/ligand complex. In each calculation, the electrostatic contribution of the solvation-free energy is calculated by solving the Generalized Born equation. All of the calculations were made with the module implemented in the AMBER 18 program from the last 100 ns of the production stage of each dynamic.

The thermodynamic integration method was used to calculate the affinities of XenA and XenB for the TNT and TNF ligands. This method calculates the free energy difference between an initial state (enzyme/TNT) and a final one (enzyme/TNF), coupling them through a nonspatial parameter λ (0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 values). The cycle used considers three steps to transform the methyl group of TNT into the hydroxyl of TNF through a double topology approach. All calculations were performed in the AMBER 18 program.[32]

Trajectory Analysis. Two trajectories were obtained for each model, one of 300 ns with 15 000 position data and another of 50 ns with 5000 position data. Both were processed with the CPPTRAJ program.[29] The flexibility of the different regions of the enzyme was measured by normal-mode analysis of the quadratic fluctuations of the alpha carbons of the protein, considering the three first normal modes. In addition, the deviation of the root-mean-square (RMSD) of the carbon skeleton of the protein and of each substrate in complex with the enzyme was quantified. To determine the RMSD for TNT and TNF, the carbons of the aromatic ring were used (C1−C5). In the case of TNG, the carbons of the skeleton (C1−C3) were used for the analysis.

The HAD distances were measured in the trajectories. To simplify the notation, the name assigned for a hydride transfer distance begins with the hydride donor and then with the hydride acceptor. For example, the carbon reduction distance 3 of the aromatic ring was defined as N5:FMN−C3:TNT. In the first part, it is indicated that the hydride donor is nitrogen N5, and after the line it is indicated that the hydride acceptor is the carbon 3 of TNT (Figure 1). Thus, the hydride donor for all reactions was the nitrogen of the central aromatic ring (FMN:N5) of FMN cofactor, while the hydride acceptor can be the nitrogen of the nitro groups of the ligands or a carbon of the aromatic ring that is adjacent to the methyl (TNT) or hydroxyl (TNF) radical. To measure the reduction distance of the nitro groups of TNT and TNF, the nitrogens bound to carbon 2 (N5:FMN−N2:TNT or N5:FMN−N2:TNF), 4 (N5:FMN−N4:TNT or N5:FMN−N4:TNF), and 6 (N5:FMN−N6:TNT or N5:FMN−N6:TNF) were considered to accept hydrides. In the case of the TNG ligand, measurements were made from N5:FMN to the nitrogen of the nitro groups attached to carbons 1 (N1), 2 (N2), or 3 (N3) of the aliphatic chain.

■ ASSOCIATED CONTENT

Supporting Information

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Figures S1−S5 and Table S1 (PDF)

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Chromatic pupillometry for the characterization of the pupillary light reflex in *Octodon degus*

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ABSTRACT

The common degu (*Octodon degus*) is an emerging model in biomedical science research due to its longevity and propensity to develop human-like conditions. However, there is a lack of standardized techniques for this non-traditional laboratory animal. In an effort to characterize the model, we developed a chromatic pupillometry setup and analysis protocol to characterize the pupillary light reflex (PLR) in our animals. The PLR is a biomarker to detect early signs for central nervous system deterioration. Chromatic pupillometry is a non-invasive and anesthesia-free method that can evaluate different aspects of the PLR, including the response of intrinsically photosensitive retinal ganglion cells (ipRGCs), the disfunction of which has been linked to various disorders. We studied the PLR of 12 degus between 6 and 48 months of age to characterize responses to LEDs of 390, 450, 500, 525 and 605 nm, and used 5 with overall better responses to establish a benchmark for healthy PLR (PLR+) and deteriorated PLR (PLR-). Degu pupils contracted up to 65% of their horizontal resting size before reaching saturation. The highest sensitivity was found at 500 nm, with similar sensitivities at lower tested intensities for 390 nm, coinciding with the medium wavelength and short wavelength cones of the degu. We also tested the post-illumination pupillary response (PIPR), which is driven exclusively by ipRGCs. PIPR was largest in response to 450 nm light, with the pupil preserving 48% of its maximum constriction 9 s after the stimulus, in contrast with 24% preserved in response to 525 nm, response driven mainly by cones. PLR- animals showed maximum constriction between 40% and 50% smaller than PLR+, and their PIPR almost disappeared, pointing to a disfunction of the iPRGCs rather than the retinal photoreceptors. Our method thus allows us to non-invasively estimate the condition of experimental animals before attempting other procedures.

1. Introduction

Octodon degus, or degu, is a hystricomorph rodent endemic to central Chile with growing interest as an animal model (for an overview, see Ardiles et al., 2013; Hurley et al., 2018). One of their more salient characteristics is their longevity. Degus can live up to 8 years in captivity (Lee, 2004), which makes them good candidates for studies on the effects of aging and the associated plethora of degenerative and metabolic disorders like diabetes, amyloidosis and atherosclerosis (Edwards, 2009; Homan et al., 2010). Moreover, they are highly social animals with a mostly diurnal circadian profile (Lee, 2004), which makes them more appropriate models for the study of the circadian rhythms, its disorders and possible treatments than the exclusively nocturnal mice and rats.

During ageing, degus also develop signs of inflammation at the brain and retinal level as well as cognitive deterioration (van Groen et al., 2011; Inestrosa et al., 2015). Particularly, older degus have been shown to develop molecular hallmarks of Alzheimer's Disease (AD) (Tarragon et al, 2013; Inestrosa et al., 2005; van Groen et al., 2011; Ardiles et al., 2012, Cisternas et al., 2018). Although the proportion of affected animals still a subject of debate (Steffen et al., 2016; Bourdenx et al., 2017; Hurley et al., 2018), there is very strong evidence for the presence of these symptoms, which is further reinforced by the fact that the aforementioned disorders commonly found in degus (diabetes, circadian disruption, atherosclerosis etc.) are usually comorbid with AD, and frequently considered risk factors for the development of the disease (i.e. Musiek et al., 2015; De Reuck et al., 2016; Ribe and Lovestone, 2016).

Due to their diurnal and social nature, degus are good candidates for models in the study of the melanopsin system. Photoreception in mammals is not limited to conscious visual perception, but also comprises a range of "non-image forming responses" (NIFRs) that include,

Abbreviations: PLR, Pupillary light reflex; ipRGC, Intrinsically-photosensitive reti-nal ganglion cells; PIPR, Post-illumination pupillary response

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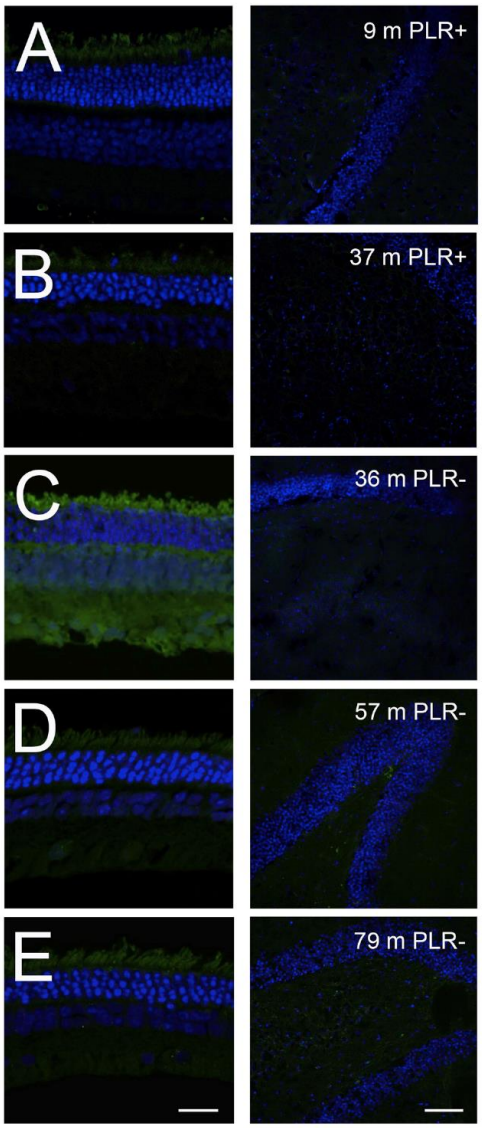

Fig. 5. Increased neuronal death marker expression in retinas from PLR- degus. Representative immunostaining from retina (left) and hippocampus (right) of some degus tested with our method. Each row represents a different degu: 9 m PLR+ (A), 37 m PLR+ (B), 36 m PLR- (C), 57 m PLR- (D) and 79 m PLR- (E).

first stages of deterioration. For this reason, we separated as PLR + those degus with the absolute best sensitivity to create a benchmark. Our intention is to use a very strict standard as we start screening our colony, as we would rather our test created false positives than leave deteriorated animals unnoticed. Therefore, in future studies we will consider as PLR- any animal that presents either maximum constriction or $PIPR_{10}$ below the average of our PLR+ animals minus one standard deviation. Were we to find a large number of false positives, the test parameters can be relaxed to average minus two standard deviations, more in line with usual conventions.

In conclusion, we were able to develop a non-invasive and affordable method for carrying out chromatic pupillometry in awake degus and performed the first characterization of the pupillary light reflex on this animal. Moreover, we developed a protocol to classify our animals according to PLR performance, which gives us an easy way to screen our colony for animals showing potential ipRGC dysfunction. Although selective deterioration of ipRGCs is suggested to be present in AD, and chromatic pupillometry is considered a promising diagnostic method (Chougule et al., 2019), it is as yet unknown how consistent is ipRGC loss, especially at earlier stages in the disease. Another complicating factor is that some other conditions, like glaucoma, can result in melanopsin cell dysfunction (Kankipati et al., 2011). Our preliminary histological study of animals evaluated using our methods found signs of neuronal cell death in either the retina or the hippocampus of PLR- animals, while PLR+ animals showed no such signs. However, PLR- animals never showed cell death consistently in retina, hippocampus or both. Due to the low number of sacrificed animals since the method was implemented, we have been unable to assess whether neurodegeneration in retina and hippocampus affects PLR deterioration in different ways. Future studies will continue to test the strength of the relationship between a deteriorated PLR and the presence of neurodegenerative disorders, and the specificity of diagnosis that can be achieved using different PLR characteristics. Although much work remains to be done, we consider the development and implementation of our chromatic pupillometry test a promising advance towards the establishment of *O. degus* as a more standardized animal model for comparative and biomedical studies.

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Appendix A. Supplementary data

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Ketamine-Treatment During Late Adolescence Impairs Inhibitory Synaptic Transmission in the Prefrontal Cortex and Working Memory in Adult Rats

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Schizophrenia (SZ) is associated with changes in the structure and function of several brain areas. Several findings suggest that these impairments are related to a dysfunction in γ-aminobutyric acid (GABA) neurotransmission in brain areas such as the medial prefrontal cortex (mPFC), the hippocampus (HPC) and the primary auditory cortex (A1); however, it is still unclear how the GABAergic system is disrupted in these brain areas. Here, we examined the effect of ketamine (Ket) administration during late adolescence in rats on inhibition in the mPFC-, ventral HPC (vHPC), and A1. We observe that Ket treatment reduced the expression of the calcium-binding protein parvalbumin (PV) and the GABA-producing enzyme glutamic acid decarboxylase 67 (GAD67) as well as decreased inhibitory synaptic efficacy in the mPFC. In addition, Ket-treated rats performed worse in executive tasks that depend on the integrity and proper functioning of the mPFC. Conversely, we do not find such changes in vHPC or A1. Together, our results provide strong experimental support for the hypothesis that during adolescence, the function of the mPFC is more susceptible than that of HPC or A1 to NMDAR hypofunction, showing apparent structure specificity. Thus, the impairment of inhibitory circuitry in mPFC could be a convergent primary site of SZ-like behavior during the adulthood.

Keywords: ketamine, late adolescence, PV-INs, GABA, mPFC, schizophrenia-like behavior

Abbreviations: ACSF, artificial cerebrospinal fluid; A1, Primary auditory cortex; GABA, γ-aminobutyric acid; Amph, amphetamine; ANOVA, analysis of variance; CNQX, 7-nitro-2,3-dioxo-1,4-dihydroquinoxaline-6-carbonitrile; D-AP5, 2-amino-5-phosphonopentanoic acid; EPSCs, excitatory postsynaptic currents; GAD67, glutamic acid decarboxylase 67; HPC, hippocampus; IPSCs, inhibitory postsynaptic currents; Ket, ketamine; mPFC, medial prefrontal cortex; mIPSC, miniature inhibitory postsynaptic currents; MK-801, Dizocilpine; NMDAR, N-methyl-D-aspartate receptor; PV+, calcium-binding protein parvalbumin; PFC, prefrontal cortex; PND, postnatal days; PPR, paired-pulse ratio; SZ, schizophrenia; sIPSC, spontaneous inhibitory postsynaptic currents; TTX, tetrodotoxin. Veh, vehicle; WM, working memory.

a reduction in frequency of miniature events could occur by release failures (Chen and Roper, 2003). Indeed, short-term depression has been attributed to an increased neurotransmitter release probability from presynaptic terminals (O'Donovan and Rinzel, 1997). By analyzing two forms of short–term synaptic plasticity, paired pulse depression (PPD) and use-dependent depression during a 10 Hz stimulus train. We found evidence for a decrease in GABA neurotransmitter release. In input-output curves, we found that for similar stimulus intensities the Ket-treated mPFC slices exhibited lower IPSC amplitude than control slices, suggesting that Ket treatment diminished evoked inhibitory synaptic transmission. Surprisingly, we observed that Ket treatment increased the amplitude of both sIPSC and mIPSC, which might be the result of Ket-induced changes of GABA vesicular content, postsynaptic sensitivity, or both. It has been shown that in layer II/III of individuals with SZ, $GABA_A$ α2 subunit mRNA levels are elevated, which could explain this increase in the amplitude of the sIPSC and mIPSC (Volk et al., 2002). The increase in postsynaptic $GABA_A$ α2 receptor protein levels has been interpreted as coordinated compensations in response to a PV deficit in synthesis and release of GABA to regain normal GABA signaling (Lewis et al., 2005; Beneyto et al., 2011). In addition, the reduction in PV and GAD67 levels that result from chronic NMDAR blockade are believed to represent maladaptive mechanisms in a faulty effort to maintain a correct excitatory/inhibitory in the cortical network (Behrens et al., 2007; Lisman et al., 2008). Interestingly, it has been shown that NMDA receptors are essential to regulate the peri-adolescent maturation of the GABAergic networks (Thomases et al., 2013). Thus, it is possible that NMDA antagonist Ket/MK-801 treatment may impede the normal function of GABA synaptic efficacy.

Collectively, our data show that NMDAR antagonist Ket and MK801 administration during late adolescence impairs in a specific manner PFC over HPC or A1 cortex, and that this discrete mPFC disruption is enough to induce SZ-like behaviors and corresponding changes on PV and GAD67 levels, as well as GABAergic impairment on layer II/III of the mPFC. Therefore, administration of Ket during the late adolescent period appears to be a useful model for cognitive and negative symptoms of SZ. Moreover, reductions in PV and GAD67 in the mPFC are related to an impairment of the inhibitory synaptic transmission. The mechanisms underlying late adolescent impairment in the PFC and their potential relationship to the pathophysiology of SZ clearly warrants further investigation.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The Institutional Animal bioethics Committee for research with experimental animals (CIBICA), Universidad de Valparaío declares that he has evaluated the protocol of experimentation used in the present work entitled: Ketamine-treatment during late adolescence impairs inhibitory synaptic transmission in the prefrontal cortex and working memory in adult rats of the researcher, Dr. Marco Fuenzalida Núñez, University of Valparaíso. The Institutional Animal bioethics Committee of the Universidad de Valparaíso and according to standards outlined in the National Institute of Health (United States) guidelines approves the experimental protocol used in this investigation.

AUTHOR CONTRIBUTIONS

MP, CM, OS, FG, IG, and VP-S conducted the experiments and data analysis. AD-S, PF, PM, and MF contributed to experimental design, data discussion, and writing.

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SUPPLEMENTARY MATERIAL

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FIGURE S1 | Schematic drawing of the experimental design, structure of the basic two-alternative choice task (2-ACT), and locomotor activities during adulthood. **(A)** The arrow represents the postnatal days of the animals (PND). The behavioral tasks and electrophysiological tools were measured during adulthood between PND 60 and 70. The locomotor activities in reaction to Ket were done during adolescence (PND 51). **(B)** With auditory attention training beginning after weaning, the rats were trained in the 2-ACT for 20 days. The rat initiated a trial by inserting its nose into the center nose-poke, which triggers the computer to present two types of acoustic stimuli at random: one was a low-frequency tone at 1 kHz, and the other was a high-frequency 15 kHz tone. The rats were trained to respond with right pokes for low tones and left pokes for high tones. To analyze the ketamine effects on auditory attention, the animals were subjected to 50 2-ACT trials 1 day after 2-ACT training (baseline) during adolescence (1 day after Ket treatment, PND 51) and during adulthood (PND 60).

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β-catenin aggregation in models of ALS motor neurons: GSK3β inhibition effect and neuronal differentiation

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by motor neuron death. A 20% of familial ALS cases are associated with mutations in the gene coding for superoxide dismutase 1 (SOD1). The accumulation of abnormal aggregates of different proteins is a common feature in motor neurons of patients and transgenic ALS mice models, which are thought to contribute to disease pathogenesis. Developmental morphogens, such as the Wnt family, regulate numerous features of neuronal physiology in the adult brain and have been implicated in neurodegeneration. β-catenin is a central mediator of both, Wnt signaling activity and cell-cell interactions. We previously reported that the expression of mutant SOD1 in the NSC34 motor neuron cell line decreases basal Wnt pathway activity, which correlates with cytosolic β-catenin accumulation and impaired neuronal differentiation. In this work, we aimed a deeper characterization of β-catenin distribution in models of ALS motor neurons. We observed extensive accumulation of β-catenin supramolecular structures in motor neuron somas of pre-symptomatic mutant SOD1 mice. In cell-cell appositional zones of NSC34 cells expressing mutant SOD1, β-catenin displays a reduced co-distribution with E-cadherin accompanied by an increased association with the gap junction protein Connexin-43; these findings correlate with impaired intercellular adhesion and exacerbated cell coupling. Remarkably, pharmacological inhibition of the glycogen synthase kinase-3β (GSK3β) in both NSC34 cell lines reverted both, β-catenin aggregation and the adverse effects of mutant SOD1 expression on neuronal differentiation. Our findings suggest that early defects in β-catenin distribution could be an underlying factor affecting the onset of neurodegeneration in familial ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the selective death of motor neurons in the spinal cord, brainstem and motor cortex. This progressive disease causes atrophy of limb, axial and respiratory muscles (Kiernan et al., 2011). Most ALS cases are considered sporadic (sALS), while 10% are familial (fALS), involving mutations in superoxide dismutase 1 (SOD1), transactive response DNA binding protein 43 (TARDBP or TDP-43), fused in sarcoma/translocated in sarcoma (FUS/TLS), and the hexanucleotide repeat expansions in C9ORF72 as the most common alterations (Al-Chalabi and Hardiman, 2013; Turner et al., 2013). > 150 mutations in SOD1 have been linked to ALS with varying degree of aggressiveness and aggregation propensity, which explains around 20% of fALS cases (Valentine et al., 2005). Importantly, several studies have reported the presence of abnormal $SOD1^{WT}$ species in post-mortem sALS tissue highlighting the relevance of the protein in disease pathogenesis (Bosco et al., 2010; Ciechanover and Kwon, 2015; Forsberg et al., 2010;

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β-catenin destruction complex that becomes disassembled upon Wnt pathway activation (Ciani and Salinas, 2005). In these studies, stabilized β-catenin binding to APC blocks the cytoskeleton-dependent ability of APC to induce neurite outgrowth (Votin et al., 2005). Therefore, addressing transcriptional or local signaling responses elicited by GSK3β inhibition will be essential to define its impact on the disassembly of the macromolecular structures formed by β-catenin as well as on neuronal differentiation. These studies will also allow to test for possible pharmacological strategies to manipulate β-catenin accumulation and its potential impact in ALS progression.

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Role of TRPM8 channels in altered cold sensitivity of corneal primary sensory neurons induced by axonal damage

Abbreviated title: TRPM8 channels in altered corneal cold sensitivity

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RP, GU, AI, MC, EO, PO, RM: acquisition, analysis and interpretation of the data. EO, PO: analysis and development of the mathematical model. RM, JB, CB: Conceptualization and supervision. RP, GU, AI, MC, EO, PO and RM contributed to the preparation of figures. RM directed the study and drafted the paper. All the authors reviewed the manuscript and approved the final version.

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NMDA receptor modulation by NOX2 drives synaptic plasticity and spatial memory impairments in rats exposed pre and postnatally to ethanol

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test. Significance was set at $P < 0.05$, and is indicated as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. The behavioral data were analyzed with Two-Way Anova repeated measures, followed by Tukey's post-hoc test. The partial eta squared (η^2p) or Cohen's d was used to inform effect size and the alpha level was kept at 0.05 across tests.

All statistical analyses were performed using OriginPro 9.3 software (OriginLab Corporation).

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Authors' contributions

PH, EFO, AEC, GS and CH designed research; WP, SFE, CG, EFO, PH and GS performed research; WP, SFE, CG, EFO, PH and GS analyzed data; and PH, EFO, SFE, CH and AEC wrote the paper.

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Molecular modelling predicts that 2-methoxyestradiol disrupts HIF function by binding to the PAS-B domain

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ABSTRACT

An estradiol metabolite, 2-methoxyestradiol (2ME), has emerged as an important regulator of ovarian physiology. 2ME is recognized as a potent anti-angiogenic agent in clinical trials and laboratory studies. However, little is known about its molecular actions and its endogenous targets. 2ME is produced by human ovarian cells during the normal menstrual cycle, being higher during regression of the corpus luteum, and is postulated to be involved in the anti-angiogenic process that plays out during luteolysis.

We utilized cell biology techniques to understand the molecular mechanism of 2ME anti-angiogenic effects on human granulosa luteal cells. The principal effect of 2ME was to alter Hypoxia Inducible Factor 1A (HIF1A) subcellular localization. Molecular modelling and multiple bioinformatics tools indicated that 2ME impairs Hypoxia Inducible Factor complex (HIF) nuclear translocation by binding to a buried pocket in the HIF1A Per Arnt Sim (PAS)-B domain. Binding of 2ME to HIF1A protein is predicted to perturb HIF1A-Hypoxia Inducible Factor B (HIFB) interaction, a key step in HIF nuclear translocation, preventing the transcriptional actions of HIF, including Vascular Endotelial Growth Factor (VEGF) gene activation. To our knowledge, 2ME is the first putative HIF endogenous ligand characterized with anti-angiogenic activity. This postulate has important implications for reproduction, because angiogenic processes are critical for ovarian follicular development, ovulation and corpus luteum regression. The present research could contribute to the development of novel pharmacological approaches for controlling HIF activity in human reproductive diseases.

1. Introduction

The ovarian follicle and the corpus luteum are the main sources of estradiol (E2) throughout the ovarian cycle [1]. Estradiol has diverse biological effects in numerous human tissues, and its actions are mainly mediated by estradiol nuclear receptors. Estradiol can also be metabolized by several metabolic pathways, undergoing various chemical modifications such as hydroxylation, glucuronidation, sulfonation and methylation [2]. Interestingly, some of these E2 metabolites have biological actions that do not involve the traditional pathways associated with the estradiol nuclear receptor.

2-methoxyestradiol (2ME) is one of the E2 metabolites produced by human luteinized granulosa cells. It does not exhibit estrogenic activity and has a low binding affinity for the estrogen receptor [3,4]. 2ME is normally present in luteal tissue throughout the luteal phase, being maximum at the time of luteolysis [5,6]. This compound has compelling anti-angiogenic and anti-proliferative activities [7–10]. Based on these features, 2ME has been tested in oncology trials [11,12].

Abbreviations: GC, Human lutein granulosa cells; 2ME, 2-methoxyestradiol; E2, Estradiol; hCG, human gonadotrophic chorionic hormone; HIF, Hypoxia Inducible Factor complex; HIF1A, Hypoxia Inducible Factor 1A protein; HIF2A, Hypoxia Inducible Factor 2A protein; HIFB, Hypoxia Inducible Factor B protein, VEGF, Vascular Endothelial Growth Factor; VEGF, Vascular Endothelial Growth Factor; PAS-B, Per Arnt Sim B domain; PAS-A, Per Arnt Sim A domain, bHLH, basic helix-loop-helix; VHL, von Hippel-Lindau tumor suppressor protein; HRE, hypoxia response element

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Interestingly, in cell lines from mice and rats, 2ME at pharmacological concentrations can act through GPR30 [46,47], and we cannot rule out the possibility that 2ME could have an effect through this receptor in addition to its antiangiogenic actions through HIF1A. The proposal of a direct interaction between 2ME and HIF is an attractive hypothesis because there is evidence supporting the ligand binding capacity of HIF PAS domains [35,40,41].

To obtain insights into this hypothesis, we modeled 2ME binding in both PAS domains of HIF1A in HIF1A-HIFB heterodimers and simulated them with molecular dynamics. Protein movements, the ligand movements, the interaction between them and the motion correlations were analyzed. As a control, we modelled the binding of E2 in the same pockets. E2 is very similar in structure to 2ME, but does not exert any anti-angiogenic effect, or reduce VEGF levels [9,48]. On the contrary, E2 has pro-angiogenic and proliferative effects on several tissues [49–51]. We found that the 2ME molecule is stable inside the pockets, and can establish some interactions such as hydrogen bonds and π-π interactions, especially when it is modeled inside the PAS-B cavity (Fig. 3). Additionally, in simulations without ligand, the PAS-B domain maintained a stable number of water molecules inside (Fig. 2), which have a particular dynamic behavior, comparable with functional cavities of other proteins [52,53]. On the contrary, the PAS-A domain was not able to retain water molecules inside during the simulation, suggesting that this domain is not stable enough to hold a ligand.

Afterwards, we analyzed whether the interactions between HIF1A and HIFB were altered in the simulations with bound ligands. In all four conditions, these interactions do not change, and the overall structure of the dimer remained very similar after the simulation (Figure S2). This finding is not entirely surprising as it has been previously reported that the protein-protein surface between HIF1A (or HIF2A) and HIFB does not change when other ligands are simulated inside the PAS domain [40,54–56]. Remarkably, these other ligands were previously identified as inhibitors of HIF function and their ability to bind to the protein has been confirmed experimentally [40,56,57].

We looked for a method that can identify perturbations in protein regions that do not necessarily imply conformational changes. One such method is calculation of generalized motion correlations, which has proven useful in detection of allosteric networks in proteins [58]. When 2ME was placed inside HIF1A PAS-B cavity, we found higher correlations with other regions of the dimer, far from the domain where the 2ME molecule resides (Fig. 4). This observation indicates an improved capacity of 2ME to perturb the rest of the structure, in comparison with E2 or 2ME itself in PAS-A domain.

Given these findings, we argue that the PAS-B domain is a suitable candidate for binding ligands such as 2ME due to its stability and number of interactions established by both protein and ligand. The high correlation between the movement of 2ME and the movement of the rest of the dimer, even in distant regions of the structure, suggest that the binding of 2ME to the PAS-B domain may perturb the protein-protein interface between HIF1A-HIFB over a period of time that exceeds reasonable simulation times (normally nanoseconds).

As mentioned above, several studies have demonstrated that there are cavities within the PAS-A and PAS-B domains of HIF2A. These cavities, characterized as hydrophobic pockets, can bind to several small artificial compounds [40,41,42]. Nuclear Magnetic Resonance and calorimetric analyses have established that the PAS-B cavity has optimal conditions for ligand binding, and it has been proposed as a target for chemical design of new drugs. HIF2A and HIF1A are homologues that are expressed in different tissues, but their structure, size and properties are quite similar (Figure S4). These similarities support the idea that the PAS domains in HIF1A have a similar behavior and also can hold a ligand.

Crystallographic work on isolated HIF PAS domains [35,40,56] found dimers interacting through their β-sheets, just like prokaryotic PAS domains [59]. A more complete heterodimer crystal was released in 2015 [39], showing a different HIF protein interaction interface, where PAS domains are part of it, but not as reported in the previous crystal structure. It is tempting to speculate that the PAS domains are important in the initial interaction of both proteins, acting as reconnaissance domains that promote the full formation of the protein-protein interface. In the final conformation, the dimer changes the position of PAS domains breaking the β-sheets interaction interface (reported originally for isolated PAS domains or other PAS-containing proteins) and favors the interaction detected in the full-dimer crystal [39]. Our results from steered molecular dynamics support this idea because when we pull one protein with respect to the other, the PAS domains do not have the strongest interactions, compared to the C-terminal region of bHLH, which is full of inter-protein salt bridges (Table 1).

In summary, the structural and physiological data provided by our research on the function and expression of HIF1A protein in GC indicate that anti-angiogenic actions of 2ME occur by a mechanism distinct from changes in protein expression, e.g. altering HIF1A subcellular distribution. Physiological concentrations of 2ME do not affect cellular HIF1A protein levels. After analysis of dynamic simulations of HIF1A-HIFB dimer bound to 2ME, we postulate that 2ME produces its anti-angiogenic effects by binding directly to PAS-B cavity of HIF1A. The 2ME-HIF1A interaction could modify the HIF1A-HIFB heterodimer interface and consequently prevent HIF nuclear translocation and gene activation.

To our knowledge, we are the first group to suggest that HIF1A could bind a small endogenous molecule like 2ME, and the site of this binding could be the pocket formed by the PAS-B domain. These data contribute to our understanding of the complexity of HIF protein regulation mechanisms and the physiological functions of 2ME during the corpus luteum regression.

Ethical approval

The study was approved by the Clinical Hospital San Borja Arriarán Medical Ethics Committee. All women enrolled in this study signed an informed consent prior to participation.

Acknowledgements

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Authors Roles.

AP, SH, PK and LD design the work. AP, FV and AM collecting experimental data. AP and FV analyzed and interpreted experimental data, and drafting the work. SH, PK, JFS and LD revising it critically. LD approval the final version to be published.

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Conflict of Interest.

The authors declare no conflicts of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2019.02.004.

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SCIENTIFIC REPORTS

OPEN

Speed-Selectivity in Retinal Ganglion Cells is Sharpened by Broad Spatial Frequency, Naturalistic Stimuli

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César R. Ravello[1], Laurent U. Perrinet[2], María-José Escobar[3] & Adrián G. Palacios[1]

Motion detection represents one of the critical tasks of the visual system and has motivated a large body of research. However, it remains unclear precisely why the response of retinal ganglion cells (RGCs) to simple artificial stimuli does not predict their response to complex, naturalistic stimuli. To explore this topic, we use Motion Clouds (MC), which are synthetic textures that preserve properties of natural images and are merely parameterized, in particular by modulating the spatiotemporal spectrum complexity of the stimulus by adjusting the frequency bandwidths. By stimulating the retina of the diurnal rodent, Octodon degus with MC we show that the RGCs respond to increasingly complex stimuli by narrowing their adjustment curves in response to movement. At the level of the population, complex stimuli produce a sparser code while preserving movement information; therefore, the stimuli are encoded more efficiently. Interestingly, these properties were observed throughout different populations of RGCs. Thus, our results reveal that the response at the level of RGCs is modulated by the naturalness of the stimulus - in particular for motion - which suggests that the tuning to the statistics of natural images already emerges at the level of the retina.

Motion detection is essential for animal survival, and studies show that many retinal cells[1–3] and a considerable portion of the cortex[4] are involved in its processing. However, most of these studies have focused on the detection of artificial stimuli, such as random dots, lines, or moving gratings, while ignoring the natural signals from the environment in which animals have evolved and to which their visual system would be attuned to[5–9]. Moreover, computational models based on the response to simple, artificial stimuli fail to predict the response to naturalistic images[10,11]. Nevertheless, working with natural images directly is not always possible due to their complexity regarding critical parameters of signals processing, including visual content and its variability. The latter makes it hard to control the motion information that is effectively being presented to the visual system[12,13].

In the visual cortex, motion is detected at different levels and by populations of cells attuned to different parameters of the stimulus (see[4] for a review). It has been reported that visual neurons stimulated with complex images, which should be closer to natural images, change their tuning curves compared to the response obtained with simple stimuli, becoming narrower and thus more selective to speed[14]. In turn, stimulation with naturalistic images generates sparser code[15,16] and eye tracking responses become more precise[17].

In the retina, the most basic form of motion detection is the response to moving textures in which Y-like *Retinal Ganglion Cells* (RGCs) increase their firing rate when sub-regions of their *Receptive Field* (RF) alternate between light and dark luminosity levels through time, regardless of the direction of motion[18,19]. It has been demonstrated that these mechanisms arises from the RF properties of ON and OFF *Bipolar Cells* (BCs) that connect to the RGCs[20] and transmit their activity in a non-linear fashion. This non-linear integration of the activity of BCs allows that the alternating activation of cells of both polarities do not cancel each other upon reaching RGCs[21]. These BCs can generate fast transient responses when there is a luminance change of the corresponding polarity (dark to light or light to dark respectively), so each time that BCs detect a temporal change in luminance,

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Author Contributions
M.J.E., L.U.P., A.P. conceived the study. A.P. provided the animals, equipment for electrophysiology and data analysis. C.R.R. designed the stimulation protocols and conducted the experiments. C.R.R. and L.U.P. analyzed the results. C.R.R. and M.J.E. prepared the figures. All authors reviewed the manuscript.

Additional Information
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Article

Zoledronic Acid Modulation of TRPV1 Channel Currents in Osteoblast Cell Line and Native Rat and Mouse Bone Marrow-Derived Osteoblasts: Cell Proliferation and Mineralization Effect

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check for updates

Abstract: Bisphosphonates (BPs) reduce bone pain and fractures by balancing the osteoblast/osteoclast ratio. The behavior of ion channels in the presence of BPs is not known. To investigate this, the effect of zoledronic acid BP (ZOL) (3×10^{-8} to 5×10^{-4} M) treatment, on ion channels, cell proliferation, and mineralization, has been investigated on preosteoclast-like cells, RAW264.7, preosteoblast-like cells MC3T3-E1, and rat/mouse native bone marrow-derived osteoblasts. In whole-cell patch clamp on cell line- and bone marrow-derived osteoblasts, ZOL potentiated outward currents. On RAW264.7, ZOL (10^{-4} M)-evoked current was reduced by the Kv channel blocker tetraethylammonium hydrochloride (TEA), but not by the selective TRPV1-channel antagonist capsazepine. On MC3T3-E1 cells and bone marrow-derived osteoblasts, ZOL-evoked current (5×10^{-8} to 10^{-4} M) was reduced by capsazepine, whereas the selective TRPV1-channel agonist capsaicin potentiated the control current. In the cell proliferation assay, 72 h incubation of RAW264.7 and MC3T3-E1 cells with ZOL reduced proliferation, with IC_{50} values of 2.62×10^{-7} M and 2.02×10^{-5} M, respectively. Mineralization of MC3T3-E1 cells and bone marrow-derived osteoblasts was observed in the presence of capsaicin and ZOL (5×10^{-8}–10^{-7} M); ZOL effects were antagonized by capsazepine. In summary, the ZOL-induced activation of TRPV1 channel mediates the mineralization of osteoblasts and counterbalances the antiproliferative effects, increasing the IC_{50}. This mechanism is not operative in osteoclasts lacking the TRPV1 channel.

Keywords: TRPV1 channel; zoledronic acid; voltage dependent potassium channel; osteoblast; osteoclast; bisphosphonates; cell proliferation; mineralization

1. Introduction

Bone tissue is one of the preferred sites for metastasis in patients with advanced malignancy, especially in case of prostate, breast cancer, and multiple myeloma. Tumor cells prefer to migrate into heavily vascularized areas of the skeleton, such as the red marrow of the long bones, sternum, pelvis, ribs, and vertebrae [1], because of the support of their microenvironment. The bone microenvironment is considered a fertile "soil", containing cytokines and growth factors that stimulate the growth of

pulled in a horizontal pipette puller (Sutter Instruments) from borosilicate capillary glass (World Precision Instruments; 1B150F-4), and pipette tips were fire-polished using a heating filament under a microscope to promote high-resistance seal formation [48,49].

Inside-out excised patch recordings were performed at 20° using patch pipettes with a tip size of 15–25 µm (pipette resistance <1 MΩ after polishing). Currents were obtained in response to voltage pulses from −60 to +260 mV (Vm) in 10 mV steps and were recorded with an *Axopatch 200B* system (Axon Instruments) using Clampex 10 (Axon Instruments) acquisition software. Leak subtraction was performed based on a P/4 protocol [50]. Electrophysiological data were analyzed with Clampfit 10.5 software (Molecular devices corporation, Sunnyvale, CA, USA).

4.10. Data Analysis and Statistics

Data were collected and analyzed using Excel software (Microsoft Office 2010); curve fitting was made using SigmaPlot 10.0. The statistical results are presented as mean ± SEM. The number of replicates relative to each experimental dataset was reported in the results paragraph and in the figure description. The Student *t*-test was used to evaluate the significance of differences between the means of two groups. p values < 0.05 were considered to indicate statistical significance.

For patch clamp experiments on cells, the percentage of activation was calculated as (I drug − I CTRL)/(I max − I CTRL), where I max was evaluated at the maximum voltage applied in the control (CTRL) condition. For patch clamp experiments on *Xenopus* oocytes, the percentage of activation of ZOL was calculated as (I drug − I CTRL)/(I Caps − I CTRL), referring to the amount of currents activated by the TRPV1-agonist capsaicin (10^{-5} M) at the same voltages.

5. Conclusions

TRPV1 channel can be a novel target for zoledronic acid and structurally related BP compounds, mediating the proliferative cytoprotective action of these drugs on osteoblasts. Further investigations are needed to clarify the binding site/s of this class of drugs on the TRPV1 channel. This mechanism may contribute to the anti-osteoporotic effect of zoledronic acid, as well as the pain-relieving effect. Beneficial effects of zoledronic acid can be expected in skeletal muscle where TRPV1 channel appear to play a role in activating hypertrophic signaling and cytoprotection [51,52].

6. Patents

Currently no patents are resulting from the work reported in this manuscript.

Author Contributions: Conceptualization, D.T. and R.S.; methodology, R.S., F.M., M.A., M.G.P.; software, F.M.; validation, A.S. and R.L.; investigation, R.S. and M.A.; drugs synthesis, M.G.P. and A.S.; writing—review and editing, all the co-authors; visualization, F.M., R.S.; supervision, R.L., A.S. and D.T.; project administration, A.S.; funding acquisition, A.S., R.L. and D.T.

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Neuroendocrinology of reproduction: Is gonadotropin-releasing hormone (GnRH) dispensable?

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ABSTRACT

Gonadotropin releasing hormone (GnRH) is a highly conserved neuroendocrine decapeptide that is essential for the onset of puberty and the maintenance of the reproductive state. First identified in mammals, the GnRH signaling pathway is found in all classes of vertebrates; homologues of GnRH have also been identified in invertebrates. In addition to its role as a hypothalamic releasing hormone, GnRH has multiple functions including modulating neural activity within specific regions of the brain. These various functions are mediated by multiple isoforms, which are expressed at diverse locations within the central nervous system. Here we discuss the GnRH signaling pathways in light of new reports that reveal that some vertebrate genomes lack GnRH1. Not only do other isoforms of GnRH not compensate for this gene loss, but elements upstream of GnRH1, including kisspeptins, appear to also be dispensable. We discuss routes that may compensate for the loss of the GnRH1 pathway.

1. Introduction

Gonadotropin-releasing hormone (GnRH) is the pivotal neuropeptide regulating fertility and reproduction in vertebrates (Gore, 2002). In vertebrates, at least fifteen GnRH isoforms have been identified (Lethimonier et al., 2004), where any given species has two or three forms of GnRH, each encoded by different genes. The different forms of GnRH are expressed in both neuronal and non-neuronal tissues and serve a number of distinct functions. Historically, GnRH has been known primarily as a hypothalamic hormone controlling the onset of puberty and the maintenance of fertility through the release of the gonadotropes, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the pituitary. For the purposes of this review the name "hypophysiotropic" will be used to refer to the hypothalamic reproductive form of GnRH.

GnRH has been extensively studied in mammals and fishes and as different isoforms of GnRH were discovered they were named according to the animal in which they were found, thus leading to a confusing body of literature. However, when the corresponding genes were identified, the different GnRH peptides could be classified based on the sequence of the corresponding genes and their location in the genome, clarifying the naming of GnRH isoforms.

In considering the function of the hypothalamic GnRH neurons, it is important to note that there are significant differences between the anatomy of fish and mammalian brains, both important model systems used to study the neuroendocrinology of GnRH. In mammals, GnRH is released from nerve endings into the hypophyseal portal system in a pulsatile manner to stimulate the synthesis of the gonadotropes, FSH and LH, from the anterior pituitary. Thus, the communication between the hypothalamic GnRH cells and the pituitary occurs *via* a localized circulatory system called the median eminence in which hypothalamic-releasing and -inhibiting hormones converge onto the portal capillary system of the ventral hypothalamus (Fig. 1A) (Kitahashi et al., 2013; Biran et al., 2015). However, this elaborate pituitary vasculature connecting the hypothalamus to the anterior pituitary gland typical of tetrapods is notably lacking in teleost fish (Peter et al., 1990), in which axons of the GnRH neurons project directly to post-synaptic sites in the pituitary (Fig. 1B). In addition, the neurohypophysis is greatly reduced in size. While structurally different from mammals, the teleost fish brain contains all the hypothalamic cell types identified in mammals (Machluf et al., 2011), including the GnRH containing cells localized to the parvocellular nucleus (Gomes et al., 2013). The lack of a median eminence in teleost fish is believed to be a derived trait because primitive fishes such as coelacanth, sturgeons, and lungfish (Ball, 1981; Gorbman, 1995), as well as cartilaginous fishes (Gaillard et al., 2018), contain a median eminence. Although hypothalamic structures differ between teleosts and mammals, it is generally thought that GnRH has remained the principal peptide controlling fertility, reproduction, and

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Yet these ligands differ in the number of amino acids: The GnRH peptides (in vertebrates and urochordates) are composed of 10 amino acids, whereas protostome, echinoderm, and amphioxus GnRH-like peptides are an 11- or 12-residue peptide that contains 2 amino acids after an N-terminal pyro-Glu. The protostome peptides, corazonin and adipokinetic hormones, are thought to be orthologous to the GnRH family, due to the presence of several amino acids typical of GnRHs and sequence similarity among their receptors and GnRH receptors, but the evolutionary relationship remains controversial (9, 10). The naming of the GnRH-like peptides used in the paper of Andreatta (ref. 3, tables S1–S3) is subject to discussion, as the use of "GnRH" to describe invertebrate peptides may imply a closer evolutionary relationship than exists [see Tsai (11) for a discussion on naming of invertebrate peptides].

In vertebrates, the regulation of many endocrine processes originates in the hypothalamus of the brain; this includes control of appetite, energy balance, and puberty. While a coordinated link between energy balance and reproduction is essential to ensure successful propagation of the species, vertebrates and invertebrates have evolved distinct mechanisms. Strikingly, only in the lineage leading to vertebrates (Fig.1), has GnRH ultimately been coopted into an endocrine function specialized for the stimulation of gonadotropin release from the anterior pituitary (12). This function does not appear to exist in the lineage of protostomes (Fig. 1); thus it is not surprising that Andreatta et al. (3) do not find a defect in reproductive function in the *crz1*$^{-/-}$ mutant animals. Yet the elucidation of downstream target genes of CRZ1 signaling involved with glycoprotein and carbohydrate metabolism harkens back to the ancestral necessity of guaranteeing sufficient resources (metabolic) to ensure successful reproduction. This is made more crucial in the marine worm, a uniparous animal whose "reproduce and die" strategy of survival places great pressure on the single reproductive event.

Recently, my laboratory (13) and others have shown that the zebrafish, *Danio rerio*, lacks the *gnrh1* gene and appears not to require GnRH as a reproductive peptide, leaving the mystery of what peptide has been coopted to control reproduction. A potential candidate, the gonadotropin-inhibitory hormone (GnIH), may also be a candidate for circalunar reproductive pathway in *Platynereis* (14, 15). In the grass puffer, a fish with a semilunar spawning cycle, genes encoding GnIH and its receptor (as well as Kiss2) showed distinct seasonal, daily, and circadian variations in the brain, suggesting common regulatory mechanisms involving melatonin, circadian clock, and water temperature (16).

The marine bristleworm is a nonstandard model system that represents a large group of animals, the polychaetes, with more than 10,000 described species encompassing a wide variety of reproductive strategies. Studies in the marine bristleworm will allow us to better understand the evolution and cooption of peptide-receptor signaling pathways that regulate environmental control of reproduction. The advent of gene-editing techniques allowed for the generation of the *crz1*$^{-/-}$ mutant, and bioinformatic techniques underlie the data mining necessary to elucidate metabolic pathways that appear to "cue" from the lunar cycle, thus allowing these widely dispersed marine animals to coordinate their only spawning event. What is lacking are data collected from the field that potentially could be extremely revealing, as "domestication" (maintaining animals in captivity) and social context strongly affect hormonal states and thus behavior. This is evidenced in attempts to explain individual variation in circulating hormone levels above breeding baselines in vertebrates (17) where cellular components of target tissues such as receptors, metabolizing enzymes, and other factors clearly modulate effects of peptide hormones. Ideally, samples of circulating CRZ1 peptide in premetamorphosis and postmetamorphosis bristleworms coordinated with the natural lunar cycle would confirm the results obtained in the laboratory, although authors (3) do acknowledge both the necessity and difficulty of obtaining such data. In the face of the climate crisis, the marine bristleworm may prove to be an indicator species we can use to assess hormonal and metabolic variations in response to environmental stress already evidenced in our oceans, with the potential of avoiding future catastrophes in the natural world (18).

In PNAS, Andreatta et al. build on previous work showing that the marine bristleworm *Platynereis dumerilii* has a circalunar (monthly) clock that interacts in an independent manner with the circadian clock (4).

The role of the moon in the coordination of reproduction is most obvious in aquatic species where it is important to coordinate broadcast spawning over large areas, as has been observed with spawning of coral reefs, some marine fishes, and crabs (19) (20). In the modern world illuminated by artificial lighting at all hours of the circadian clock, we often forget that the moon exerts a huge effect on the planet's ecosystems: The moon pulls the oceans, creating the tides (many animals respond to the tides as natural cues coordinating reproduction); the moon stabilizes Earth's climate by reducing variability in the axial tilt; and, finally, important for this paper, the moon can act as an environmental cue (zeitgeber) setting the genetically encoded biological clock that most organisms possess. The polychaete *Platynereis* has been shown previously to express circadian rhythm genes that can be modulated either directly or indirectly by the circalunar clock, yet the circalunar clock is independent of these gene oscillations (4). A striking finding in this paper (3) is that the CRZ1-like peptide appears to act as a link coordinating the sensation of the lunar cycle with the metabolic resources necessary for spawning. Future research will hopefully elucidate the mechanisms by which lunar cycle is transduced to a communal sensory signal coordinating the brain–reproductive axis in diverse populations of animals.

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Annex 12.- Organization of Scientific Events

CENTRO INTERDISCIPLINARIO DE Neurociencia de Valparaíso

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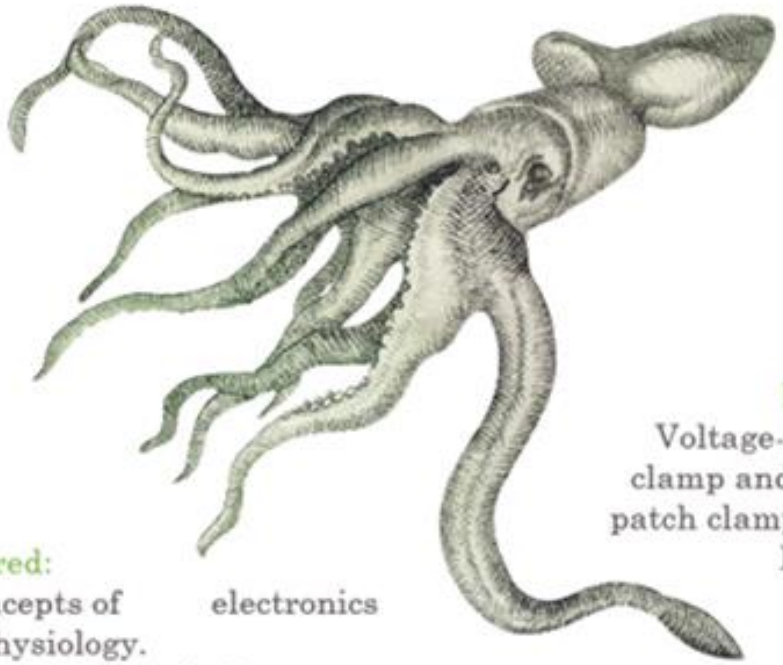

Lab activities

Voltage-clamp, patch clamp and voltage- and patch clamp fluorometry
Luminiscence
Resonance energy Transfer
Expression, purification and reconstitution of ion channels
Structural analysis
Electrophysiology and two-photon microscopy in neurons.

Topics covered:

General concepts of electronics for electrophysiology.
Expression and biophysical characterization of ion channels
Molecular rulers
Ion channel Structure
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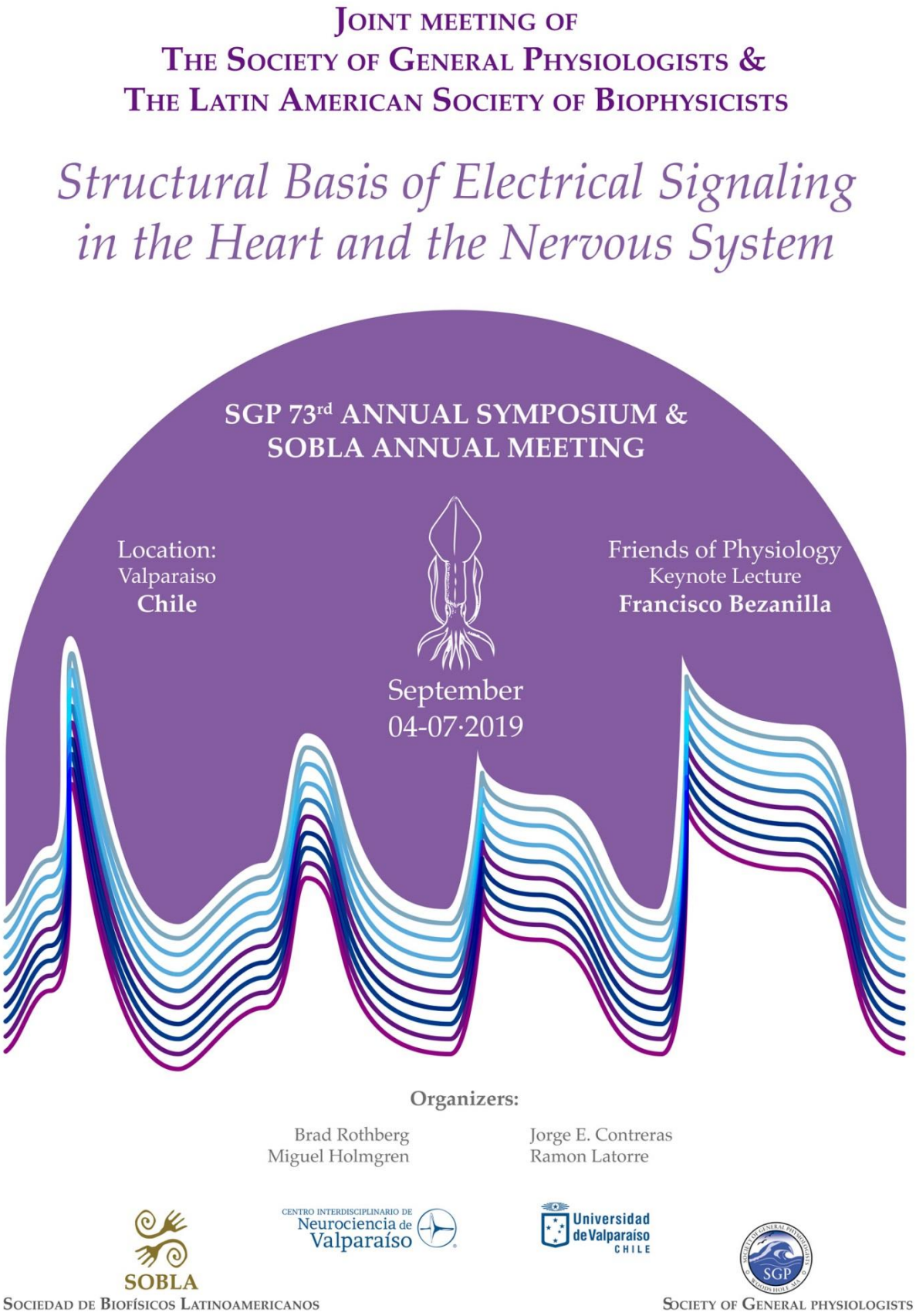
JOINT MEETING OF
THE SOCIETY OF GENERAL PHYSIOLOGISTS &
THE LATIN AMERICAN SOCIETY OF BIOPHYSICISTS
Structural Basis of Electrical Signaling in the Heart and the Nervous System
SGP 73rd ANNUAL SYMPOSIUM &
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Location:
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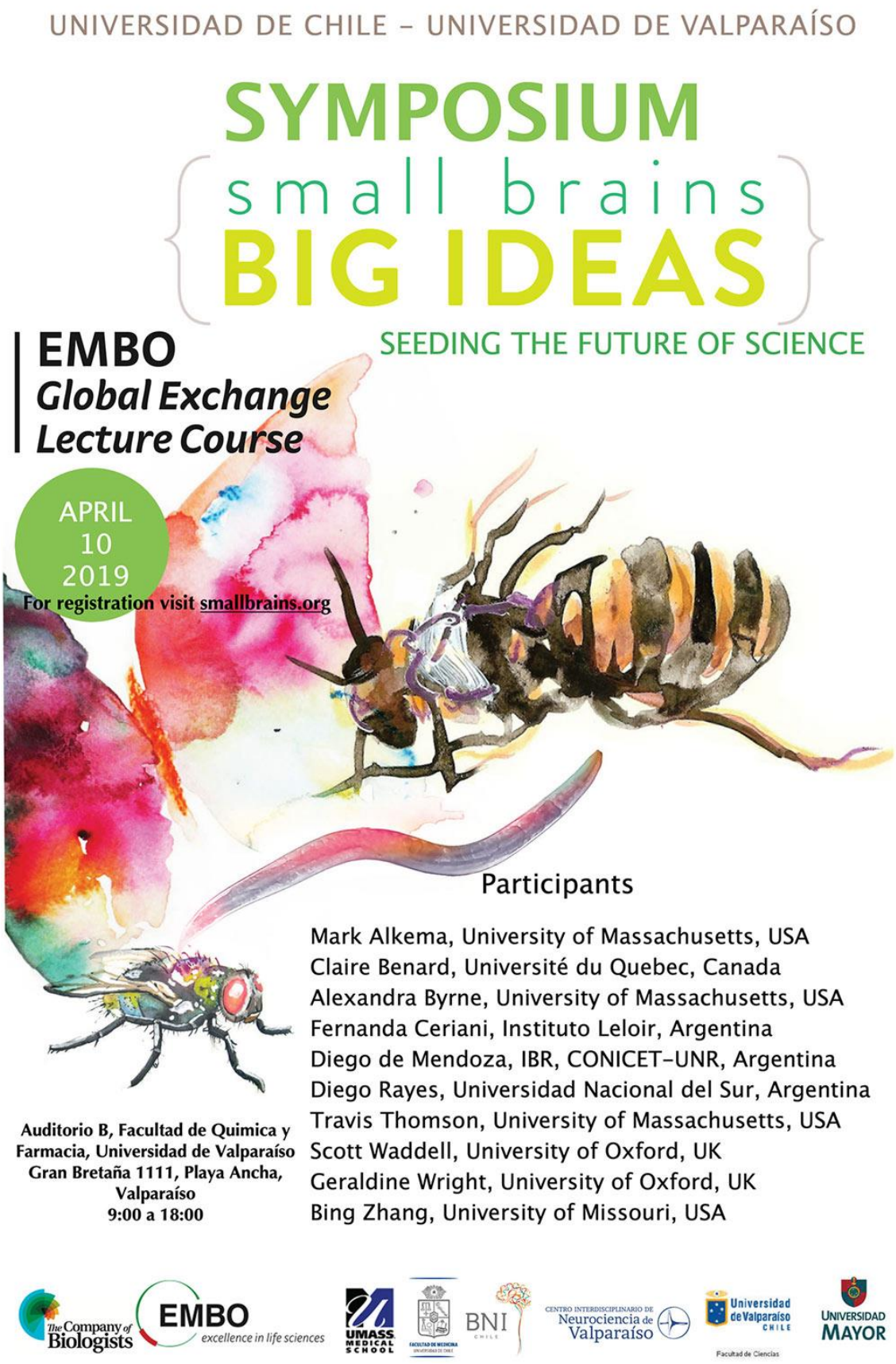
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Diego de Mendoza, IBR, CONICET–UNR, Argentina
Diego Rayes, Universidad Nacional del Sur, Argentina
Travis Thomson, University of Massachusetts, USA
Scott Waddell, University of Oxford, UK
Geraldine Wright, University of Oxford, UK
Bing Zhang, University of Missouri, USA
Auditorio B, Facultad de Quimica y Farmacia, Universidad de Valparaíso
Gran Bretaña 1111, Playa Ancha, Valparaíso
9:00 a 18:00
The Company of Biologists
EMBO
excellence in life sciences
UMASS MEDICAL SCHOOL
BNI
CENTRO INTERDISCIPLINARIO DE Neurociencia de Valparaíso
Universidad de Valparaíso CHILE
Facultad de Ciencias
UNIVERSIDAD MAYOR


"La interpretación de la realidad en el cerebro"
Ranulfo Romo
PhD Neurociencia
Jueves 11 de abril, 19:00 horas
Biblioteca Santiago Severín. Plaza Simón Bolívar 1653, Valparaíso.
Abierto a todo público.
Compartiremos un vino de honor con el expositor.
FESTIVAL
PUERTO DE IDEAS
CENTRO INTERDISCIPLINARIO DE
Neurociencia de
Valparaíso


Reunión Conjunta CINV-AC3E
Jueves 22 de Agosto, 2019
9:00 a 18:00 horas
CENTRO INTERDISCIPLINARIO DE
Neurociencia de
Valparaíso
Advanced Center
for Electrical and Electronic Engineering
Universidad
de Valparaíso
CHILE
UNIVERSIDAD TECNICA
FEDERICO SANTA MARIA

LACONEU

Latin American Summer School in Computational Neuroscience

LACONEU2019, V Latin American Summer School in Computational Neuroscience, tiene el agrado de invitar a la Comunidad Universitaria a las siguientes charlas

Psychedelics: therapeutic mechanisms
Dr. Robin Carthart-Harris
Head of Psychedelic Research
Center for Neuropsychopharmacology
Division of Brain Sciences, Imperial College London

Lunes 7 de enero 2019, 18:00hrs, C228, Avda. España 1680, Valparaíso, Universidad Técnica Federico Santa María

---

A Fundamental Novel View of Brain Function: Complexity, Criticallity & Consciousness
Dr. Dante Chialvo
Center of Complex Systems and Brain Sciencies
Universidad Nacional de San Martin, Argentina

Blindfold Learning of a Neural Metric for Discrimination
Dr. Thierry Mora
Laboratoire de Physique Statistique
École Normale Supérieure, France

Martes 8 de enero 2019, 17:00hrs, C228, Avda. España 1680, Valparaíso, Universidad Técnica Federico Santa María

---

What is Complexity Science and why it is important?
Dr. Henrik Jensen
Complex System Science
Imperial College London, England

Criticality as a Signature of Healthy Neural Systems
Dr. Dietmar Plenz
Chief of Sectio on Critical Brain Dynamics
National Institute of Mental Health, USA

Jueves 10 de enero 2019, 17:00hrs, C228, Avda. España 1680, Valparaíso, Universidad Técnica Federico Santa María

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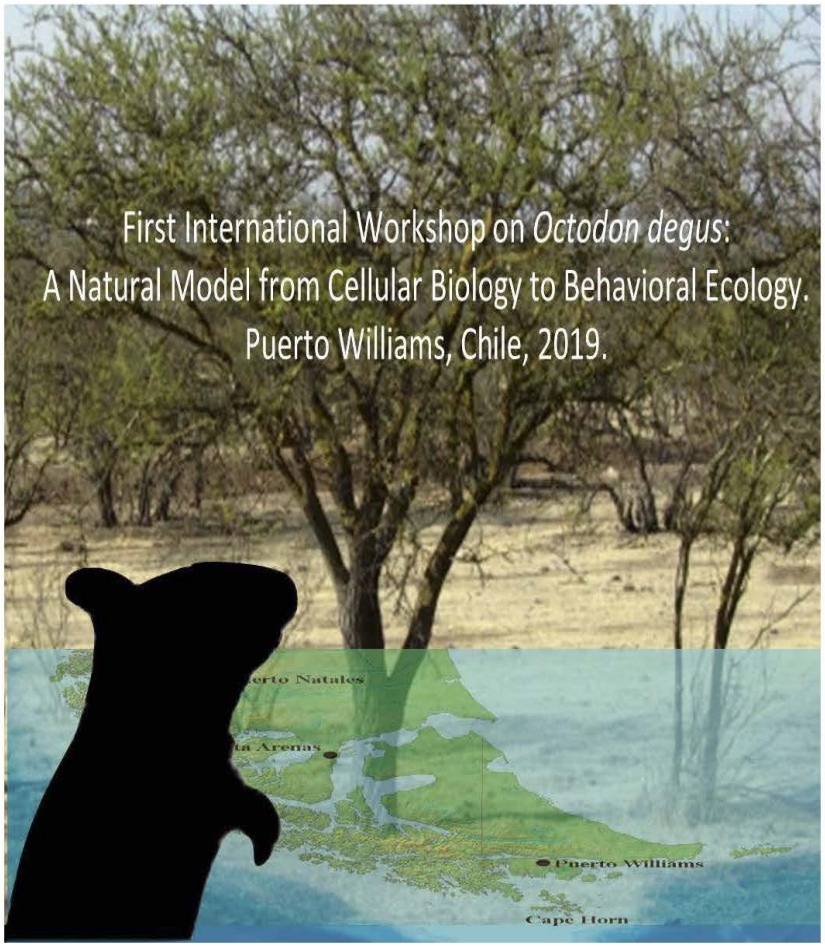
The Physics of Sleep and Alertness
Dr. Peter Robinson
Brain Dynamics Group, Complex System Group
The University of Sydney, Australia

Miércoles 9 de enero 2019, 17:00hrs, C228, Avda. España 1680, Valparaíso, Universidad Técnica Federico Santa María

AC3E CINV UNIVERSIDAD TECNICA FEDERICO SANTA MARIA CONICYT Universidad de Valparaíso CHILE INTERNATIONAL BRAIN IBRO RESEARCH ORGANIZATION ISCV


First International Workshop on *Octodon degus*:
A Natural Model from Cellular Biology to Behavioral Ecology.
Puerto Williams, Chile, 2019.
Puerto Williams
Cape Horn

Annex 13.- Outreach activities throughout the period


Tertulias Porteñas
Centro Interdisciplinario de Neurociencia de Valparaíso
INTELIGENCIA artificial
¿BENEFICIO O CONDENA?
MODERA
Patricio Fernández
Escritor, fundador del semanario The Clinic
INVITADOS
Tomás Pérez-Acle
Biólogo y Doctor en Biotecnología CINV
Andrés Claro
Doctor en filosofía y literatura
María Fernanda Pérez Trautmann
PhD en ciencias biológicas
Jueves 23 MAYO
19:00 hrs.
Aula Magna UV
Errazuriz 2120
Valparaiso
ORGANIZA
Centro Interdisciplinario de Neurociencia de Valparaíso
Universidad de Valparaíso CHILE
CON EL RESPALDO
milenio
MEDIA PATNERS
RVL 97 3fm
Empresa El Mercurio de Valparaíso
AUSPICIAN
Cirilo Hotel Armstrong
Tres Peces
Cocinamar
Casa Gourmet Altamira Valparaíso


Tertulias Porteñas
Centro Interdisciplinario de Neurociencia de Valparaíso
EL CEREBRO
y la eterna
JUVENTUD
INVITADOS
Claudio Hetz
Director del Instituto de Neurociencia Biomédica (BNI) de la U. de Chile
Paulina Urrutia
Actriz, directora del Teatro Camilo Henríquez
José Luis Dinamarca
Geriatra, Programa de Ortogeriatría en Hospital Dr. Gustavo Fricke
MODERA
Patricio Fernández
Escritor y columnista. Colaborador en New York Times, Diario El País y otros medios latinoamericanos.
Jueves
01
AGOSTO
19:00 hrs.
Hall Central del CENTEX,
Sotomayor 233,
Valparaíso.
ORGANIZA
Centro Interdisciplinario de Neurociencia de Valparaíso
Universidad de Valparaíso CHILE
AUSPICIAN
Cirilo Hotel Apostólico
Casa Ciencia Altamira Valparaíso
Cocinamar
Tres Peces
CON EL RESPALDO
milenio
MEDIA PATNERS
RVL 97.3fm
UCV
Empresa El Mercurio de Valparaíso


Tertulias Porteñas
Neurociencia de Valparaíso
AGUA
CAMBIO CLIMÁTICO
y el
futuro
de la especie humana
INVITADOS
Gino Casassa
Glaciólogo, Ingeniero Civil Hidráulico, Doctor en Glaciología.
Claudio Latorre
PhD en Biología Evolutiva, con especialización en Paleoecología, Paleoclimatología y Biogeografía/Botánica.
Rodrigo Mundaca
Vocero Movimiento de Defensa por el acceso al Agua, la Tierra y la protección del Medio Ambiente, MODATIMA.
MODERA
Patricio Fernández
Escritor y columnista. Colaborador en New York Times, Diario El País y otros medios latinoamericanos.
Sábado
30
NOVIEMBRE
11:00 am
Parque Cultural, EX CÁRCEL, Valparaíso.
Universidad de Valparaíso
Neurociencia de Valparaíso
milenio
Parque Cultural Valparaíso
ALTAMIRA
RVL 97.3fm
UCV
EL MERCURIO DE VALPARAÍSO


PRESENTACIÓN
SALA CONFERENCIAS
SURA
Corporación
del Libro y la Lectura
FAS
FESTIVAL
DE AUTORES
SANTIAGO
CINV
DEMENTE. EL CEREBRO
UN HUESO DURO DE ROER
Sábado 28 sept.
15:30 hrs
MEDIA PARTNER
ADN 91.7
EN ALIANZA
SEGUROS
sura

Annex 14.- Articles and Interviews

1. "They create a mathematical model to fight Fake news".
Newspaper: Las Últimas Noticias
April 29, 2019
Link: https://www.lun.com/Pages/NewsDetail.aspx?dt=2019-04-29&PaginaId=26&bodyid=0
Scope: National

Es un software diseñado para medir el comportamiento de las sociedades y que estudió la difusión del Ébola

Crean modelo matemático para luchar contra las fake news

CAMILA FIGUEROA

La vida transcurre en el clúster de capturo de Tomás Pérez-Acle. Un conglomerado de exactamente 256 procesadores que equivalen al poder de 256 cerebros computacionales unidos entre sí. Una verdadera bestia virtual.

En esa vida diseñada por el biólogo computacional habitan once millones de personas impalpables. Todas con emociones, características físicas y accesos a la información diferentes.

"Acá los días se llaman tiempo de cómputo. 120 días equivalen a 45 minutos", explica el investigador que cautivó al Premio Nacional de Ciencias Aplicadas Pablo Valenzuela, quien lo llevó al Laboratorio de Biología Computacional de la Fundación Ciencia y Vida, institución que dirige.

Simulación de sociedades

Pérez-Acle, también investigador del Instituto Milenio Centro Interdisciplinario de Neurociencia de Valparaíso, creó un software con el que puede simular el comportamiento de sociedades frente a distintas reglas.

El año pasado, cuenta, junto a su estudiante Alejandro Bernardin quisieron observar cómo se comportaban once millones de personas ante una enfermedad pandémica infecciosa: el Ébola. Así fue como lo envío a una pasantía de tres meses con el matemático canadiense Jonathan Dushoff, parte del equipo de respuesta al Ébola de la Organización Mundial de la Salud (OMS).

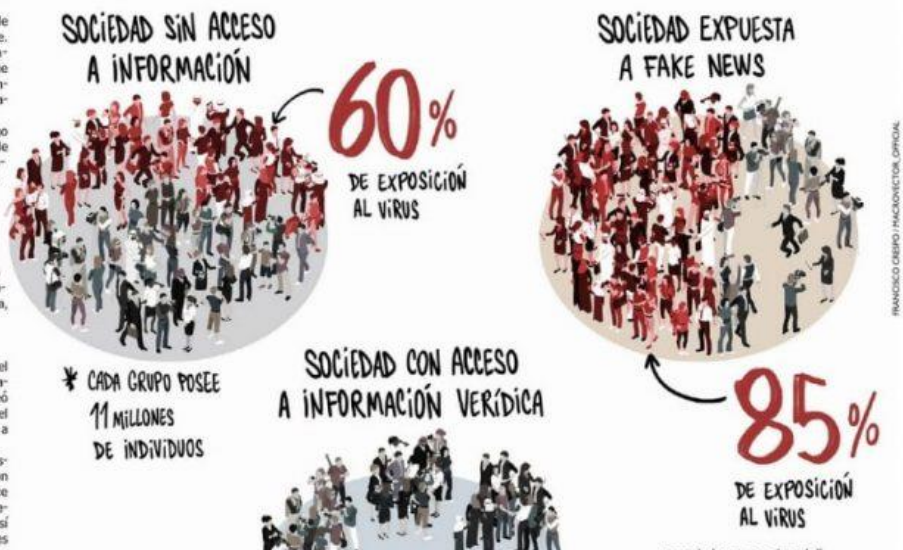
"Una pandemia es una enfermedad, un número reproductivo básico (R0) mayor a 2. Es decir, que una persona puede infectar en promedio a más de dos personas. Si eso ocurre, puede llegar a todo el mundo", explica.

Tomás Pérez-Acle analizó el comportamiento de la enfermedad en una población bien informada, en otra con noticias falsas y en una sin acceso a la información.

Para realizar el experimento utilizó tres tipos de civilizaciones: una sin acceso a la información, otra con acceso a información verídica y una con acceso a rumores, los llamados fake news.

"El Ébola tiene un R0 de alrededor de 2,2, es bajo pero pandémico. Es importante ese número", detalla. Y da un ejemplo. "El año 2003 apareció en China una supuesta pandemia llamada Síndrome Respiratorio Agudo (SARS). Pensaban que iba a morir mucha gente. Pero la OMS le encargó a tipos que hicieran los cálculos. Determinaron que el R0 era menor a uno. O sea, no era pandemia", asegura.

Agrega que el Ébola tiene una tasa de mortalidad entre un 25 y 90 por ciento, lo que es muy variable. "Uno espera que además de la biología, existan factores externos que influyen en cómo la enfermedad se dispersa. Ahí aparece nuestra hipótesis sobre cómo la información puede ser usada como una forma de modular la dispersión de una enfermedad infecciosa", explica.



FRANCISCO CRESPO / MACKXVICTOR_OFFICIAL

Dispersión

Pérez-Acle explica que la información se distribuye dependiendo de si su valencia es positiva o negativa. El rumor tiene valencia negativa y la información verdadera, positiva.

"La cantidad de personas que conoce información con valencia negativa (rumores) es mayor a la que conoce la positiva. Eso se ve con curvas. Con la información de valencia negativa la curva sube mucho y cae rápidamente. Los rumores son como montañas rusas. ¿Cuánto más alta es? Depende del valor de la información. Entre más negativo es el rumor, más es la cantidad de personas que lo sabe", dice.

El investigador también habla de canales de información. "Los rumores tienden a agrupan más rápido a los canales de información. O sea, un fake news está más rápido en todas las redes sociales que la noticia real. También se mide con curvas. La bajada de la curva del rumor es fome, lenta. La de la información fidedigna cae rápido", detalla.

Pérez-Acle modeló a los tres tipos de sociedades 1.000 veces en el súper computador que puede hacerlo todo al mismo tiempo. "Modelamos una sociedad como la chilena, conectada con la carretera Panamericana. El 20 por ciento de las once millones de personas tenía auto. Dejamos que la simulación corriera y vimos lo que pasó después de se entregan los mensajes", dice.

El científico demostró que la información fidedigna disminuye la probabilidad de que una persona con información positiva acepte los rumores como válidos. "No afecta la tasa de contagio, sino que la forma en que la información se transmite. Pero una persona que tiene más información positiva tiene menos probabilidades de contagiarse porque sabe que no debe estornudar sin taparse la boca, por ejemplo".

¿Y las conclusiones? Según Pérez-Acle, después de 120 días con el virus, el 69% de la sociedad que no tuvo acceso a la información estuvo expuesta a la enfermedad. O sea, estuvo en contacto con un infectado. "Cuando agregamos los rumores, esa tasa llegó al 85% y con la información fidedigna diminuyó al 20%. Los flujos información afectan la dispersión de esta enfermedad", finaliza.

Imitar fake news

El biólogo computacional está seguro que para combatir las fake news hay que usar la misma técnica. "Cuando se quiere combatir rumores, primero hay que entender cómo funcionan y combatirlos de la misma manera. No saco nada con decir que los antivacunas están equivocados porque sabemos cómo es la curva de la información de valencia negativa. Va a ser difícil que cambie de opinión porque las fake news apelan a las emociones, a lo inesperado y también siempre muestran algo que parece ser evidencia", cuenta.

Por ejemplo, cuenta, si una institución pretende realizar una campaña de vacunación, debiese utilizar la técnica del fake news para abarcar a más personas. "En vez de titular con que empezó la campaña, debiesen apelar a las emociones e indicar cuántos niños han muerto por no vacunarse contra el sarampión. Además deben usar todos los canales, Facebook, Twitter, Instagram, Youtube", destaca.

2. "Chilean enters the United States Academy of Sciencies"
2. Newspaper: Las Últimas Noticias
May 8, 2019
Link: http://www.lun.com/Pages/NewsDetail.aspx?dt=2019-05-08&PaginaId=6&bodyid=0
Scope: National

6 EL DÍA Miércoles 8 de mayo de 2019 / Las Últimas Noticias

Juan Carlos Sáez es el director alterno del CINV

Juan Carlos Sáez investiga las enfermedades crónicas

Chileno ingresa a la Academia de Ciencias de Estados Unidos

CAMILA FIGUEROA

La originalidad de la investigación de Juan Carlos Sáez, doctor en neurociencias del Albert Einstein College of Medicine of Yeshiva University, cautivó a los miembros de la Academia de Ciencias de Estados Unidos, quienes lo nombraron como nuevo científico asociado de la agrupación.

"Destacaron mis estudios sobre la inflamación en las enfermedades crónicas", celebra el director alterno del Instituto Milenio Centro Interdisciplinario de Neurociencias de la Universidad de Valparaíso.

Neurocientífico investiga un posible tratamiento a base de boldina, molécula extraída del boldo.

Inflamación

Hace quince años, cuenta Sáez, se le ocurrió demostrar la existencia de los hemicanales: unos agujeros que tienen las células de las personas que padecen enfermedades crónicas. Aunque sus estudios, aclara, los realiza solo con animales.

"Nadie había comprobado su existencia hasta que logramos hacerlo. Era como lo del agujero negro: estaba la hipótesis, pero faltaba la evidencia, la foto. Eso fue lo que hicimos con los hemicanales", grafica Sáez, también académico de la Universidad Católica.

-¿Dónde están exactamente esos agujeros, doctor Sáez?

-En las membranas de las células. Comunican el interior con el exterior. Son como unos hoyitos, por ahí salen y entran muchas cosas.

-¿Como cuáles?

-Por ahí se pierden moléculas muy útiles para la vida. Y también les entran cosas en exceso que no les hacen bien. Por ejemplo, el calcio.

-¿Qué hace el calcio?

-Cuando tenemos mucho de algo, nos mata. Si la célula se sobrecarga de calcio, se inflama y no puede manejar ese proceso. Eso provoca la muerte celular. Si mueren muchas células en un tejido, se genera la disfunción de un órgano. Por ejemplo, si es en el cerebro, se van perdiendo funciones. Si es en los músculos, puede provocar una miopatía y la persona no puede moverse.

-¿Los sanos tienen esos hoyos en las células?

-Sí, pero muy pocos. Recién estamos estudiando la razón de por qué tienen menos.

-¿Se pueden cerrar esos agujeros?

-Tenemos algunas moléculas que encajan perfectamente en esos hemicanales, que son estos agujeros de las células. Los tapan e impiden la entrada excesiva de calcio. Con la boldina (molécula derivada del boldo), por ejemplo, logramos cerrar los hemicanales. Aunque este reconocimiento de la academia no es por la boldina, es por toda la investigación de esos agujeros.

-¿De qué le sirve ser parte de la Academia de Ciencias de Estados Unidos, doctor?

-Es un tremendo honor. Creo que va a repercutir en que la gente le va a dar más credibilidad a mi trabajo cuando lo lea.

-¿Le pidieron algo a cambio?

-El requisito es no bajar la calidad. Esto es como un juicio. Hay que demostrar que lo que uno dice es verdad y para eso debe existir un análisis de los datos muy sólido. Acá los "podrían" no sirven, solo se requieren evidencias.

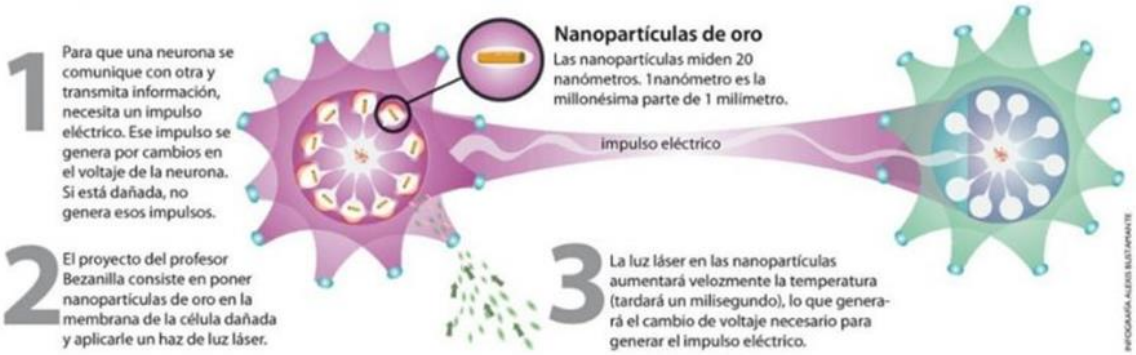
» "La gente le va a dar más credibilidad a mi trabajo cuando lo lea"
Juan Carlos Sáez

3: "Great idea: use gold nanoparticles to repair damaged neurons"
Newspaper: Las Últimas Noticias
September 4, 2019
Link: http://www.lun.com/Pages/NewsDetail.aspx?dt=2019-09-04&PaginaId=4&bodyid=0
Scope: National

Han testeado en retinas de ratas y en partes del cerebro

Macanuda idea: usar nanopartículas de oro para reparar neuronas dañadas

El biofísico Francisco Bezanilla creó un método para estimular el impulso nervioso. Usa un láser para cambiar el voltaje de las células.



Camila Figueroa

El imaginario del doctor en Biofísica Francisco Bezanilla es tan amplio que tuvo que inventar un concepto para describir lo que había descubierto. Lo llamó, dice, optocapacitancia. Un método que permite producir impulsos nerviosos entre las neuronas, pero de manera artificial. O sea, puede generar la electricidad necesaria, en caso de que fuese inexistente, para que la información se traspase de una célula cerebral a otra.

La técnica, asegura el académico del Departamento de Bioquímica de la Universidad de Chicago, consiste en adosarle a la membrana de las neuronas una serie de nanométricas partículas de oro.

Esas nanopartículas, explica Bezanilla, son capaces de absorber la luz y transformarla en calor en un tiempo extremadamente breve. "Un milisegundo como máximo", destaca el investigador del Instituto Milenio Centro Interdisciplinario de Neurociencia de la Universidad de Valparaíso (CINV).

Un cambio veloz de temperatura en la membrana de una neurona, dice el biofísico, provoca un cambio en el voltaje y justamente eso es lo que produce el impulso eléctrico que transmite la información entre neuronas.

Ramón Latorre, Premio Nacional de Ciencias Naturales, elogia el trabajo de Bezanilla. "Tiene una trayectoria muy exitosa en Estados Unidos, es de los biofísicos más reconocidos a nivel mundial. Toda su vida se ha dedicado a entender cómo se transmite el impulso nervioso. Esa es su contribución y está involucrado en un proyecto para activar a las neuronas con nanopartículas de oro, creó un área novedosa", destaca.

> **"Tiene una trayectoria muy exitosa en Estados Unidos"**
> Ramón Latorre, Premio Nacional de Ciencias Naturales, alabando al autor del proyecto

Electricidad e información

Bezanilla cuenta que como todas las células, las neuronas tienen una membrana muy delgada que separa su interior del exterior. "Mide unos cinco nanómetros (un nanómetro equivale a la millonésima parte de un milímetro)", explica el investigador.

La membrana de una neurona, dice Bezanilla, siempre tiene un potencial eléctrico. "Actúa como un capacitor, que es lo que separa la carga de un lado de otro. La membrana funciona como aislante y separa la carga negativa de la positiva. Eso se descubrió hace rato, pero nadie le había dado un uso hasta ahora", detalla.

El biofísico explica que para que el impulso eléctrico se genere y la información de una neurona sea traspasada a otra, es necesario un cambio de temperatura en la membrana, que a su vez, cambia el voltaje. En caso de que la neurona no pueda hacer aquel juego, dice Bezanilla, es posible realizarlo de manera artificial. Y justamente es lo que han probado en rebanadas de cerebro de ratas. "Si uno cambia la temperatura bruscamente, en un milisegundo, cambias el potencial de la membrana y estimulas el impulso nervioso. Todo es velocidad", menciona.


Francisco Bezanilla.

Partículas de oro

Las partículas de oro mucho tienen que ver con generar artificialmente el cambio brusco de temperatura. "Si pones las nanopartículas muy cerca de la membrana y después, bruscamente les das un impulso de luz con un láser, ese oro bruscamente se va a calentar. De esa manera cambia el voltaje y se inicia el potencial nervioso. Los pulsos de luz pueden ser entre un microsegundo a un milisegundo", detalla.

Esas nanopartículas de oro miden 20 nanómetros, tienen forma de cilindro y se caracterizan por absorber muy bien la longitud de onda de la luz verde. "Toda la energía que viene del láser se transforma en calor. También podemos usar infrarrojo", menciona.

Impulsos y ceguera

Una posible aplicación, dice, es en quienes han perdido los fotorreceptores que tiene la retina. "Son células que toman los fotones de la luz y los transforman en un impulso eléctrico, que se traslada por el nervio óptico hasta el cerebro que construye la imagen", aclara.

Dice Bezanilla que cuando los fotorreceptores mueren, las personas quedan ciegas. Sin embargo, a veces, las células que toman la información de las fotorreceptoras permanecen vivas. "Podemos fabricar un nuevo receptor con el oro. Lo hemos probado en retinas aisladas de ratas. En un futuro, es posible que se inyecten las partículas a través del globo del ojo, pero las personas tendrían que usar unos anteojos con un láser que capte la luz", dice el científico. "Me gusta la electricidad".

-¿Ha hecho más cosas, doctor?

-Fabriqué un televisor para el mundial de 1962. No sé si usted estaba viva, pero en esa época nadie tenía tele y había que ver los partidos. Como yo era un radioaficionado, fabriqué un transmisor. Desde chiquitito que me gusta este cuento.

Bezanilla expondrá este miércoles, a las 17.30 horas, en el Parque Cultural de Valparaíso (https://bit.ly/2lQjvj4).

4. “Books quote: “DeMente. El cerebro un hueso duro de roer”. When scientist helps to understand neuroscience”.
Web: El Mostrador/Podcast: Spotify
July 14, 2019
Link: https://www.elmostrador.cl/cultura/2019/07/14/cita-de-libros-demente-el-cerebro-un-hueso-duro-de-roer-los-cientificos-nos-ayudan-a-entender-la-neurociencia/
Scope: International

5. "TVN launches new neuroscience program".
Newspaper: El Mercurio
September 23, 2019
Link: https://cinv.uv.cl/neuropolis/
Scope: National

Bruno Grossi se someterá a distintas pruebas para resolver dudas sobre la neurociencia.

TVN estrena nuevo programa de neurociencia

MARTÍN CIFUENTES

Un ex chico *reality* recorrerá Valparaíso, Viña del Mar, Quintero, Maitencillo y Santiago, entre otras paradas, develando algunas de las principales dudas de la neurociencia. Cómo se generan las memorias en el cerebro o cómo afectan las drogas y adicciones al organismo son solo algunas de las temáticas que podrán verse a partir de este sábado, a las 14:30 horas, en "Neurópolis" (TVN) y que tendrán como protagonista a Bruno Grossi, exparticipante de "1810" y doctor en Ecología y Biología Evolutiva.

El programa, que ganó un fondo del Consejo Nacional de Televisión en 2015, fue realizado por la productora Puerto Visual en conjunto con el Centro Interdisciplinario de Neurociencia de Valparaíso (CINV) y demoró cerca de dos años en realizarse. Ignacia Imboden, productora ejecutiva, afirma intentaron hacer que el espacio se diferenciara de cualquier otro. "Todos son muy científicos y no tienen mix de voces. Este es interdisciplinario y cultural", dice sobre la producción en la que participarán neurocientíficos, artistas, deportistas y masajistas, además de gente de otras áreas. La sexóloga Raffaella Di Girolamo, el físico César Hidalgo, el director de orquesta Paolo Bortolameolli y la actriz pornográfica Amarna Miller son algunos de los entrevistados.

"Neurópolis" debuta el sábado a las 14:30 horas y tratará temas vinculados a la generación de memorias y efectos de las drogas, entre otros.

Al inicio de cada capítulo, Grossi planteará un problema y buscará la respuesta a través de las distintas voces. El también premio Nobel Alternativo en 2015 realizará experimentos ciudadanos y se someterá a sí mismo a distintas pruebas físicas. Para explicar las temáticas, Imboden ejemplifica: "En el capítulo de la memoria, hay una mujer que explica el efecto de haber perdido sus fotos familiares en el incendio de Valparaíso. Y después hay un neurocientífico que profundiza en dónde se generan esas memorias y cómo se altera. Entonces son cosas simples, enfocadas a orientar el tema a lo científico".

Según comenta Juan Carlos García, director ejecutivo del CINV, el proyecto nace de uno previo realizado con la productora Cábala ("Neuromantes"). "Quisimos transformar esto en una nueva forma de hacer TV abierta", comenta el ejecutivo, y explica que el motivo de la elección de Valparaíso se debe a motivación por la consolidación de la ciudad como "el lugar de conocimiento".

La serie, que contará con ocho episodios de 50 minutos, será la primera transmitida en TV abierta en ser concebida de la mano de un centro científico.

6. "Scientists call for ending schedule changes"
Newspaper: La Tercera
August 29, 2019
Link: https://cinv.uv.cl/john-ewer-ley-cambio-horario/
Scope: National

NACIONAL

Científicos piden terminar con los cambios de hora

Agrupación que reúne a médicos internacionales publicó un informe que exhibe las consecuencias de esta modificación.

C. Yáñez / P. Sepúlveda

La discusión por el uso de los horarios de verano o invierno ya ha sido zanjada en muchas partes. Por ejemplo, en Europa, Rusia decidió en 2014 quedarse solo con el horario de invierno, sin modificaciones, mientras que la Unión Europea acordó que el último domingo de marzo de 2021 será el último cambio de hora.

En Chile, el sábado 7 de septiembre habrá un nuevo cambio para adoptar el horario de verano. Cuando sean las 0 horas, los relojes deberán avanzar hasta la 1.00.

Eso, pese a que médicos y científicos ya fijaron una posición: "El horario de verano se debe abolir", dijo la Sociedad de Investigación sobre Ritmos Biológicos (SRBR, por sus siglas en inglés), agrupación científica que reúne a médicos e investigadores de todo el mundo y que promueve la investigación del reloj humano.

Un estudio del SRBR, publicado en junio, recomendó a los gobiernos elegir "un horario estándar permanente y eliminar los cambios de horario de verano o eliminar el horario de verano permanente".

"La elección del horario de verano es política y, por lo tanto, se puede cambiar", señala el artículo.

John Ewer, neurobiólogo del Centro Interdisciplinario de Neurociencia de Valparaíso (CINV), de la U. de Valparaíso, explica que se deben eliminar estos cambios, porque el cuerpo no se acostumbra. "No solo no tiene ventajas energéticas, la principal razón para adoptar el horario de verano, sino que es malo para la salud, genera muertes evitables. Es la razón de peso para dejar de cambiar la hora y adoptar, definitivamente, el horario de invierno", dice.

Luz y salud

Según el documento de SRBR, el reloj del cuerpo debe estar sincronizado con la luz solar, porque biológicamente toda la fisiología humana se activa durante el día y descansa en la noche.

El horario de verano crea problemas que pueden ser a corto plazo (agudos) o a largo plazo (crónicos).

"Los primeros días después del cambio repentino al horario hay efectos agudos que incluyen una menor duración del sueño, peor desempeño y peor salud; mayor riesgo de ataques cardíacos (en comparación con otras semanas) y más accidentes de tránsito", dice el estudio.

Los efectos crónicos pueden durar en verano, porque en muchas personas los relojes sociales (hora fijada por ley) y los corporales permanecen configurados en diferentes momentos. Esto se traduce en menos horas de sueño, disminución de la esperanza de vida, trastornos mentales, del sueño, cognitivos.

Lo lógico, dice Ewer, es adoptar el huso horario que más se acerque al reloj biológico y que en Chile debería ser el de invierno.

Evelyn Benavides, neurólogo de Clínica Vespucio, especialista en trastornos de sueño, cree que "lo ideal sería mantener un horario fijo y, dadas las características geográficas de cada zona, iniciar las actividades una hora más temprano en la zona norte, donde sale el sol antes, y en la zona sur iniciar las actividades una hora más tarde".

El neurólogo de Clínica Ciudad del Mar, Enzo Rivera, dice que los cambios de hora son negativos. "En los niños el sueño perdido afecta el rendimiento escolar. En el sueño se producen los mecanismos de consolidación de memoria y aprendizaje, y el cansancio y somnolencia tienen un impacto en la atención en clases".

Javiera Castro, doctorada en Ciencias Biomédicas de la U. de Chile, dice que dormir una hora menos un día no tiene efectos neurológicos importantes, pero sí resulta incómodo, porque el cuerpo tiene un ritmo acostumbrado con cierta luz, "y que de un día para otro se cambie, hace que el cuerpo se active más tarde".

Pedro Moya, neurólogo de Clínica Las Condes, explica que la mayoría de las personas, sobre todo de las ciudades, ya tiene una desregularización del ciclo día noche y actividad descanso que se regula con la luz del sol, debido al estrés, la luz artificial, trabajo extra, pantallas, ruidos, cambios de temperatura, entre otros factores.

¿QUIÉNES CAMBIAN DE HORA?

Algunos países africanos jamás han recurrido al horario diferenciado. Otros como los europeos, dejarán de hacerlo en 2021. Brasil, Colombia y Argentina, tampoco lo hacen.

FUENTE: TimeDate

LA TERCERA

"No solo no tiene ventajas energéticas, sino que es malo para la salud; genera muertes evitables".

JOHN EWER
U. DE VALPARAÍSO

EVIDENCIA CIENTÍFICA

ACUMULACIÓN
Según el SRBR, los problemas derivados del cambio de hora pueden ser a corto plazo (agudos) o a largo plazo (crónicos).

CORAZÓN
El estudio señala también que estos continuos cambios horarios generan un mayor riesgo de ataques cardíacos en la población.

ACCIDENTES
Estadísticamente, en las semanas posteriores al cambio se producen más accidentes de tránsito.

CEREBRO
La evidencia científica también habla de trastornos mentales, del sueño y cognitivos.