Annex 3:
Publications

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REVIEW
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Oxytocin and Vasopressin Receptor Gene Polymorphisms: Role in Social and Psychiatric Traits

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Oxytocin (OXT) and arginine-vasopressin (AVP) are two phylogenetically conserved neuropeptides that have been implicated in a wide range of social behaviors. Although a large body of research, ranging from rodents to humans, has reported on the effects of OXT and AVP administration on affiliative and trust behaviors, and has highlighted the genetic contributions of OXT and AVP receptor polymorphisms to both social behaviors and to diseases related to social deficits, the consequences of peptide administration on psychiatric symptoms, and the impact of receptor polymorphisms on receptor function, are still unclear. Despite the exciting advances that these reports have brought to social neuroscience, they remain preliminary and suffer from the problems that are inherent to monogenetic linkage and association studies. As an alternative, some studies are using polygenic approaches, and consider the contributions of other genes and pathways, including those involving DA, 5-HT, and reelin, in addition to OXT and AVP; a handful of report are also using genome-wide association studies. This review summarizes findings on the associations between OXT and AVP receptor polymorphism, social behavior, and psychiatric diseases. In addition, we discuss reports on the interactions of OXT and AVP receptor genes and genes involved in other pathways (such as those of dopamine, serotonin, and reelin), as well as research that has shed some light on the impact of gene polymorphisms on the volume, connectivity, and activation of specific neural structures, differential receptor expression, and plasma levels of the OXT and AVP peptides. We hope that this effort will be helpful for understanding the studies performed so far, and for encouraging the inclusion of other candidate genes not explored to date.

Keywords: SNP, SSR, psychiatric disorders, dopamine, serotonin, polygenic trait, quantitative traits loci, GWAS

In addition, an open challenge is to determine the exact mechanism by which neuropeptides influence psychiatric symptoms. Although some reports are shedding light on the impact of different gene polymorphisms on socially relevant behaviors and their associated psychiatric disorders, the complete pathways between genes, gene interactions and behavior is still a black dark forest. As told by Chomsky, "there's a famous joke about a drunk under a lamppost looking for a pencil dropped on the ground. Somebody comes up and asks 'What are you looking for?' He says, 'I'm looking for a pencil that I dropped.' 'Well, where did you drop it?' He says, 'Oh, I dropped it across the street.' 'Well, why are looking here?' 'This is where there is light.' That's the way the sciences work. [...] If you try to move it a little further, maybe ultimately you'll get across the street."

AUTHOR CONTRIBUTIONS

MA, MM, MR, AR, and JE had fun conceiving, drafting and critically revising this review.

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REFERENCES

Aebi, M., van Donkelaar, M. M., Poelmans, G., Buitelaar, J. K., Sonuga-Barke, E. J., Faraone, S. V., et al. (2015). Gene-set and multivariate genome-wide association analysis of oppositional defiant behavior subtypes in attention-deficit/hyperactivity disorder. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* doi: 10.1002/ajmg.b.32346. [Epub ahead of print].

Amir, R. E., Van den Veyer, I. B., Wan, M., Tran, C. Q., Francke, U., and Zoghbi, H. Y. (1999). Rett syndrome is caused by mutations in X-linked M ECP2, encoding m ethyl-CpG -binding protein 2. *Nat. Genet.* 23, 185–188. doi: 10.1038/13810

Anagnostou, E., Soorya, L., Chaplin, W., Bartz, J., Halpern, D., Wasserman, S., et al. (2012). Intranasal oxytocin versus placebo in the treatment of adults with autism spectrum disorders: a randomized controlled trial. *Mol. Autism.* 3, 16. doi: 10.1186/2040-2392-3-16

Aoki, Y., Yahata, N., Watanabe, T., Takano, Y., Kawakubo, Y., Kuwabara, H., et al. (2014). Oxytocin improves behavioural and neural deficits in inferring others' social emotions in autism. *Brain* 137, 3073–3086. doi: 10.1093/brain/awu231

Apter-Levy, Y., Feldman, M., Vakart, A., Ebstein, R. P., and Feldman, R. (2013). Impact of maternal depression across the first 6 years of life on the child's mental health, social engagement, and empathy: the moderating role of oxytocin. *Am. J. Psychiatry* 170, 1161–1168. doi: 10.1176/appi.ajp.2013.12121597

Auyeung, B., Lombardo, M. V., Heinrichs, M., Chakrabarti, B., Sule, A., Deakin, J. B., et al. (2015). Oxytocin increases eye contact during a real-time, naturalistic social interaction in males with and without autism. *Transl. Psychiatry* 5:e507. doi: 10.1038/tp.2014.146

Bakermans-Kranenburg, M. J., and Van Ijzendoorn, M. H. (2013). Sniffing around oxytocin: review and meta-analyses of trials in healthy and clinical groups with implications for pharmacotherapy. *Transl. Psychiatry* 3:e258. doi: 10.1038/tp.2013.34

Bakermans-Kranenburg, M. J., and van Ijzendoorn, M. H. (2014). A sociability gene? Meta-analysis of oxytocin receptor genotype effects in humans. *Psychiatr. Genet.* 24, 45–51. doi: 10.1097/YPG.0b013e3283643684

Bartz, J., Simeon, D., Hamilton, H., Kim, S., Crystal, S., Braun, A., et al. (2011). Oxytocin can hinder trust and cooperation in borderline personality disorder. *Soc. Cogn. Affect. Neurosci.* 6, 556–563. doi: 10.1093/scan/nsq085

Baumgartner, T., Heinrichs, M., Vonlanthen, A., Fischbacher, U., and Fehr, E. (2008). Oxytocin shapes the neural circuitry of trust and trust adaptation in humans. *Neuron* 58, 639–650. doi: 10.1016/j.neuron.2008.04.009

Beery, A. K., Lacey, E. A., and Francis, D. D. (2008). Oxytocin and vasopressin receptor distributions in a solitary and a social species of tuco-tuco (Ctenomys haigi and *Ctenomys sociabilis*). *J. Comp. Neurol.* 507, 1847–1859. doi: 10.1002/cne.21638

Bell, C. J., Nicholson, H., Mulder, R. T., Luty, S. E., and Joyce, P. R. (2006). Plasma oxytocin levels in depression and their correlation with the temperament dimension of reward dependence. *J. Psychopharmacol.* 20, 656–660. doi: 10.1177/0269881106060512

Benjamin, D. J., Cesarini, D., van der Loos, M. J. H. M., Dawes, C. T., Koellinger, P. D., Magnusson, P. K. E., et al. (2012). The genetic architecture of economic and political preferences. *Proc. Natl. Acad. Sci. U.S.A.* 109, 8026–8031. doi: 10.1073/pnas.1120666109

Bethlehem, R. A., Van Honk, J., Auyeung, B., and Baron-Cohen, S. (2013). Oxytocin, brain physiology, and functional connectivity: a review of intranasal oxytocin fMRI studies. *Psychoneuroendocrinology* 38, 962–974. doi: 10.1016/j.psyneuen.2012.10.011

Boccia, M. L., Petrusz, P., Suzuki, K., Marson, L., and Pedersen, C. A. (2013). Immunohistochemical localization of oxytocin receptors in human brain. *Neuroscience* 253, 155–164. doi: 10.1016/j.neuroscience.2013.08.048

Brookfield, J. F. (2010). Q&A: promise and pitfalls of genome-wide association studies. *BMC Biol.* 8:41. doi: 10.1186/1741-7007-8-41

Brosnan, S. F., Parrish, A., Beran, M. J., Flemming, T., Heimbauer, L., Talbot, C. F., et al. (2011). Responses to the Assurance game in monkeys, apes, and humans using equivalent procedures. *Proc. Natl. Acad. Sci. U.S.A.* 108, 3442–3447. doi: 10.1073/pnas.1016269108

Caldwell, H. K., Lee, H. J., Macbeth, A. H., and Young, W. S. (2008). Vasopressin: behavioral roles of an "original" neuropeptide. *Prog. Neurobiol.* 84, 1–24. doi: 10.1016/j.pneurobio.2007.10.007

Caldwell, H. K., Stephens, S. L., and Young, W. S. (2009). Oxytocin as a natural antipsychotic: a study using oxytocin knockout mice. *Mol. Psychiatry* 14, 190–196. doi: 10.1038/sj.mp.4002150

Cardon, L. R., and Abecasis, G. R. (2003). Using haplotype blocks to map human complex trait loci. *Trends Genet.* 19, 135–140. doi: 10.1016/S0168-9525(03)00022-2

Cardoso, C., Kingdon, D., and Ellenbogen, M. A. (2014). A meta-analytic review of the impact of intranasal oxytocin administration on cortisol concentrations during laboratory tasks: moderation by method and mental health. *Psychoneuroendocrinology* 49, 161–170. doi: 10.1016/j.psyneuen.2014.07.014

Cesarini, D., Dawes, C. T., Fowler, J. H., Johannesson, M., Lichtenstein, P., and Wallace, B. (2008). Heritability of cooperative behavior in the trust game. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3721–3726. doi: 10.1073/pnas.0710069105

Cesarini, D., Dawes, C. T., Johannesson, M., Lichtenstein, P., and Wallace, B. (2009). Genetic variation in preferences for giving and risk taking. *Q. J. Econ.* 12, 809–842. doi: 10.1162/qjec.2009.124.2.809

Chang, W. H., Lee, I. H., Chen, K. C., Chi, M. H., Chiu, N. T., Yao, W. J., et al. (2014). Oxytocin receptor gene rs53576 polymorphism modulates oxytocin-dopamine interaction and neuroticismtraits –a SPECT study. *Psychoneuroendocrinology* 47, 212–220. doi: 10.1016/j.psyneuen.2014.05.020

Chen, F. S., Kumsta, R., von Dawans, B., Monakhov, M., Ebstein, R. P., and Heinrichs, M. (2011). Common oxytocin receptor gene (OXTR) polymorphism and social support interact to reduce stress in humans. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19937–19942. doi: 10.1073/pnas.1113079108

Chen, L., Pan, H., Tuan, T. A., Teh, A. L., MacIsaac, J. L., Mah, S. M., et al. (2015). Brain-derived neurotrophic factor (BDNF) Val66Met polymorphism influences

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JOURNAL OF LIPID RESEARCH ASBMB

Dietary rescue of altered metabolism gene reveals unexpected *Drosophila* mating cues[S]

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Abstract To develop and reproduce, animals need long-chain MUFAs and PUFAs. Although some unsaturated FAs (UFAs) can be synthesized by the organism, others must be provided by the diet. The gene, *desat1*, involved in *Drosophila melanogaster* UFA metabolism, is necessary for both larval development and for adult sex pheromone communication. We first characterized *desat1* expression in larval tissues. Then, we found that larvae in which *desat1* expression was knocked down throughout development died during the larval stages when raised on standard food. By contrast pure MUFAs or PUFAs, but not saturated FAs, added to the larval diet rescued animals to adulthood with the best effect being obtained with oleic acid (C18:1). Male and female mating behavior and fertility were affected very differently by preimaginal UFA-rich diet. Adult diet also strongly influenced several aspects of reproduction: flies raised on a C18:1-rich diet showed increased mating performance compared with flies raised on standard adult diet. Therefore, both larval and adult *desat1* expression control sex-specific mating signals. A similar nutrigenetics approach may be useful in other metabolic mutants to uncover cryptic effects otherwise masked by severe developmental defects.—Bousquet, F., I. Chauvel, J. Flaven-Pouchon, J-P. Farine, and J-F. Ferveur. **Dietary rescue of altered metabolism gene reveals unexpected *Drosophila* mating cues. *J. Lipid Res.* 2016. 57: 443–450.**

Supplementary key words lipid • fitness • hydrocarbon • *cis*-vaccenyl acetate

Lipids are widely used by plants and animals. In particular, FAs are components of the cell membrane and are also involved in cellular signaling (1–3). In animals, FAs are necessary for many functions related to development and reproduction, but the link between these two aspects is not clear (4–6). FAs can vary both in their carbon chain length and in their level of unsaturations (i.e., number of double bonds on the carbon chain). FAs can either have no double bonds [called saturated FAs (SFAs)], or have one or more double-bond(s) (MUFAs and PUFAs, respectively). If animals can only synthesize some unsaturated FAs (UFAs), they are auxotroph for others, which they must therefore find in their diet. Desaturase enzymes often play a pivotal role in the FA metabolism of plants, vertebrates, and invertebrates (7–9). Genetic alteration of FA-metabolism enzymes can induce severe human diseases (10–12), underscoring their importance.

The high genetic conservation between *Drosophila melanogaster* and vertebrates makes it a valuable experimental model organism to study metabolic functions, including lipid metabolism (7, 13, 14), however with some limitation (15). This invertebrate species, which is particularly suitable to study the relationship between nutriments and gene function ("nutrigenetics, nutrigenomics"), is also used as a translational model (16). A good picture of nutrigenetic conservation is provided by a peculiar dietary treatment ("Lorenzo oil," a mixture of UFAs), which can partially cure adrenoleukodystrophy, a fatal X chromosome-linked brain disease resulting in the accumulation of very long chain FAs (VLCFAs) (17). Interestingly, a *Drosophila* type of Lorenzo oil made with UFAs can cure an adrenoleukodystrophy-related neuronal degenerative model in the fly (18).

In *D. melanogaster*, the Δ^9-desaturase gene, *desat1*, is required both for development and reproduction; null *desat1* mutants die during larval life (19, 20), whereas hypomorphic *desat1* mutant flies show altered sex pheromone production and perception. The pleiotropic effect

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Abbreviations: AEL, after egg laying; C16:0, palmitic acid; C16:1, palmitoleic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, linolenic acid; CH, cuticular hydrocarbon; CS, Canton-S; cVA, *cis*-vaccenyl acetate; dSREBP, *Drosophila* sterol regulatory element-binding protein; fR, *desat1* RNAi knockdown; L1, L2, L3, first, second, and third larval instars; PRR, putative regulatory region; SFA, saturated FA; UFA, unsaturated FA; VLCFA, very long chain FA; 7,11-HD, 7,11-heptacosadiene; 7-T, 7-tricosene.

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Original Article

Original Article

Changes in Olfactory Receptor Expression Are Correlated With Odor Exposure During Early Development in the zebrafish (*Danio rerio*)

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Abstract

We have previously shown that exposure to phenyl ethyl alcohol (PEA) causes an increase in the expression of the transcription factor *otx2* in the olfactory epithelium (OE) of juvenile zebrafish, and this change is correlated with the formation of an odor memory of PEA. Here, we show that the changes in *otx2* expression are specific to βPEA: exposure to αPEA did not affect *otx2* expression. We identified 34 olfactory receptors (ORs) representing 16 families on 4 different chromosomes as candidates for direct regulation of OR expression via Otx2. Subsequent in silico analysis uncovered Hnf3b binding sites closely associated with Otx2 binding sites in the regions flanking the ORs. Analysis by quantitative polymerase chain reaction and RNA-seq of OR expression in developing zebrafish exposed to different isoforms of PEA showed that a subset of ORs containing both Otx2/Hnf3b binding sites were downregulated only in βPEA-exposed juveniles and this change persisted through adult life. Localization of OR expression by in situ hybridization indicates the downregulation occurs at the level of RNA and not the number of cells expressing a given receptor. Finally, analysis of immediate early gene expression in the OE did not reveal changes in *c-fos* expression in response to either αPEA or βPEA.

Key words: *c-fos*, *otx2*, phenyl ethyl alcohol, RNA-seq

Introduction

How the activation of olfactory receptors (ORs) leads to odor recognition is still unknown due to the large number of ORs and the wide variety of compounds that can form an odor (Mombaerts 1999; DeMaria and Ngai 2010), but clearly experience and neural plasticity play a role in odor perception and discrimination (for review, see Wilson and Stevenson 2003). Olfactory imprinting, a type of olfactory memory that is formed during early development and retained throughout life without reinforcement, has been described in invertebrates (McCall and Eaton 2001; Remy and Hobert 2005) and vertebrates (Hasler and Scholz 1983; Hudson and Distel 1998; Harden et al. 2006). This behavior is dependent upon local olfactory cues experienced during early development and has been studied extensively in pacific salmon, animals that retain a memory of home stream odors for life and use this memory to return and spawn (Hasler and Scholz 1983). Studies using phenyl ethyl alcohol (PEA) as an artificial odorant to imprint juveniles and bait the adults to PEA-marked sites (Hasler and Scholz 1983; Nevitt et al. 1994) demonstrated that the olfactory epithelia in PEA-imprinted fish showed a strong physiological response to PEA in comparison to the nonimprinted animals (Nevitt et al. 1994), supporting a role for peripheral

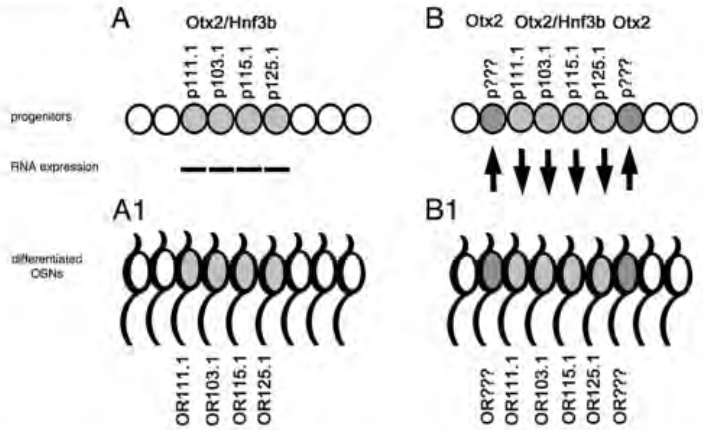

Figure 9. Regulation of OR expression may enhance signal-to-noise ratio though altering levels of OR RNA. (**A**) In control animals, progenitors of OSNs express *OR111-1*, *OR103-1* (chromosome 15), *OR115-1*, and *OR125-1* (chromosome 21) at a baseline level (dashed lines) in differentiated OSNs. (**A1**) The same ORs in animals exposed to βPEA (**B**) are repressed by the Otx2/Hnf3b transcription factors and show decreased levels of OR RNA (arrows) in differentiated OSNs (**B1**). Otx2 alone may mediate the upregulation of ORs responding to βPEA (B, B1, OR???), yet to be identified.

motifs (Figure 9A, control) are further repressed in the presence of βPEA (Figure 9B, arrows, βPEA). Whether this model has overlying control elements active at the level of the chromosome has yet to be determined.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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References

Acampora D, Boyl PP, Signore M, Martinez-Barbera JP, Ilengo C, Puelles E, Annino A, Reichert H, Corte G, Simeone A. 2001. OTD/OTX2 functional equivalence depends on 5′ and 3′ UTR-mediated control of Otx2 mRNA for nucleo-cytoplasmic export and epiblast-restricted translation. *Development*. 128(23):4801–4813.

Acampora D, Mazan S, Lallemand Y, Avantaggiato V, Maury M, Simeone A, Brûlet P. 1995. Forebrain and midbrain regions are deleted in Otx2-/- mutants due to a defective anterior neuroectoderm specification during gastrulation. *Development*. 121(10):3279–3290.

Alioto TS, Ngai J. 2005. The odorant receptor repertoire of teleost fish. *BMC Genomics*. 6:173.

Bailey TL, Elkan C. 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc Int Conf Intell Syst Mol Biol*. 2:28–36.

Bailey TL, Noble WS. 2003. Searching for statistically significant regulatory modules. *Bioinformatics*. 19(Suppl 2):ii16–ii25.

Balbuena E, Llorens J. 2003. Comparison of *cis*- and *trans*-crotononitrile effects in the rat reveals specificity in the neurotoxic properties of nitrile isomers. *Toxicol Appl Pharmacol*. 187(2):89–100.

Barth AL, Justice NJ, Ngai J. 1996. Asynchronous onset of odorant receptor expression in the developing zebrafish olfactory system. *Neuron*. 16(1):23–34.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc B*. 57:289–300.

Boyl PP, Signore M, Annino A, Barbera JP, Acampora D, Simeone A. 2001. Otx genes in the development and evolution of the vertebrate brain. *Int J Dev Neurosci*. 19(4):353–363.

Bruni P, Ilengo C, Bobola N, Corte G. 1999. Binding properties of the human homeodomain protein OTX2 to a DNA target sequence. *FEBS Lett*. 445(1):160–164.

Buck L, Axel R. 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell*. 65(1):175–187.

Byrd CA, Jones JT, Quattro JM, Rogers ME, Brunjes PC, Vogt RG. 1996. Ontogeny of odorant receptor gene expression in zebrafish, *Danio rerio*. *J Neurobiol*. 29(4):445–458.

Cadiou H, Aoudé I, Tazir B, Molinas A, Fenech C, Meunier N, Grosmaitre X. 2014. Postnatal odorant exposure induces peripheral olfactory plasticity at the cellular level. *J Neurosci*. 34(14):4857–4870.

Chess A, Simon I, Cedar H, Axel R. 1994. Allelic inactivation regulates olfactory receptor gene expression. *Cell*. 78(5):823–834.

Coppola DM, Waggener CT. 2012. The effects of unilateral naris occlusion on gene expression profiles in mouse olfactory mucosa. *J Mol Neurosci*. 47(3):604–618.

DeMaria S, Ngai J. 2010. The cell biology of smell. *J Cell Biol*. 191(3):443–452.

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Review Article

Dysferlin function in skeletal muscle: Possible pathological mechanisms and therapeutical targets in dysferlinopathies

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ABSTRACT

Mutations in the dysferlin gene are linked to a group of muscular dystrophies known as dysferlinopathies. These myopathies are characterized by progressive atrophy. Studies in muscle tissue from dysferlinopathy patients or dysferlin-deficient mice point out its importance in membrane repair. However, expression of dysferlin homologous proteins that restore sarcolemma repair function in dysferlinopathy animal models fail to arrest muscle wasting, therefore suggesting that dysferlin plays other critical roles in muscle function. In the present review, we discuss dysferlin functions in the skeletal muscle, as well as pathological mechanisms related to dysferlin mutations. Particular focus is presented related the effect of dysferlin on cell membrane related function, which affect its repair, vesicle trafficking, as well as Ca^{2+} homeostasis. Such mechanisms could provide accessible targets for pharmacological therapies.

Contents

Abbreviations: LGMD, limb girdle muscular dystrophy; CK, creatine kinase levels; MG53, mitsugumin 53; Cx, connexins; TRPV2, transient receptor potential vanilloid type 2; STB, syncytiotrophoblasts.

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1. Introduction

The hereditary myopathies comprise a large family of degenerative muscular disorders genetically determined by over 350 different mutations in distinct genes, for which novel causing mutations and genes are identified each year (Kaplan and Hamroun, 2014). The pathological

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In the meantime, what alternatives could be suggested? Pharmacological approaches are certainly attractive, as they can yield potential therapies much faster, and have the added value of reversibility. Yet, results are still disappointing. Use of glucocorticoids has not yielded beneficial effects, in light of the use of such compounds in many patients due to initial misdiagnosis. Yet, controlled studies with deflazacort have clearly demonstrated no beneficial effects (Walter et al., 2013). Vitamin D supplementation has been proposed as a potential therapy, in light of studies showing increased dysferlin expression in carriers of one mutation in the dysferlin gene (De Luna et al., 2012), yet studies in actual patients are still lacking. Lately, the inflammatory response in muscles of dysferlinopathy patients has been linked to upregulated interleukin 1β, and in this regard, the inhibition of this cytokine or the use of interleukin 4 to counteract the M1-mediated immune response in dysferlin-deficient mouse muscle (Cohen et al., 2015). Another, open label study, showed improvements in the mobility of pelvic and shoulder girdles in two patients after treatment with rixutimab, which also correlated with a dramatic decrease in B-cell number (Lerario et al., 2010). Hence, specific targets within the immune system appear much more effective, compared to a generalized immune and inflammatory modulation sought with glucocorticoids. Also, promoting hypertrophy in dysferlinopathy has to be weighed carefully, as Lee et al. (2015) demonstrated that myostatin inhibition in mouse models of the disease, which induces hypertrophy, results in increased CK levels, suggesting increased muscle destruction.

The latter evidence suggests that, initially, pharmacological treatment targeting specific aspects of the immune response, perhaps associated with controlled stimulation of muscle trophism, may be an interesting path in the quest for therapeutical alternatives for these patients. The final cure will necessarily involve gene therapy, and the recovery of full dysferlin expression, yet a lengthier road is anticipated in the achievement of this goal.

12. Conclusions, future directions

In the recent years, great advances have been reached in the comprehension of the role played by dysferlin in the different tissues where it is expressed. The best understood function of dysferlin is its role in the repair machinery that reseals injured cell membranes. This role is particularly relevant in skeletal muscles where the repair and regeneration of wounded plasma membrane constitute critical phenomena to maintain muscle integrity and function. In fact, dysferlinopathy causing mutations seem to impair the sarcolemmal resealing processes in injured myofibers, strongly suggesting that defects in membrane repair underlie the pathological mechanisms that lead to muscular dystrophy. However, other processes that require dysferlin participation could be deregulated in dysferlin-deficient tissues and hence contribute to the pathophysiology of dysferlinopathy. The clarification of these mechanisms is pivotal in the quest for therapeutic targets in patients harboring mutations in the dysferlin gene. The novel suggested functions of dysferlin in vesicle trafficking and membrane remodeling events could open up interesting research fields in the search of therapies that soften the dystrophic phenotypes and slow down the progression of the disease. Finally, the persistent expression of connexin in several dystrophic conditions presents an interesting therapeutical target, readily accessible to address pharmacologically, and where lead compounds do exist (Cea et al., 2016b).

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References

Abdullah, N., Padmanarayana, M., Marty, N.J., Johnson, C.P., 2014. Quantitation of the calcium and membrane binding properties of the C2 domains of dysferlin. Biophys. J. 106, 382–389.

Ampong, B.N., Imamura, M., Matsumiya, T., Yoshida, M., Takeda, S., 2005. Intracellular localization of dysferlin and its association with the dihydropyridine receptor. Acta Myol. 24, 134–144.

Anderson, L.V., Davison, K., Moss, J.A., Young, C., Cullen, M.J., Walsh, J., Johnson, M.A., Bashir, R., Britton, S., Keers, S., Argov, Z., Mahjneh, I., Fougerousse, F., Beckmann, J.S., Bushby, K.M., 1999. Dysferlin is a plasma membrane protein and is expressed early in human development. Hum. Mol. Genet. 8, 855–861.

Aoki, M., Liu, J., Richard, I., Bashir, R., et al., 2001. Genomic organization of the dysferlin gene and novel mutations in Miyoshi myopathy. Neurology 57, 271–278.

Araya, R., Eckardt, D., Maxeiner, S., Krüger, O., Theis, M., Willecke, K., Sáez, J.C., 2005. Expression of connexins during differentiation and regeneration of skeletal muscle: functional relevance of connexin43. J. Cell Sci. 118, 27–37.

Assadi, M., Schindler, T., Muller, B., Porter, J., Ruegg, M., Langen, H., 2008. Identification of proteins interacting with dysferlin using the tandem affinity purification method. Open Cell Dev. Biol. J 1, 17–23.

Azakir, B.A., Di Fulvio, S., Therrien, C., Sinnreich, M., 2010. Dysferlin interacts with tubulin and microtubules in mouse skeletal muscle. PLoS One 5, e10122.

Azakir, B., Di Fulvio, S., Kinter, J., Sinnreich, M., 2012. Proteasomal inhibition restores biological function of mis-sense mutated dysferlin in patient-derived muscle cells. J. Biol. Chem. 287, 10344–10354.

Bai, J., Chapman, E.R., 2004. The C2 domains of synaptotagmin–partners in exocytosis. Trends Biochem. Sci. 29, 143–151.

Bansal, D., Miyake, K., Vogel, S.S., Groh, S., Chen, C.C., Williamson, R., McNeil, P.L., Campbell, K.P., 2003. Defective membrane repair in dysferlin-deficient muscular dystrophy. Nature 423, 168–172.

Barohn, R.J., Miller, R.G., Griggs, R.C., 1991. Autosomal recessive distal dystrophy. Neurology 41, 1365–1370.

Barohn, R.J., Amato, A.A., Griggs, R.C., 1998. Overview of distal myopathies: from the clinical to the molecular. Neuromuscul. Disord. 8, 309–316.

Bashir, R., Britton, S., Strachan, T., et al., 1998. A gene related to *Caenorhabditis elegans* spermatogenesis factor fer-1 is mutated in limb-girdle muscular dystrophy type 2B. Nat. Genet. 20, 37–42.

Blandin, G., Marchand, S., Charton, S., Danièle, N., Gicquel, E., Boucheteil, J., Bentaïb, A., Barrault, L., Stockholm, D., Bartoli, M., Richard, I., 2013. A human skeletal muscle interactome centered on proteins involved in muscular dystrophies: LGMD interactome. Skelet. Muscle 3, 3.

Bushby, K.M., 1999. Making sense of the limb-girdle muscular dystrophies. Brain 122, 1403–1420.

Cai, C., Masumiya, H., Weisleder, N., Matsuda, N., Nishi, M., Hwang, M., Ko, J.K., Lin, P., Thornton, A., Zhao, X., Pan, Z., Komazaki, S., Brotto, M., Takeshima, H., Ma, J., 2009. MG53 nucleates assembly of cell membrane repair machinery. Nat. Cell Biol. 11, 56–64.

Cea, L.A., Riquelme, M.A., Cisterna, B.A., Puebla, C., Vega, J.L., Rovegno, M., Sáez, J.C., 2012. Connexin- and pannexin-based channels in normal skeletal muscles and their possible role in muscle atrophy. J. Membr. Biol. 245, 423–436.

Cea, L.A., Cisterna, B.A., Puebla, C., Frank, M., Figueroa, X.F., Cardozo, C., Willecke, K., Latorre, R., Sáez, J.C., 2013. De novo expression of connexin hemichannels in denervated fast skeletal muscles leads to atrophy. Proc. Natl. Acad. Sci. U. S. A. 110, 16229–16234.

Cea, L.A., Puebla, C., Cisterna, B.A., et al., 2016a. Fast skeletal myofibers of mdx mouse, model of Duchenne muscular dystrophy, express connexin hemichannels that lead to apoptosis. Cell. Mol. Life Sci. 73, 2583–2599.

Cea, L.A., Bevilacqua, J.A., Arriagada, C., Cárdenas, A.M., Bigot, A., Mouly, V., Sáez, J.C., Caviedes, P., 2016b. The absence of dysferlin induces the expression of functional connexin-based hemichannels in human myotubes. BMC Cell Biol. 17 (15) (Suppl 1).

Cenacchi, G., Fanin, M., De Giorgi, L.B., Angelini, C., 2005. Ultrastructural changes in dysferlinopathy support defective membrane repair mechanism. J. Clin. Pathol. 58, 190–195.

Chasserot-Golaz, S., Vitale, N., Umbrecht-Jenck, E., Knight, D., Gerke, V., Bader, M.F., 2005. Annexin 2 promotes the formation of lipid microdomains required for calcium-regulated exocytosis of dense-core vesicles. Mol. Biol. Cell 16, 1108–1119.

Chiu, Y.H., Hornsey, M.A., Klinge, L., Jørgensen, L.H., Laval, S.H., Charlton, R., Barresi, R., Straub, V., Lochmüller, H., Bushby, K., 2009. Attenuated muscle regeneration is a key factor in dysferlin-deficient muscular dystrophy. Hum. Mol. Genet. 18, 1976–1989.

Choi, J.H., Park, Y.E., Kim, S.I., Kim, J.I., Lee, C.H., Park, K.H., Kim, D.S., 2009. Differential immunohistological features of inflammatory myopathies and dysferlinopathy. J. Korean Med. Sci. 24, 1015–1023.

Codding, S., Marty, M., Abdullah, N., Johnson, C., 2016. Dysferlin binds snares and stimulates membrane fusion in a calcium sensitive manner. J. Biol. Chem. http://dx.doi.org/10.1074/jbc.M116.727016 jbc.M116.727016.

Cohen, T.V., Many, G.M., Fleming, B.D., Gnocchi, V.F., Ghimbovschi, S., Mosser, D.M., Hoffman, E.P., Partridge, T., 2015. Upregulated IL-1β in dysferlin-deficient muscle attenuates regeneration by blunting the response to pro-inflammatory macrophages. Skelet. Muscle 5, 24.

Corbalan-Garcia, S., Gómez-Fernández, J.C., 2014. Signaling through C2 domains: more than one lipid target. Biochim. Biophys. Acta 1838, 1536–1547.

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REVIEW

How the stimulus defines the dynamics of vesicle pool recruitment, fusion mode, and vesicle recycling in neuroendocrine cells

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Abstract

The pattern of stimulation defines important characteristics of the secretory process in neurons and neuroendocrine cells, including the pool of secretory vesicles being recruited, the type and amount of transmitters released, the mode of membrane retrieval, and the mechanisms associated with vesicle replenishment. This review analyzes the mechanisms that regulate these processes in chromaffin cells, as well as in other neuroendocrine and neuronal models. A common factor in these mechanisms is the spatial and temporal distribution of the Ca^{2+} signal generated during cell stimulation. For instance, neurosecretory cells and neurons have pools of vesicles with different locations with respect to Ca^{2+} channels, and those pools are therefore differentially recruited following different patterns of stimulation. In this regard, a brief stimulus will induce the exocytosis of a small pool of vesicles that is highly coupled to voltage-dependent Ca^{2+} channels, whereas longer or more intense stimulation will provoke a global Ca^{2+} increase, promoting exocytosis irrespective of vesicle location. The pattern of stimulation, and therefore the characteristics of the Ca^{2+} signal generated by the stimulus also influence the mode of exocytosis and the type of endocytosis. Indeed, low-frequency stimulation favors kiss-and-run exocytosis and clathrin-independent fast endocytosis, whereas higher frequencies promote full fusion and clathrin-dependent endocytosis. This latter type of endocytosis is accelerated at high-frequency stimulation. Synaptotagmins, calcineurin, dynamin, complexin, and actin remodeling, appear to be involved in the mechanisms that determine the response of these processes to Ca^{2+}.

Keywords: calcium, endocytosis, exocytosis, immediately releasable pool, kiss-and-run, secretion.

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The release of hormones is a highly regulated process that requires adjustment to maintain the body's internal balance and its response to the environment. This regulation is particularly required for the release of catecholamines and neuropeptides from the adrenal chromaffin cells. These neuroendocrine cells are innervated by cholinergic terminals of the splanchnic nerve, which release acetylcholine and peptides such as the vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptide, activating ionotropic and metabotropic receptors present in the plasma membrane of the chromaffin cells (Chowdhury *et al.* 1994). The activation of the ionotropic nicotinic receptor deals with a series of regulated events that occur in a perfect sequence:

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Abbreviations used: AP, action potential; BAPTA, 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; IRP, immediately releasable pool; P/Q-KO, α1A-deficient mice; PACAP, pituitary adenylate cyclase-activating polypeptide; RRP, ready releasable pool; SNARE, SNAP (soluble NSF attachment protein) Receptor; synprint, synaptic protein interaction site; VDCC, voltage dependent calcium channels; VIP, vasoactive intestinal polypeptide; WT, wild-type.

at 1–10 Hz, or single depolarization prolonged to 200 ms, promotes a new rapid endocytotic component (Wu and Wu 2014). These authors proposed that the pattern of the cytosolic Ca^{2+} signal defines the kinetics of endocytosis. Thus, endocytosis is triggered and facilitated by a large, transient Ca^{2+} increase localized at a micro/nano domain, but hindered by prolonged, small, and global Ca^{2+} signals (Wu and Wu 2014; Wu *et al.* 2014).

Different authors have proposed that rapid and slow endocytosis correlate with the mode of exocytosis – rapid endocytosis being associated with the kiss-and-run exocytosis, whereas slow endocytosis is associated with full fusion (Elhamdani *et al.* 2006; Wu *et al.* 2014). These two mechanisms would impact the kinetics of vesicle recycling, as well as the economy of the cell, since a slow recycling process implies the synthesis and refilling of new vesicles.

The stimulus pattern also influences the replenishment of vesicles after depletion

The maintenance of secretion under AP firing requires the continuous refilling of releasable pools of vesicles at rates that match the exocytotic activity (Smith *et al.* 1998; Sorensen 2004). In this regard, high-frequency stimulation of chromaffin cells provokes an important global Ca^{2+} increase (Klingauf and Neher 1997; Marengo and Monck 2003; Fulop and Smith 2006), which triggers exocytosis of secretory vesicles irrespectively of their location and mobilizes vesicles from upstream to downstream pools (Voets *et al.* 1999; Marengo 2005). On the other hand, in rest conditions the firing frequency is low, Ca^{2+} does not markedly accumulate (Fulop and Smith 2006), and exocytosis would be limited to a group of vesicles that are closely coupled to $VDCC_s$ that is, the IRP (Horrigan and Bookman 1994; Voets *et al.* 1999; Marengo 2005; Oré and Artalejo 2005; Álvarez *et al.* 2008).

How is the IRP refilled in order to maintain exocytosis during repetitive stimulation? In mouse chromaffin cells, it has been proposed that IRP is refilled directly from upstream vesicle pools in a sequential scheme (Chan *et al.* 2005a). Different factors may regulate this process, such as cytosolic Ca^{2+} (Voets *et al.* 1999; Marengo 2005), neural cell adhesion molecule (Chan *et al.* 2005a), and protein kinase C (Voets *et al.* 1999). A second possibility is that a fast endocytotic process, which is directly coupled to the exocytosis induced by APs at low frequencies, might be the first step of a short cycle of vesicle replenishment (Chan and Smith 2001, 2003). Indeed, at low physiological frequencies, chromaffin cells release catecholamines through Ω-shape kiss-and-run fusion events (Fulop *et al.* 2005; Fulop and Smith 2006), suggesting that the replenishment of vesicles could be associated with a short local cycling mechanism. Additionally, in bovine chromaffin cells, it was reported that kiss-and-run is directly associated with fast endocytosis (Elhamdani *et al.* 2006). Therefore, it is possible that application of AP_1s at low frequencies promotes a kiss-and-run like process, which might result in rapid replenishment of releasable vesicles. Fast endocytosis and/or fast recycling processes were also documented in other systems such as cultured hippocampal neurons (Klingauf *et al.* 1998; Deak *et al.* 2004), calyx of Held (Wu and Wu 2009), auditory hair cells (Cho *et al.* 2011), and synaptic terminals of retinal bipolar neurons (von Gersdorff and Matthews 1994). However, other studies performed in neurons argue against a major contribution of kiss-and-run in overall endocytosis (Fernandez-Alfonso and Ryan 2004; Balaji and Ryan 2007; Granseth *et al.* 2009). Consistently, Ling-Gang Wu and collaborators (Wu and Wu 2009; Wu *et al.* 2014), working in the calyx of Held, reported that rapid endocytosis does not recycle vesicles within the RRP. Based on that finding, they proposed an alternative explanation about the importance of fast endocytosis on rapid vesicle recycling. Rapid endocytosis may quickly restore the structure of release sites after exocytosis. In other words, the clearance of the release sites by endocytosis, through the removal of exocytosed vesicle membranes and proteins, would facilitate vesicle replenishment. In addition, rapid endocytosis would recycle vesicles in a recycling pool beyond the RRP to prevent vesicle exhaustion (Wu and Wu 2009).

Final remarks

Accumulated data from different laboratories indicate that the pattern of stimulation defines the pools of vesicles being recruited, the mode of exocytosis, and the type of endocytosis in neuroendocrine cells. The latter two determine, respectively, the types and amount of transmitters released, and the mechanisms associated with vesicle replenishment. In this regard, high-frequency stimulation promotes the massive full fusion of vesicles, irrespective of their location, followed by clathrin-dependent endocytosis and vesicle replenishment through vesicle mobilization from upstream to downstream pools. On the other hand, low-frequency stimulation with action potentials or very brief stimuli triggers the secretion of vesicles that are highly coupled to Ca^{2+} channels, apparently through a kiss-and-run mechanism of fusion, which is followed by a clathrin-independent fast endocytosis process. This fast endocytosis would facilitate the rapid replenishment of releasable vesicles. This tight stimulus–secretion coupling and fast recycling could help chromaffin cells maintain a basal secretory tone with little metabolic cost (Voets *et al.* 1999; Oré and Artalejo 2005).

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

References

Albillos A., Dernick G., Horstmann H., Almers W., Alvarez de Toledo G. and Lindau M. (1997) The exocytotic event in chromaffin cells revealed by patch amperometry. *Nature* **389**, 509–512.

Albillos A., Neher E. and Moser T. (2000) R-Type Ca^{2+} channels are coupled to the rapid component of secretion in mouse adrenal slice chromaffin cells. *J. Neurosci.* **20**, 8323–8330.

Aldea M., Jun K., Shin H. S., Andres-Mateos E., Solis-Garrido L. M., Montiel C., Garcia A. G. and Albillos A. (2002) A perforated patch-clamp study of calcium currents and exocytosis in chromaffin cells of wild-type and alpha(1A) knockout mice. *J. Neurochem.* **81**, 911–921.

Ales E., Tabares L., Poyato J. M., Valero V., Lindau M. and Alvarez d. T. (1999) High calcium concentrations shift the mode of exocytosis to the kiss-and-run mechanism. *Nat. Cell Biol.* **1**, 40–44.

Alvarez Y. D. and Marengo F. D. (2011) The immediately releasable vesicle pool: highly coupled secretion in chromaffin and other neuroendocrine cells. *J. Neurochem.* **116**, 155–163.

Alvarez Y. D., Ibanez L. I., Uchitel O. D. and Marengo F. D. (2008) P/Q Ca^{2+} channels are functionally coupled to exocytosis of the immediately releasable pool in mouse chromaffin cells. *Cell Calcium* **43**, 155–164.

Alvarez Y. D., Belingheri A. V., Perez Bay A. E., Javis S. E., Tedford H. W., Zamponi G. and Marengo F. D. (2013) The immediately releasable pool of mouse chromaffin cell vesicles is coupled to P/Q-type calcium channels via the synaptic protein interaction site. *PLoS ONE* **8**, e54846.

Andres-Mateos E., Renart J., Cruces J., Solis-Garrido L. M., Serantes R., Lucas-Cerrillo A. M., Aldea M., Garcia A. G. and Montiel C. (2005) Dynamic association of the Ca^{2+} channel alpha1A subunit and SNAP-25 in round or neurite-emitting chromaffin cells. *Eur. J. Neurosci.* **22**, 2187–2198.

Artalejo C. R., Adams M. E. and Fox A. P. (1994) Three types of Ca^{2+} channel trigger secretion with different efficacies in chromaffin cells. *Nature* **367**, 72–76.

Artalejo C. R., Elhamdani A. and Palfrey H. C. (2002) Sustained stimulation shifts the mechanism of endocytosis from dynamin-1-dependent rapid endocytosis to clathrin- and dynamin-2-mediated slow endocytosis in chromaffin cells. *Proc. Natl Acad. Sci. USA* **99**, 6358–6363.

Ashery U., Varoqueaux F., Voets T., Betz A., Thakur P., Koch H., Neher E., Brose N. and Rettig J. (2000) Munc13-1 acts as a priming factor for large dense-core vesicles in bovine chromaffin cells. *EMBO J.* **19**, 3586–3596.

Bacaj T., Wu D., Yang X., Morishita W., Zhou P., Xu W., Malenka R. C. and Sudhof T. C. (2013) Synaptotagmin-1 and synaptotagmin-7 trigger synchronous and asynchronous phases of neurotransmitter release. *Neuron* **80**, 947–959.

Balaji J. and Ryan T. A. (2007) Single-vesicle imaging reveals that synaptic vesicle exocytosis and endocytosis are coupled by a single stochastic mode. *Proc. Natl Acad. Sci. USA* **104**, 20576–20581.

Barg S., Galvanovskis J., Gopel S. O., Rorsman P. and Eliasson L. (2000) Tight coupling between electrical activity and exocytosis in mouse glucagon-secreting alpha-cells. *Diabetes* **49**, 1500–1510.

Barg S., Ma X., Eliasson L., *et al.* (2001) Fast exocytosis with few Ca(2+) channels in insulin-secreting mouse pancreatic B cells. *Biophys. J.* **81**, 3308–3323.

Barg S., Eliasson L., Renstrom E. and Rorsman P. (2002) A subset of 50 secretory granules in close contact with L-type Ca^{2+} channels accounts for first-phase insulin secretion in mouse beta-cells. *Diabetes* **51**(Suppl 1), S74–S82.

Berberian K., Torres A. J., Fang Q., Kisler K. and Lindau M. (2009) F-actin and myosin II accelerate catecholamine release from chromaffin granules. *J. Neurosci.* **29**, 863–870.

Bhalla A., Tucker W. C. and Chapman E. R. (2005) Synaptotagmin isoforms couple distinct ranges of Ca^{2+}, Ba^{2+}, and Sr^{2+} concentration to SNARE-mediated membrane fusion. *Mol. Biol. Cell* **16**, 4755–4764.

Brandt B. L., Hagiwara S., Kikodoro Y. and Miyazaki S. (1976) Action potentials in the rat chromaffin cell and effects of acetylcholine. *J. Physiol.* **263**, 417–439.

Breiou M., Jouannot O., Fanget I. *et al.* (2014) Cdc42 controls the dilation of the exocytotic fusion pore by regulating membrane tension. *Mol. Biol. Cell* **25**, 3195–3209.

Cai H., Reim K., Varoqueaux F., Tapechum S., Hill K., Sorensen J. B., Brose N. and Chow R. H. (2008) Complexin II plays a positive role in Ca^{2+}-triggered exocytosis by facilitating vesicle priming. *Proc. Natl Acad. Sci. USA* **105**, 19538–19543.

Cardenas A. M. and Marengo F. D. (2010) Rapid endocytosis and vesicle recycling in neuroendocrine cells. *Cell. Mol. Neurobiol.* **30**, 1365–1370.

Catterall W. A., Perez-Reyes E., Snutch T. P. and Striessnig J. (2005) International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. *Pharmacol. Rev.* **57**, 411–425.

Ceccarelli B., Hurlbut W. P. and Mauro A. (1973) Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. *J. Cell Biol.* **57**, 499–524.

Chan S. A. and Smith C. (2001) Physiological stimuli evoke two forms of endocytosis in bovine chromaffin cells. *J. Physiol.* **537**, 871–885.

Chan S. A. and Smith C. (2003) Low frequency stimulation of mouse adrenal slices reveals a clathrin-independent, protein kinase C-mediated endocytic mechanism. *J. Physiol.* **553**, 707–717.

Chan S. A., Chow R. and Smith C. (2003) Calcium dependence of action potential-induced endocytosis in chromaffin cells. *Pflugers Arch.* **445**, 540–546.

Chan S. A., Polo-Parada L., Landmesser L. T. and Smith C. (2005a) Adrenal chromaffin cells exhibit impaired granule trafficking in NCAM knockout mice. *J. Neurophysiol.* **94**, 1037–1047.

Chan S. A., Polo-Parada L. and Smith C. (2005b) Action potential stimulation reveals an increased role for P/Q-calcium channel-dependent exocytosis in mouse adrenal tissue slices. *Arch. Biochem. Biophys.* **435**, 65–73.

Chapman E. R. (2008) How does synaptotagmin trigger neurotransmitter release? *Annu. Rev. Biochem.* **77**, 615–641.

Cho S., Li G. L. and von Gersdorff H. (2011) Recovery from short-term depression and facilitation is ultrafast and Ca^{2+} dependent at auditory hair cell synapses. *J. Neurosci.* **31**, 5682–5692.

Chow R. H., von Ruden L. and Neher E. (1992) Delay in vesicle fusion revealed by electrochemical monitoring of single secretory events in adrenal chromaffin cells. *Nature* **356**, 60–63.

Chow R. H., Klingauf J. and Neher E. (1994) Time course of Ca^{2+} concentration triggering exocytosis in neuroendocrine cells. *Proc. Natl Acad. Sci. USA* **91**, 12765–12769.

Chowdhury P. S., Guo X., Wakade T. D., Przywara D. A. and Wakade A. R. (1994) Exocytosis from a single rat chromaffin cell by cholinergic and peptidergic neurotransmitters. *Neuroscience* **59**, 1–5.

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BIOPHYSICS AND COMPUTATIONAL BIOLOGY

β1-subunit–induced structural rearrangements of the Ca^{2+}- and voltage-activated K^+ (BK) channel

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Large-conductance Ca^{2+}- and voltage-activated K^+ (BK) channels are involved in a large variety of physiological processes. Regulatory β-subunits are one of the mechanisms responsible for creating BK channel diversity fundamental to the adequate function of many tissues. However, little is known about the structure of its voltage sensor domain. Here, we present the external architectural details of BK channels using lanthanide-based resonance energy transfer (LRET). We used a genetically encoded lanthanide-binding tag (LBT) to bind terbium as a LRET donor and a fluorophore-labeled iberiotoxin as the LRET acceptor for measurements of distances within the BK channel structure in a living cell. By introducing LBTs in the extracellular region of the α- or β1-subunit, we determined (*i*) a basic extracellular map of the BK channel, (*ii*) β1-subunit–induced rearrangements of the voltage sensor in α-subunits, and (*iii*) the relative position of the β1-subunit within the α/β1-subunit complex.

lanthanide resonance energy transfer | BK channels | β1-subunit

Important physiological processes involve Ca^{2+} entry into cells mediated by voltage-dependent Ca^{2+} channels. This divalent cation influx is essential for life because it permits, for example, the adequate functioning of smooth muscle or neurosecretion to occur. Some mechanism must be put into action, however, to control Ca^{2+} influx, either to dampen or to stop the physiological effects of the cytoplasmic increase in Ca^{2+}. In many cases, this dampening mechanism is accomplished by one of the most broadly expressed channels in mammals: the large-conductance Ca^{2+}- and voltage-activated K^+ (BK) channel (1–3). Because there is a single gene coding for the BK channel (*Slowpoke* KNCMA1), channel diversity must be a consequence of alternative splicing and/or interaction with regulatory subunits. In fact, both mechanisms account for BK channel diversity, but the most dramatic changes in BK channel properties are brought about through the interaction with regulatory subunits, membrane-integral proteins, denominated BK β-subunits (β1–β4) (4–7) and the recently discovered γ-subunits (γ1–γ4) (8, 9).

Structurally, the BK channel is a homotetramer of its pore-forming α-subunit and is a member of the voltage-dependent potassium (Kv) channel family. Distinct from Kv channels, however, BK channel subunits are composed of seven transmembrane domains S0–S6 (10, 11). Little is known about the detailed structure of the membrane-spanning portion of the BK channel, or of the α/β1-subunit complex. Here, we used a variant of Förster resonance energy transfer (FRET), called lanthanide-based resonance energy transfer (LRET), to determine the positions of the N terminus (NT) and S0, S1, and S2 transmembrane segments of the α-subunit of the BK channel, as well as the position of the β1-subunit in the α/β1-subunit complex. LRET uses luminescent lanthanides (e.g., Tb^{3+}) as donor instead of conventional fluorophores. This technique has been successfully used to measure intramolecular distances and to track voltage-dependent structural changes in voltage-dependent K^+ and Na^+ channels (12–16). The advantages of this technique over FRET have been discussed in detail elsewhere (17), and only its highlights will be given here. Briefly, the isotropic emission of Tb^{3+} ensures that the maximum error in distance estimation due to the orientation factor (κ^2) does not exceed ±10% in the range of 10–120 Å (17). Because Tb^{3+} has a spiked emission spectrum, it is possible to isolate the sensitized emission (SE) of the acceptor with relative ease by using an adequate optical filter. Additionally, we used the genetically encoded lanthanide-binding tag (LBT) to chelate Tb^{3+} donor within the protein structure (18) (Fig. 1*A*). Because the LBT-Tb^{3+} emission decay has a well-defined time constant of ∼2.4 ms, the donor-only (DO) emission (i.e., the donor emission in the absence of the acceptor) is very specific and distinguishable from background (13, 18, 19). Also, because the LBT is incorporated into the backbone of the protein, the donor becomes tied to the structure of the channel, likely decreasing the uncertainty of the donor position. Any distortion of the LBT structure that modifies Tb^{3+} accessibility to water will decrease its decay time constant. Therefore, by measuring the Tb^{3+} decay, it is possible to infer whether the LBT remains intact in the final structure. Moreover, the crystal structure of the LBT has been determined (18), making it possible to perform molecular dynamics (MD) simulations of the LBT-BK chimeric channels using homology-based models. The flexibility of the LBT–Tb^{3+} complex would place the Tb^{3+} atom at 5–8 Å from the LBT insertion site, giving us a concise

Significance

Large-conductance Ca^{2+}- and voltage-activated K^+ (BK) channels play many physiological roles, ranging from the maintenance of smooth muscle tone to the modulation of alcohol tolerance. In most cases, this physiological versatility of the BK channel is due to the association of the pore-forming α-subunit with β-subunits. Therefore, it is of importance to know what the structural consequences of this association are. Here, using lanthanide-based resonance energy transfer, we were able to determine the extracellular position of transmembrane segments S0–S2 with and without the β1-subunit and the position of the two transmembrane segments of the β1 subunit in the α/β1-subunit complex. We concluded that β1 produces rearrangements of the BK voltage sensor domain.

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bilayer with 150 mM KCl. After 20 ns, the obtained structure was collected and used to build the initial BK α/β1-complex.

Both α- and β1-subunits were embedded in a POPC hydrated bilayer with 150 mM KCl. To represent the β1-subunit TM position calculated by LRET experiments, the α-carbon of the first residue of each segment was restricted to the *x*–*y* coordinates of its corresponding experimentally determined position (Ser45 for TM1 and Pro164 for TM2). This conformation was selected over other β1-subunits to minimize possible steric clashes among the extracellular loops. The final system containing BK α/β1-complex (final size of 210 × 210 × 111 Å^3, total of 371,560 atoms) was submitted to a 20-ns MD protocol using secondary and symmetry restraints as described above.

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1. Contreras GF, et al. (2013) A BK (Slo1) channel journey from molecule to physiology. *Channels (Austin)* 7(6):442–458.
2. Cui J, Yang H, Lee US (2009) Molecular mechanisms of BK channel activation. *Cell Mol Life Sci* 66(5):852–875.
3. Latorre R, Brauchi S (2006) Large conductance Ca2+ activated K+ (BK) channels: Activation by Ca2+ and voltage. *Biol Res* 39(3):385–401.
4. Orio P, Rojas P, Ferreira G, Latorre R (2002) New disguises for an old channel: MaxiK channel beta-subunits. *News Physiol Sci* 17:156–161.
5. Sun X, Zaydman MA, Cui J (2012) Regulation of Voltage-Activated K(+) Channel Gating by Transmembrane β Subunits. *Front Pharmacol* 3:63.
6. Toro L, Wallner M, Meera P, Tanaka Y (1998) Maxi-K(Ca), a Unique Member of the Voltage-Gated K Channel Superfamily. *News Physiol Sci* 13:112–117.
7. Torres YP, Morera FJ, Carvacho I, Latorre R (2007) A marriage of convenience: Beta-subunits and voltage-dependent K+ channels. *J Biol Chem* 282(34):24485–24489.
8. Yan J, Aldrich RW (2010) LRRC26 auxiliary protein allows BK channel activation at resting voltage without calcium. *Nature* 466(7305):513–516.
9. Yan J, Aldrich RW (2012) BK potassium channel modulation by leucine-rich repeat-containing proteins. *Proc Natl Acad Sci USA* 109(20):7917–7922.
10. Meera P, Wallner M, Song M, Toro L (1997) Large conductance voltage- and calcium-dependent K+ channel, a distinct member of voltage-dependent ion channels with seven N-terminal transmembrane segments (S0-S6), an extracellular N terminus, and an intracellular (S9-S10) C terminus. *Proc Natl Acad Sci USA* 94(25):14066–14071.
11. Wallner M, Meera P, Toro L (1996) Determinant for beta-subunit regulation in high-conductance voltage-activated and Ca(2+)-sensitive K+ channels: An additional transmembrane region at the N terminus. *Proc Natl Acad Sci USA* 93(25):14922–14927.
12. Cha A, Snyder GE, Selvin PR, Bezanilla F (1999) Atomic scale movement of the voltage-sensing region in a potassium channel measured via spectroscopy. *Nature* 402(6763):809–813.
13. Hyde HC, et al. (2012) Nano-positioning system for structural analysis of functional homomeric proteins in multiple conformations. *Structure* 20(10):1629–1640.
14. Posson DJ, Ge P, Miller C, Bezanilla F, Selvin PR (2005) Small vertical movement of a K+ channel voltage sensor measured with luminescence energy transfer. *Nature* 436(7052):848–851.
15. Posson DJ, Selvin PR (2008) Extent of voltage sensor movement during gating of shaker K+ channels. *Neuron* 59(1):98–109.
16. Richardson J, et al. (2006) Distance measurements reveal a common topology of prokaryotic voltage-gated ion channels in the lipid bilayer. *Proc Natl Acad Sci USA* 103(43):15865–15870.
17. Selvin PR (2002) Principles and biophysical applications of lanthanide-based probes. *Annu Rev Biophys Biomol Struct* 31:275–302.
18. Nitz M, et al. (2004) Structural origin of the high affinity of a chemically evolved lanthanide-binding peptide. *Angew Chem Int Ed Eng*, 4328, pp 3682–3685.
19. Sandtner W, Bezanilla F, Correa AM (2007) In vivo measurement of intramolecular distances using genetically encoded reporters. *Biophys J* 93(9):L45–L47.
20. Candia S, Garcia ML, Latorre R (1992) Mode of action of iberiotoxin, a potent blocker of the large conductance Ca(2+)-activated K+ channel. *Biophys J* 63(2):583–590.
21. Galvez A, et al. (1990) Purification and characterization of a unique, potent, peptidyl probe for the high conductance calcium-activated potassium channel from venom of the scorpion Buthus tamulus. *J Biol Chem* 265(19):11083–11090.
22. Banerjee A, Lee A, Campbell E, Mackinnon R (2013) Structure of a pore-blocking toxin in complex with a eukaryotic voltage-dependent K(+) channel. *eLife* 2:e00594.
23. Heyduk T, Heyduk E (2001) Luminescence energy transfer with lanthanide chelates: Interpretation of sensitized acceptor decay amplitudes. *Anal Biochem* 289(1):60–67.
24. Liu G, et al. (2008) Locations of the beta1 transmembrane helices in the BK potassium channel. *Proc Natl Acad Sci USA* 105(31):10727–10732.
25. Liu G, et al. (2010) Location of modulatory beta subunits in BK potassium channels. *J Gen Physiol* 135(5):449–459.
26. Diaz L, et al. (1998) Role of the S4 segment in a voltage-dependent calcium-sensitive potassium (hSlo) channel. *J Biol Chem* 273(49):32430–32436.
27. Cui J, Aldrich RW (2000) Allosteric linkage between voltage and Ca(2+)-dependent activation of BK-type mSlo1 K(+) channels. *Biochemistry* 39(50):15612–15619.
28. Ma Z, Lou XJ, Horrigan FT (2006) Role of charged residues in the S1-S4 voltage sensor of BK channels. *J Gen Physiol* 127(3):309–328.
29. Pantazis A, Gudzenko V, Savalli N, Sigg D, Olcese R (2010) Operation of the voltage sensor of a human voltage- and Ca2+-activated K+ channel. *Proc Natl Acad Sci USA* 107(9):4459–4464.
30. Pantazis A, Kohanteb AP, Olcese R (2010) Relative motion of transmembrane segments S0 and S4 during voltage sensor activation in the human BK(Ca) channel. *J Gen Physiol* 136(6):645–657.
31. Pantazis A, Olcese R (2012) Relative transmembrane segment rearrangements during BK channel activation resolved by structurally assigned fluorophore-quencher pairing. *J Gen Physiol* 140(2):207–218.
32. Koval OM, Fan Y, Rothberg BS (2007) A role for the S0 transmembrane segment in voltage-dependent gating of BK channels. *J Gen Physiol* 129(3):209–220.
33. Semenova NP, Atkarca-Heidemann K, Lorinc E, Rothberg BS (2009) Bimane fluorescence scanning suggests secondary structure near the S3-S4 linker of BK channels. *J Biol Chem* 284(16):10684–10693.
34. Wang L, Sigworth FJ (2009) Structure of the BK potassium channel in a lipid membrane from electron cryomicroscopy. *Nature* 461(7261):292–295.
35. Liu G, et al. (2008) Position and role of the BK channel alpha subunit S0 helix inferred from disulfide crosslinking. *J Gen Physiol* 131(6):537–548.
36. Knaus HG, et al. (1994) Primary sequence and immunological characterization of beta-subunit of high conductance Ca(2+)-activated K+ channel from smooth muscle. *J Biol Chem* 269(25):17274–17278.
37. Wallner M, et al. (1995) Characterization of and modulation by a beta-subunit of a human maxi KCa channel cloned from myometrium. *Receptors Channels* 3(3):185–199.
38. Orio P, Latorre R (2005) Differential effects of beta 1 and beta 2 subunits on BK channel activity. *J Gen Physiol* 125(4):395–411.
39. Nimigean CM, Magleby KL (2000) Functional coupling of the beta(1) subunit to the large conductance Ca(2+)-activated K(+) channel in the absence of Ca(2+). Increased Ca(2+) sensitivity from a Ca(2+)-independent mechanism. *J Gen Physiol* 115(6):719–736.
40. Meera P, Wallner M, Jiang Z, Toro L (1996) A calcium switch for the functional coupling between alpha (hslo) and beta subunits (Kv,cabeta) of maxi K channels. *FEBS Lett* 385(1-2):127–128.
41. Dworetzky SI, et al. (1996) Phenotypic alteration of a human BK (hSlo) channel by hSlobeta subunit coexpression: Changes in blocker sensitivity, activation/relaxation and inactivation kinetics, and protein kinase A modulation. *J Neurosci* 16(15):4543–4550.
42. Cox DH, Aldrich RW (2000) Role of the beta1 subunit in large-conductance Ca(2+)-activated K(+) channel gating energetics. Mechanisms of enhanced Ca(2+) sensitivity. *J Gen Physiol* 116(3):411–432.
43. Bao L, Cox DH (2005) Gating and ionic currents reveal how the BKCa channel's Ca2+ sensitivity is enhanced by its beta1 subunit. *J Gen Physiol* 126(4):393–412.
44. McManus OB, et al. (1995) Functional role of the beta subunit of high conductance calcium-activated potassium channels. *Neuron* 14(3):645–650.
45. Contreras GF, Neely A, Alvarez O, Gonzalez C, Latorre R (2012) Modulation of BK channel voltage gating by different auxiliary β subunits. *Proc Natl Acad Sci USA* 109(46):18991–18996.
46. Morrow JP, et al. (2006) Defining the BK channel domains required for beta1-subunit modulation. *Proc Natl Acad Sci USA* 103(13):5096–5101.
47. Brenner R, Jegla TJ, Wickenden A, Liu Y, Aldrich RW (2000) Cloning and functional characterization of novel large conductance calcium-activated potassium channel beta subunits, hKCNMB3 and hKCNMB4. *J Biol Chem* 275(9):6453–6461.
48. Orio P, et al. (2006) Structural determinants for functional coupling between the beta and alpha subunits in the Ca2+-activated K+ (BK) channel. *J Gen Physiol* 127(2):191–204.
49. Castillo K, et al. (2015) Molecular mechanism underlying β1 regulation in voltage- and calcium-activated potassium (BK) channels. *Proc Natl Acad Sci USA* 112(15):4809–4814.
50. McGuffin LJ, Bryson K, Jones DT (2000) The PSIPRED protein structure prediction server. *Bioinformatics* 16(4):404–405.
51. Jones DT (2007) Improving the accuracy of transmembrane protein topology prediction using evolutionary information. *Bioinformatics* 23(5):538–544.
52. Krogh A, Larsson B, von Heijne G, Sonnhammer EL (2001) Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *J Mol Biol* 305(3):567–580.
53. Mitchell AR, Kent SBH, Engelhard M, Merrifield RB (1978) A new synthetic route to tert-butyloxycarbonylaminoacyl-4-(oxymethyl)phenylacetamidomethyl-resin, an improved support for solid-phase peptide synthesis. *J Org Chem* 43(14):2845–2852.
54. Rosenthal J, Lippard SJ (2010) Direct detection of nitroxyl in aqueous solution using a tripodal copper(II) BODIPY complex. *J Am Chem Soc* 132(16):5536–5537.
55. Schnölzer M, Alewood P, Jones A, Alewood D, Kent SB (1992) In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int J Pept Protein Res* 40(3-4):180–193.
56. Schnölzer M, Alewood P, Jones A, Alewood D, Kent SH (2007) In situ neutralization in Boc-chemistry solid phase peptide synthesis. *Int J Pept Res Ther* 13(1-2):31–44.
57. Hackeng TM, Griffin JH, Dawson PE (1999) Protein synthesis by native chemical ligation: Expanded scope by using straightforward methodology. *Proc Natl Acad Sci USA* 96(18):10068–10073.

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Cellular and Molecular Life Sciences

ORIGINAL ARTICLE

Fast skeletal myofibers of *mdx* mouse, model of Duchenne muscular dystrophy, express connexin hemichannels that lead to apoptosis

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Abstract Skeletal muscles of patients with Duchenne muscular dystrophy (DMD) show numerous alterations including inflammation, apoptosis, and necrosis of myofibers. However, the molecular mechanism that explains these changes remains largely unknown. Here, the involvement of hemichannels formed by connexins (Cx HCs) was evaluated in skeletal muscle of *mdx* mouse model of DMD. Fast myofibers of *mdx* mice were found to express three connexins (39, 43 and 45) and high sarcolemma permeability, which was absent in myofibers of *mdx* $Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre mice (deficient in skeletal muscle Cx43/Cx45 expression). These myofibers did not show elevated basal intracellular free Ca^{2+} levels, immunoreactivity to phosphorylated p65 (active NF-κB), eNOS and annexin V/active Caspase 3 (marker of apoptosis) but presented dystrophin immunoreactivity. Moreover, muscles of *mdx* $Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre mice exhibited partial decrease of necrotic features (big cells and high creatine kinase levels). Accordingly, these muscles showed similar macrophage infiltration as control *mdx* muscles. Nonetheless, the hanging test performance of *mdx* $Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre mice was significantly better than that of control *mdx* $Cx43^{fl/fl}$-$Cx45^{wt/wt}$ mice. All three Cxs found in skeletal muscles of *mdx* mice were also detected in fast myofibers of biopsy specimens from patients with muscular dystrophy. Thus, reduction of Cx expression and/or function of Cx HCs may be potential therapeutic approaches to abrogate myofiber apoptosis in DMD.

Keywords Connexons · Evans blue uptake · $P2X_7$ receptors · Pannexin1 · NF-κB · Cell death

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hanging time was recorded. For four limb hanging test, the mouse was led to grasp in the wire with four limbs, for which the maximum hanging time was recorded.

Selection of DMD patients and mutation and biopsy studies

Following ethical guidelines, the healthy volunteers and DMD patients who participated in this study signed an informed consent approved by the local Ethics Committee in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Diagnosis of Duchenne/Becker muscular dystrophy was achieved based on clinical examination, muscular biopsy and mutation analysis (Table 1). Genomic DNA extracted from peripheral blood leucocytes by standardized procedures (kit Purogene, Qiagen GmbH, Hilden, Germany) were screened for DMD gene duplications/deletions using the MLPA technique (SALSA MLPA probemixes P034/P035 DMD, MRC-Holland, Amsterdam, The Netherlands). In those cases without duplications/deletions, the 79 exons of the *DMD* gene and part of the intronic regions were sequenced by NGS technology by the Illumina MiSeq team. The amplified fragments were performed using the Multiplicom MASTR DMD kit. All variants detected were confirmed by Sanger sequencing (amplified DNA products were sequenced by the dideoxy termination method, BigDye Sequencing Kit, Applied Biosystems, Foster City, CA, USA). Human biopsies from controls and DMD patients consisted of small fragments of quadriceps muscle and were quickly frozen with isopentane in a container immersed in liquid nitrogen. Cryosections of 10 μm fixed in 4 % paraformaldehyde were processed for histological (H–E and trichrome), histochemical stains [oxidative (NADH, SDH and COX), ATPasa (pH 4.2; 4.6 and 9.4), Oil-red-O and PAS] and immunohistochemistry (dystrophin 1, 2 and 3, alpha, beta, delta and gamma sarcoglycans, merosin and beta-spectrin and Cxs).

Statistical analysis

Results are presented as mean ± standard error (SE). Two populations were compared using the logarithm of ratio and posterior Student's *t* test. For multiple comparisons with a single control, a non-parametric one-way ANOVA followed by the Tukey's multiple comparison test was used. Analyses were carried out using GRAPHPAD software. $P < 0.05$ was considered statistically significant.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors certify that the experiments comply with the current laws of Chile, where the experiments were performed. All protocols were approved by the Bioethics Committee of the Pontificia Universidad Católica de Chile (Protocol No. 176) in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All efforts were made to minimize animal suffering, reduce the number of animals used, and alternatives to in vivo techniques, if available.

References

1. Emery AE (2002) Muscular dystrophy into the new millennium. Neuromuscul Disord 12:343–349
2. Ervasti JM, Campbell KP (1993) A role for the dystrophin–glycoprotein complex as a transmembrane linker between laminin and actin. J Cell Biol 122:809–823
3. Pifers D (2014) A new day for Duchenne's? The time has come for newborn screening. Mol Genet Metab 113:11–13
4. Bulfield G, Siller WG, Wight PA, Moore KJ (1984) X chromosome-linked muscular dystrophy (mdx) in the mouse. Proc Natl Acad Sci USA 81:1189–1192
5. Bahnave CD, Allen DG (1995) Intracellular calcium and force in single mouse muscle fibres following repeated contractions with stretch. J Physiol 488:25–36
6. Yeung EW, Head SI, Allen DG (2003) Gadolinium reduces short-term stretch-induced muscle damage in isolated mdx mouse muscle fibres. J Physiol 552:449–458
7. Matsuda R, Nishikawa A, Tanaka H (1995) Visualization of dystrophic muscle fibers in mdx mouse by vital staining with Evans blue: evidence of apoptosis in dystrophin-deficient muscle. J Biochem 118:959–964
8. Hamer PW, McGeachie JM, Davies MJ, Grounds MD (2002) Evans Blue Dye as an in vivo marker of myofibre damage: optimising parameters for detecting initial myofibre membrane permeability. J Anat 200:69–79
9. Cea LA, Cisterna BA, Puebla C, Frank M, Figueroa XF, Cardozo C et al (2013) De novo expression of connexin hemichannels in denervated fast skeletal muscles leads to atrophy. Proc Natl Acad Sci USA 110:16229–16234
10. Sáez JC, Leybaert L (2014) Hunting for connexin hemichannels. FEBS Lett 588:1205–1211
11. Eskandari S, Zampighi GA, Leung DW, Wright EM, Loo DD (2002) Inhibition of gap junction hemichannels by chloride channel blockers. J Membr Biol 185:93–102
12. Franco A Jr, Lansman JB (1990) Calcium entry through stretch-inactivated ion channels in mdx myotubes. Nature 344:670–673
13. Bao L, Sachs F, Dahl G (2004) Connexins are mechanosensitive. Am J Physiol Cell Physiol 287:C1389–C1395

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RESEARCH Open Access

The absence of dysferlin induces the expression of functional connexin-based hemichannels in human myotubes

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Abstract

Background: Mutations in the gene encoding for dysferlin cause recessive autosomal muscular dystrophies called dysferlinopathies. These mutations induce several alterations in skeletal muscles, including, inflammation, increased membrane permeability and cell death. Despite the fact that the etiology of dysferlinopathies is known, the mechanism that explains the aforementioned alterations is still elusive. Therefore, we have now evaluated the potential involvement of connexin based hemichannels in the pathophysiology of dysferlinopathies.

Results: Human deltoid muscle biopsies of 5 Chilean dysferlinopathy patients exhibited the presence of muscular connexins (Cx40.1, Cx43 and Cx45). The presence of these connexins was also observed in human myotubes derived from immortalized myoblasts derived from other patients with mutated forms of dysferlin. In addition to the aforementioned connexins, these myotubes expressed functional connexin based hemichannels, evaluated by ethidium uptake assays, as opposed to myotubes obtained from a normal human muscle cell line, RCMH. This response was reproduced in a knock-down model of dysferlin, by treating RCMH cell line with small hairpin RNA specific for dysferlin (RCMH-sh Dysferlin). Also, the presence of $P2X_7$ receptor and the transient receptor potential channel, TRPV2, another Ca^{2+} permeable channels, was detected in the myotubes expressing mutated dysferlin, and an elevated resting intracellular Ca^{2+} level was found in the latter myotubes, which was in turn reduced to control levels in the presence of the molecule D4, a selective Cx HCs inhibitor.

Conclusions: The data suggests that dysferlin deficiency, caused by mutation or downregulation of dysferlin, promotes the expression of Cx HCs. Then, the *de novo* expression Cx HC causes a dysregulation of intracellular free Ca^{2+} levels, which could underlie muscular damage associated to dysferlin mutations. This mechanism could constitute a potential therapeutical target in dysferlinopathies.

Keywords: Dysferlinopathy, Membrane permeability, Calcium

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Competing interests
PC declares patent protection for the RCMH cell line. LAC, JAB, CA, AMC, AB, VM and JCS, declare no financial and non-financial competing interests.

Authors' contribution
LAC designed research, performed experiments, analyzed data and wrote the paper. JAB, CA, AMC, AB, VM, JCS and PC designed research, analyzed the data, and wrote the paper. All authors read and approved the final manuscript.

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References

1. Matsuda C, Hayashi YK, Ogawa M, Aoki M, Murayama K, Nishino I, Nonaka I, Arahata K, Brown RH Jr. The sarcolemmal proteins dysferlin and caveolin-3 interact in skeletal muscle. Hum Mol Genet. 2001;10:1761–6.
2. Klinge L, Laval S, Keers S, Haldane F, Straub V, Barresi R, Bushby K. From T-tubule to sarcolemma: damage-induced dysferlin translocation in early myogenesis. FASEB J. 2007;21:1768–76.
3. Kerr JP, Ziman AP, Mueller AL, Muriel JM, Kleinhans-Welte E, Gumerson JD, Vogel SS, Ward CW, Roche JA, Bloch RJ. Dysferlin stabilizes stress-induced Ca2+ signaling in the transverse tubule membrane. Proc Natl Acad Sci U S A. 2013;110:20831–6.
4. Bansal D, Miyake K, Vogel SS, Groh S, Chen CC, Williamson R, McNeil PL, Campbell KP. Defective membrane repair in dysferlin-deficient muscular dystrophy. Nature. 2003;423:168–72.
5. Klinge L, Harris J, Sewry C, Charlton R, Anderson L, Laval S, Chiu YH, Hornsey M, Straub V, Barresi R, Lochmüller H, Bushby K. Dysferlin associates with the developing T-tubule system in rodent and human skeletal muscle. Muscle Nerve. 2010;41:166–73.
6. Ceriacchi G, Fanin M, De Giorgi LB, Angelini E. Ultrastructural changes in dysferlinopathy support defective membrane repair mechanism. J Clin Pathol. 2005;58:190–5.
7. Urtizberea JA, Bassez G, Leturcq F, Nguyen K, Krahn M, Levy N. Dysferlinopathies. Neurol India. 2008;56:289–97.
8. Han R. Muscle membrane repair and inflammatory attack in dysferlinopathy. Skelet Muscle. 2011;1:10.
9. Nagaraju K, Rawat R, Veszelovszky E, Thapliyal R, Kesari A, Sparks S, Raben N, Plotz P, Hoffman EP. Dysferlin deficiency enhances monocyte phagocytosis: a model for the inflammatory onset of limb-girdle muscular dystrophy 2B. Am J Pathol. 2008;172(3):774–85.
10. Rawat R, Cohen TV, Ampong B, Francia D, Henriques-Pons A, Hoffman EP, Nagaraju K. Inflammasome up-regulation and activation in dysferlin-deficient skeletal muscle. Am J Pathol. 2010;176(6):2891–900.
11. Cea LA, Cisterna BA, Puebla C, Frank M, Figueroa XF, Cardozo C, Willecke K, Latorre R, Sáez JC. De novo expression of connexin hemichannels in denervated fast skeletal muscles leads to atrophy. Proc Natl Acad Sci U S A. 2013;110:16229–34.
12. Cea LA, Riquelme MA, Cisterna BA, Puebla C, Vega JL, Rovegno M, Sáez JC. Connexin- and pannexin-based channels in normal skeletal muscles and their possible role in muscle atrophy. J Membr Biol. 2012;245:423–36.
13. Sáez JC, Leybaert L. Hunting for connexin hemichannels. FEBS Lett. 2014;588:1205–11.
14. Cea LA, Riquelme MA, Vargas AA, Urrutia C, Sáez JC. Pannexin 1 channels in skeletal muscles. Front Physiol. 2014;5:eCollection.
15. Lostal W, Bartoli M, Roudaut C, Bourg N, Krahn M, Pryadkina M, Borel P, Suel L, Roche JA, Stockholm D, Bloch RJ, Levy N, Bashir R, Richard I. Lack of correlation between outcomes of membrane repair assay and correction of dystrophic changes in experimental therapeutic strategy in dysferlinopathy. PLoS One. 2012;7:e38036.
16. Craig SW, Pardo JV. Gamma actin, spectrin, and intermediate filament proteins colocalize with vinculin at costameres, myofibril-to-sarcolemma attachment sites. Cell Motil. 1983;3:449–62.
17. Kerr JP, Ward CW, Bloch RJ. Dysferlin at transverse tubules regulates Ca(2+) homeostasis in skeletal muscle. Front Physiol. 2014;5:89. eCollection.
18. Iwata Y, Katanosaka Y, Arai Y, Shigekawa M, Wakabayashi S. Dominant-negative inhibition of Ca2+ influx via TRPV2 ameliorates muscular dystrophy in animal models. Hum Mol Genet. 2009;18:824–34.
19. Neeper MP, Liu Y, Hutchinson TL, Wang Y, Flores CM, Qin N. Activation properties of heterologously expressed mammalian TRPV2: evidence for species dependence. J Biol Chem. 2007;282:15894–902.
20. Contreras JE, Sánchez HA, Eugenín EA, Speidel D, Theis M, Willecke K, Bukauskas FF, Bennett MV, Sáez JC. Metabolic inhibition induces opening of unopposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture. Proc Natl Acad Sci U S A. 2002;99:495–500.
21. D'hondt C, Ponsaerts R, De Smedt H, Bultynck G, Himpens B. Pannexins, distant relatives of the connexin family with specific cellular functions? Bioessays. 2009;31:953–74.
22. Schalper KA, Sánchez HA, Lee SC, Altenberg GA, Nathanson MH, Sáez JC. Connexin 43 hemichannels mediate the Ca2+ influx induced by extracellular alkalinization. Am J Physiol Cell Physiol. 2010;299:C1504–15.
23. Proulx A, Merrifield PA, Naus CC. Blocking gap junctional intercellular communication in myoblasts inhibits myogenin and MRF4 expression. Dev Genet. 1997;20:133–44.
24. Tóth A, Fodor J, Vincze J, Olah T, Juhász T, Zákány R, Csernoch L, Zádor E. The Effect of SERCA1b Silencing on the Differentiation and Calcium Homeostasis of C2C12 Skeletal Muscle Cells. PLoS One. 2015;10:e0123583.
25. Turner PR, Schultz R, Ganguly B, Steinhardt RA. Proteolysis results in altered leak channel kinetics and elevated free calcium in mdx muscle. J Membr Biol. 1993;133:243–51.
26. Azakir BA, Erne B, Di Fulvio S, Stirnimann G, Sinnreich M. Proteasome inhibitors increase missense mutated dysferlin in patients with muscular dystrophy. Sci Transl Med. 2014;6:250ra112.
27. Turner PR, Fong PY, Denetclaw WF, Steinhardt RA. Increased calcium influx in dystrophic muscle. J Cell Biol. 1991;115:1701–12.
28. Azakir BA, Di Fulvio S, Kinter J, Sinnreich M. Proteasomal inhibition restores biological function of mis-sense mutated dysferlin in patient-derived muscle cells. J Biol Chem. 2012;287:10344–54.
29. Ho M, Post CM, Donahue LR, Lidov HG, Bronson RT, Goolsby H, Watkins SC, Cox GA, Brown RH Jr. Disruption of muscle membrane and phenotype divergence in two novel mouse models of dysferlin deficiency. Hum Mol Genet. 2004;13:1999–2010.
30. Philippi S, Bigot A, Marg A, Mouly V, Spuler S, Zacharias U. Dysferlin-deficient immortalized human myoblasts and myotubes as a useful tool to study dysferlinopathy. Version 2. PLoS Curr. 2012;4:RRN1298.
31. Mamchaoui K, Trollet C, Bigot A, Negroni E, Chaouch S, Wolff A, Kandalla PK, Marie S, Di Santo J, St Guily JL, Muntoni F, Kim J, Philippi S, Spuler S, Levy N, Blumen SC, Voit T, Wright WE, Aamiri A, Butler-Browne G, Mouly V. Immortalized pathological human myoblasts: towards a universal tool for the study of neuromuscular disorders. Skelet Muscle. 2011;1:34.

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Dexamethasone-induced muscular atrophy is mediated by functional expression of connexin-based hemichannels

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ABSTRACT

Long-term treatment with high glucocorticoid doses induces skeletal muscle atrophy. However, the molecular mechanism of such atrophy remains unclear. We evaluated the possible involvement of connexin-based hemichannels (Cx HCs) in muscle atrophy induced by dexamethasone (DEX), a synthetic glucocorticoid, on control ($Cx43^{fl/fl}Cx45^{fl/fl}$) and Cx43/Cx45 expression-deficient ($Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre) skeletal myofibers. Myofibers of $Cx43^{fl/fl}Cx45^{fl/fl}$ mice treated with DEX (5 h) expressed several proteins that form non-selective membrane channels (Cx39, Cx43, Cx45, Panx1, $P2X_7$ receptor and TRPV2). After 5 h DEX treatment *in vivo*, myofibers of $Cx43^{fl/fl}Cx45^{fl/fl}$ mice showed Evans blue uptake, which was absent in myofibers of $Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre mice. Similar results were obtained *in vitro* using ethidium as an HC permeability probe, and DEX-induced dye uptake in control myofibers was blocked by $P2X_7$ receptor inhibitors. DEX also induced a significant increase in basal intracellular Ca^{2+} signal and a reduction in resting membrane potential in $Cx43^{fl/fl}Cx45^{fl/fl}$ myofibers, changes that were not elicited by myofibers deficient in Cx43/Cx45 expression. Moreover, treatment with DEX induced NFκB activation and increased mRNA levels of TNF-α in control but not in Cx43/Cx45 expression-deficient myofibers. Finally, a prolonged DEX treatment (7 days) increased atrogin-1 and Murf-1 and reduced the cross sectional area of $Cx43^{fl/fl}Cx45^{fl/fl}$ myofibers, but these parameters remained unaffected in $Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre myofibers. Therefore, DEX-induced expression of Cx43 and Cx45 plays a critical role in early sarcolemma changes that lead to atrophy. Consequently, this side effect of chronic glucocorticoid treatment might be avoided by co-administration with a Cx HC blocker.

1. Introduction

Glucocorticoids are frequently used as anti-inflammatory and immunosuppressive agents [1]. However, high doses and prolonged use induces undesired lateral effects such as reduction in tetanic stimuli-induced force [2] and muscular atrophy [3]. Nonetheless, the molecular mechanisms that explain the latter undesired effect are not completely understood.

Muscle wasting after glucocorticoid treatment is highly relevant and is called "steroid myopathy". It is characterized by an insidious process that causes weakness mainly in the proximal muscles of the upper and lower limbs and in the neck flexors [4,5]. An excess of either endogenous or exogenous corticosteroids can cause this condition. The excess of endogenous corticosteroid production can arise from adrenal tumors [6,7] and an excess of exogenous corticosteroids can result from steroid treatment for asthma, chronic obstructive pulmonary disease, and inflammatory processes, such as connective tissue disorders among others [8,9].

It has been suggested that glucocorticoids increase proteasome-dependent protein degradation [10] and inhibit protein synthesis [11]. Several noxious conditions that induce skeletal muscle atrophy (*e.g.* sepsis, cachexia and starvation) are also associated with an increase in circulating glucocorticoids levels [11], suggesting that these hormones constitute a common factor in skeletal muscle atrophy associated with these conditions. Accordingly, treatment with a glucocorticoid receptor antagonist (RU-38486) reduces muscle atrophy associated with sepsis [12]. Additionally, glucocorticoids have been shown to induce muscle

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Hence, it is possible that the same occurs in other systems expressing glucocorticoids receptors like skeletal muscles [13]. Whether a similar mechanism explains the upregulation of Cx39 and Cx45 remains to be demonstrated.

The aforementioned mechanism suggests that the use of corticoids to treat Duchenne patients is controversial and perhaps inappropriate. Accordingly, it has been reported that corticoid treatment does not increase the life expectancy of Duchenne patients [43], probably because glucocorticoids induce the activation of NFκB [16], which acts as a pro-inflammatory signal for muscular tissue as demonstrated in this report. It should be kept in mind that steroids have differential effects in different tissues (*e.g.*, anti-inflammatory in the immune system and inflammatory in skeletal muscles). In particular, in non-immune systems tissues like skeletal muscles, glucocorticoids induce inflammation. It is highly relevant to describe that this pro-inflammatory response to steroids in muscle tissue is at least partially mediated by the activation of Cx HCs [22]. Since there are several drugs that have been proven to inhibit HCs and pannexins, it is desirable to test if the likely negative effects of steroids could be modulated by the inhibition of these hemichannels.

Since at least Cx43 HCs are permeable to Ca^{2+} [28], lack of atrophy in myofibers of $Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre mice treated with DEX can be explained by the lack of Ca^{2+} influx, and hence the lack of activation of protein degradation pathways (as observed by the low atrogin-1 and Murf-1 levels), which has been previously shown to be involved in DEX-induced muscle atrophy [10]. The lack of activation of protein degradation pathways is probably related to the low intracellular Ca^{2+} signal observed in myofibers of $Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre mice, as was observed in this study. Moreover, DEX treatment for 7 days in normal mice induced muscle atrophy, which did not occur in the absence of Cx43 and Cx45 expression in muscles. This suggests that DEX-induced muscle atrophy is mediated by Cx HCs. Interestingly, the absence of Cx43Cx45 HCs completely prevented DEX-induced muscular atrophy. But in other conditions, like denervation, it just prevented ~75% of muscular atrophy [22], thus suggesting the involvement of additional mechanisms that lead to muscular atrophy.

In conclusion, DEX treatment is associated with early muscle atrophy and muscle inflammation. These effects can be explained by the expression and activation of Cx HCs. Inhibition of Cx HCs during early steroid treatment could prevent the activation of protein degradation pathways, Ca^{2+} influx, and unfolding of atrophy.

Ethical standards

The authors certify that the experiments comply with the current laws within Chile, where the experiments were performed. All protocols were approved by the Bioethics Committee of the Pontificia Universidad Católica de Chile (protocol no. 176) in accordance with the ethical standards stipulated in the 1964 Declaration of Helsinki and its later amendments. All efforts were made to minimize animal suffering as well as to reduce the number of animals used, and alternatives to *in vivo* techniques were applied, if available.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Transparency document

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

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References

[1] T. Rhen, J.A. Cidlowski, Anti-inflammatory action of glucocorticoids. New mechanisms for old drugs, N. Engl. J. Med. 353 (2005) 1711–1723.
[2] Y.S. Shin, H. Fink, R. Khiroya, C. Ibebunjo, J. Martyn, Prednisolone-induced muscle dysfunction is caused more by atrophy than by altered acetylcholine receptor expression, Anesth. Analg. 91 (2000) 322–328.
[3] P.O. Hasselgren, Glucocorticoids and muscle catabolism, Curr. Opin. Clin. Nutr. Metab. Care 2 (1999) 201–205.
[4] D. Annane, What is the evidence for harm of neuromuscular blockade and corticosteroid use in the intensive care unit? Semin. Respir. Crit. Care Med. 37 (2016) 51–56.
[5] G. Hermans, G. Van den Berghe, Clinical review: intensive care unit acquired weakness, Crit. Care 19 (2015) 274.
[6] J. Stindl, P. Tauber, C. Sterner, I. Tegtmeier, R. Warth, S. Bandulik, Pathogenesis of adrenal aldosterone-producing adenomas carrying mutations of the Na(+)/K(+)-ATPase, Endocrinology 156 (2015) 4582–4591.
[7] G.M. Goddard, A. Ravikumar, A.C. Levine, Adrenal mild hypercortisolism, Endocrinol. Metab. Clin. N. Am. 44 (2015) 371–379.
[8] D.K. Ingawale, S.K. Mandlik, S.S. Patel, An emphasis on molecular mechanisms of anti-inflammatory effects and glucocorticoid resistance, J. Complement. Integr. Med. 12 (2015) 1–13.
[9] Z. Jiang, L. Zhu, Update on molecular mechanisms of corticosteroid resistance in chronic obstructive pulmonary disease, Pulm. Pharmacol. Ther. S1094-5539 (2016) 30002–30005.
[10] B.G. Li, P.O. Hasselgren, C.H. Fang, G.D. Warden, Insulin-like growth factor-I blocks dexmethasone-induced protein degradation in cultured myotubes by inhibiting multiple proteolytic pathways, J. Burn Care Rehabil. 25 (2004) 112–118.
[11] O. Schakman, H. Gilson, S. Kalista, J.P. Thissen, Mechanisms of muscle atrophy induced by glucocorticoids, Horm. Res. 72 (Suppl. 1) (2008) 36–41.
[12] M. Konagaya, P.A. Bernard, S.R. Max, Blockade of glucocorticoid receptor binding and inhibition of dexamethasone-induced muscle atrophy in the rat by RU38486, a potent glucocorticoid antagonist, Endocrinology 119 (1986) 375–380.
[13] T. Kuo, C.A. Harris, J.C. Wang, Metabolic functions of glucocorticoid receptor in skeletal muscle, Mol. Cell. Endocrinol. 380 (2013) 79–88.
[14] M.A. Minetto, R. Qaisar, V. Agoni, G. Motta, E. Longa, D. Miotti, M.A. Pellegrino, R. Bottinelli, Quantitative and qualitative adaptations of muscle fibers to glucocorticoids, Muscle Nerve 52 (2015) 631–639.
[15] O. Schakman, S. Kalista, C. Barbé, A. Loumaye, J.P. Thissen, Glucocorticoid-induced skeletal muscle atrophy, Int. J. Biochem. Cell Biol. 45 (2013) 2163–2172.
[16] S. Khan, Z. Lopez-Dee, R. Kumar, J. Ling, Activation of NFkB is a novel mechanism of pro-survival activity of glucocorticoids in breast cancer cells, Cancer Lett. 337 (2013) 90–95.
[17] M. Imae, Z. Fu, A. Yoshida, T. Noguchi, H. Kato, Nutritional and hormonal factors control the gene expression of FoxOs, the mammalian homologues of DAF-16, J. Mol. Endocrinol. 30 (2003) 253–262.
[18] H. Yang, M.J. Menconi, W. Wei, V. Petkova, P.O. Hasselgren, Dexamethasone upregulates the expression of the nuclear cofactor p300 and its interaction with C/EBPbeta in cultured myotubes, J. Cell. Biochem. 94 (2005) 1058–1067.
[19] H. Wang, N. Kubica, L.W. Ellisen, L.S. Jefferson, S.R. Kimball, Dexamethasone represses signaling through the mammalian target of rapamycin in muscle cells by enhancing expression of REDD1, J. Biol. Chem. 281 (2006) 39128–39134.
[20] A.R. Evenson, M.U. Fareed, M.J. Menconi, J.C. Mitchell, P.O. Hasselgren, GSK-3beta inhibitors reduce protein degradation in muscles from septic rats and in dexamethasone-treated myotubes, Int. J. Biochem. Cell Biol. 37 (2005) 2226–2238.
[21] S. Konno, Hydroxyl radical formation in skeletal muscle of rats with glucocorticoid-induced myopathy, Neurochem. Res. 30 (2005) 669–675.
[22] L.A. Cea, B.A. Cisterna, C. Puebla, M. Frank, X.F. Figueroa, C. Cardozo, K. Willecke, R. Latorre, J.C. Sáez, De novo expression of connexin hemichannels in denervated fast skeletal muscles leads to atrophy, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 16229–16234.
[23] L.A. Cea, C. Puebla, B.A. Cisterna, R. Escamilla, A.A. Vargas, M. Frank, P. Martínez-Montero, C. Prior, J. Molano, I. Esteban-Rodríguez, I. Pascual, P. Gallano, G. Lorenzo, H. Pian, L.C. Barrio, K. Willecke, J.C. Sáez, Fast skeletal myofibers of mdx mouse, model of Duchenne muscular dystrophy, express connexin hemichannels that lead to apoptosis, Cell. Mol. Life Sci. 73 (2016) 2583–2599.
[24] J.C. Sáez, V.M. Berthoud, M.C. Branes, A.D. Martinez, E.C. Beyer, Plasma membrane channels formed by connexins: their regulation and functions, Physiol. Rev. 83 (2003) 1359–1400.
[25] J.J. Bunim, R.L. Black, L. Lutwak, R.E. Peterson, G.D. Whedon, Studies on dexamethasone, a new synthetic steroid, in rheurheumatoid arthritis: a preliminary report; adrenal cortical, metabolic and early clinical effects, Arthritis Rheum. 1 (1958) 313–331.
[26] K. Nakazawa, M. Liu, K. Inoue, Y. Ohno, Potent inhibition by trivalent cations of ATP-gated channels, Eur. J. Pharmacol. 325 (1997) 237–243.
[27] P. Honore, D. Donnelly-Roberts, M.T. Namovic, G. Hsieh, C.Z. Zhu, J.P. Mikusa, G. Hernandez, C. Zhong, D.M. Gauvin, P. Chandran, R. Harris, A.P. Medrano, W. Carroll, K. Marsh, J.P. Sullivan, C.R. Faltynek, M.F. Jarvis, A-740003 [N-(1-{[(cyanoimino)(5-quinolinylamino) methyl]amino}-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl) acetamide], a novel and selective P2X7 receptor antagonist, dose-dependently reduces neuropathic pain in the rat, J. Pharmacol. Exp. Ther. 319 (2006) 1376–1385.

ANALES

¿CÓMO AFECTA EL COLOR AL COMPORTAMIENTO?: UNA MIRADA A LA VISIÓN ULTRAVIOLETA EN MAMÍFEROS

Andrés E. Chávez[1]

Resumen: Comprender como el color puede regular comportamientos ha sido estudiado en una gran variedad de contextos. Avances desde la fisiología y ecología sensorial han demostrado que muchos mamíferos, incluyendo el roedor chileno *Octodon degus (degú)*, presentan un sistema visual adaptado para la visión de colores (dicromática), con un tipo de fotorreceptor especializado para la detección de señales en el rango ultravioleta (UV; 360 nm). La luz UV ocupa el rango espectral de longitudes de onda ligeramente más cortos que el visible para los seres humanos. Esta asombrosa capacidad visual está involucrada en múltiples comportamientos que van desde la navegación y orientación hasta la detección de alimentos y depredadores, selección sexual, así como para tareas de alto nivel de apoyo como la evaluación de su compañero y la comunicación intraespecífica. Si bien la función de la visión UV en el comportamiento del degú no está del todo clara, se ha postulado que este tipo de señal visual podrían estar involucradas en comportamientos de navegación y comunicación entre congéneres.

Palabras claves: Ecología sensorial, visión UV, Fotorreceptores UV, retina, patrón de coloración animal, conducta..

Abstract: Understanding how colour can regulate behavior has been study in a variety of contexts. New advances from the physiology and sensory ecology have shown that most mammals, including the Chilean rodent *Octodon degus* (degu), have a visual system adapted to the dichromatic colors vision. Notably, their retinas contain a photoreceptor specialized for signal detection in the ultraviolet (UV) range (~360 nm). UV-light is localized in the spectral range of wavelengths slightly shorter than those visible to humans. This amazing visual capability of many species is known to be involved in multiple behaviors, including navigation and orientation, predator's detection, sexual selection and to supporting high-level tasks such as mate assessment and intraspecific communication. While the role of UV vision in the degu is not entirely clear, it has been suggested that this type of visual cues may be involved in navigation and conspecific communication.

Keywords: Sensory ecology, UV vision, UV cone photoreceptor, retina, animal color pattern.

El color está en todo lo que nos rodea. Es una sensación que añade emociones y pensamientos a nuestras vidas, y con ello puede regular nuestros comportamientos. No es casualidad por ejemplo, el color con el cual pintamos nuestra casa, la pieza de nuestros hijos o el color de ropa que utilizamos. Siempre tendemos a sentirnos más a gusto con un color que con otro. De hecho, durante los últimos años, los efectos funcionales del color en humanos han sido estudiados en una variedad de contextos, incluyendo razonamientos, atenciones, emociones y comportamientos (Valdez y Mehrabian, 1994, Elliot y Maier, 2014). En el reino animal, en tanto, sabemos que el patrón de coloración de una especie juega un papel fundamental en múltiples comportamientos incluidos la detección y ubicación de alimentos, detección y camuflaje de predadores, tareas sociales, selección sexual, entre otras (Endler, 1990). Pero, ¿Qué es el color? ¿Cómo percibimos el color? y ¿Cómo él puede generar o modificar conductas?.

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Consistente con esta adaptación para la vida diurna y la visión UV, la retina de los degú presentan una abundante cantidad de conos (>30%) comparado con especies nocturnas (Jacobs, *et al.*, 2003), aun cuando estas últimas también presenta un sistema visual adaptado para la visión UV(Jacobs, et al., 1991, Jacobs, *et al.*, 2001, Peichl, et al., 2005). Pero ¿Hay alguna característica en el medio ambiente o algún comportamiento que pueda explicar la visión UV en el degú?.

Experimentos conductuales demostraron que el degú es capaz de hacer discriminaciones de color, entre el UV y la luz visible (Jacobs, *et al.*, 2003), lo cual sugiere fuertemente qué los objetos presentes en su hábitat y que reflejan UV son potencialmente distinguibles para esta especie y pudieran estar regulando patrones de comportamientos. Sin embargo, mediciones de reflectancia (capacidad de un cuerpo de reflejar la luz o emitir color) de muchos substratos del hábitat del degú no mostraron patrones cercanos al UV, indicando que la visión UV en esta especie no estaría participando en la detección y búsqueda de alimentos. Por el contrario, se observó que el tórax o la zona ventral del degú, a diferencia de la zona dorsal, muestra un aumento en los niveles de reflectancia en el rango UV(Chavez, *et al.*, 2003). Dada estas características, se planteó la posibilidad que la señal UV estaría jugando un rol en los patrones de comunicación entre individuos de su misma especie. De hecho, durante una llamada de alerta, uno de los comportamientos del degú es pararse en forma vertical sobre sus patas traseras y exponer su tórax a la vista de sus congéneres(Vasquez, 1997). Notablemente, en la búsqueda de otras señales UV presentes en el hábitat de esta especie y que pudieran ser conductualmente relevantes en el contexto social para el degú, se observó que la orina, la cual estos animales utilizan para la marcación de sus senderos de forrajeo y áreas de reunión(Ebensperger y Bozinovic, 2000, Ebensperger y Caiozzi, 2002), muestran una alta reflectancia en el rango UV en su estado fresco comparado a su estado seco (Chavez, *et al.*, 2003). Por lo tanto, se planteó la posibilidad de que estas marcas pudieran representar tanto señales visuales, como olfativas, proveyendo una ventaja para la orientación de largo alcance.

De esta manera, mientras para nosotros el UV puede ser una señal de alerta o de daño, es claro que para esta y muchas otras especies de mamíferos, peces, aves, y reptiles la señal UV es fundamental para múltiples comportamientos sociales como: *(i)* selección sexual (variaciones entre machos y hembras; jóvenes y adultos); *(ii)* en el apoyo en tareas sociales; *(iii)*, en la comunicación intra-específica y/o como un distractor para potenciales predadores (Cronin y Bok, 2016). Mientras la función de la visión UV en el degú aun no es del todo clara, con esta mirada a la función de la visión UV en un roedor endémico de chile, se puede ejemplificar claramente, como el color genera múltiples adaptaciones y puede regular múltiples comportamientos.

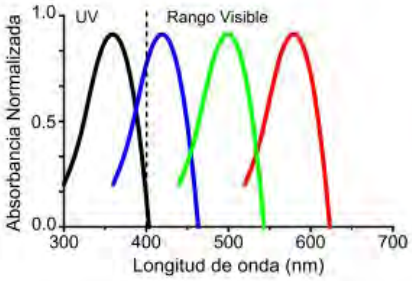

Fig. 1: Esquema del espectro de luz visible en humanos y los espectros de absorbencia para las cuatro clases de pigmentos visuales observados en vertebrados. Se puede observar un pigmento visual en el rango UV (360 nm), uno de onda corta sensible a través de 400–470 nm y otro sensible a 480-530 nm. El último posee un máximo alrededor de 570 nm.

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Multi-drug Resistance Profile of PR20 HIV-1 Protease is attributed to Distorted Conformational and Drug Binding Landscape: Molecular Dynamics Insights

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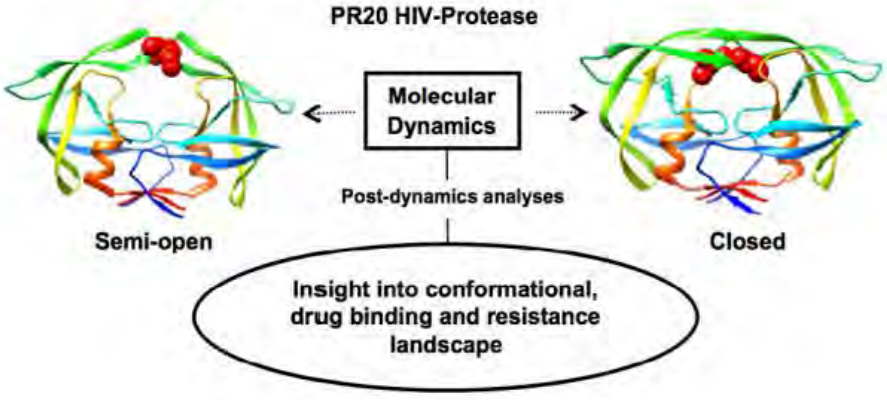
Graphical Abstract



inhibitor which can inhibit multidrug resistant strains by a great extent. Drug discovery targeting the HIV-protease still remains a key interest in modern drug discovery. The findings reported in this study will significantly help in the rational design of post darunavir novel HIV-PR inhibitors with an improved potency, targeted towards various multi-drug resistant strains.

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7. Conflicts of Interest

Authors declare no potential conflicts of interest.

8. Supplementary Materials

The RMSDs, potential energies, graphical representation of RIN profiles as well as Cytoscape sessions of RINs were presented as Supplementary Materials.

Connexin hemichannels explain the ionic imbalance and lead to atrophy in denervated skeletal muscles

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Running Head: Connexin hemichannels induce skeletal muscle atrophy

Key words: Calcium ion; sodium ion; protein synthesis; protein degradation, skeletal muscle atrophy.

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GENETICS | INVESTIGATION

The Splice Isoforms of the *Drosophila* Ecdysis Triggering Hormone Receptor Have Developmentally Distinct Roles

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ABSTRACT To grow, insects must periodically shed their exoskeletons. This process, called ecdysis, is initiated by the endocrine release of Ecdysis Trigger Hormone (ETH) and has been extensively studied as a model for understanding the hormonal control of behavior. Understanding how ETH regulates ecdysis behavior, however, has been impeded by limited knowledge of the hormone's neuronal targets. An alternatively spliced gene encoding a G-protein-coupled receptor (*ETHR*) that is activated by ETH has been identified, and several lines of evidence support a role in ecdysis for its A-isoform. The function of a second ETHR isoform (ETHRB) remains unknown. Here we use the recently introduced "Trojan exon" technique to simultaneously mutate the *ETHR* gene and gain genetic access to the neurons that express its two isoforms. We show that ETHRA and ETHRB are expressed in largely distinct subsets of neurons and that ETHRA- but not ETHRB-expressing neurons are required for ecdysis at all developmental stages. However, both genetic and neuronal manipulations indicate an essential role for ETHRB at pupal and adult, but not larval, ecdysis. We also identify several functionally important subsets of ETHR-expressing neurons including one that coexpresses the peptide Leucokinin and regulates fluid balance to facilitate ecdysis at the pupal stage. The general strategy presented here of using a receptor gene as an entry point for genetic and neuronal manipulations should be useful in establishing patterns of functional connectivity in other hormonally regulated networks.

KEYWORDS behavior; ecdysis; hormones; neural circuit; transgene targeting

HORMONES are major determinants of behavior, playing essential roles in mating, feeding, stress response, and other activities related to survival and reproduction. Identifying the neural circuits through which hormones act, however, has been complicated by the fact that many hormones directly enter the central nervous system and exert their effects at broadly dispersed sites. Establishing the "connectome" of hormonal action thus clearly requires tools different from those used to study synaptic connectivity in neural circuits. Pfaff and his colleagues pioneered the strategy of using sites of hormone binding as a guide to mapping behavioral circuits: By determining the principal sites of estrogen binding in female rat brains they elucidated the network underlying the rodent lordosis response (Pfaff and Keiner 1973; Pfaff *et al.* 1994). Receptor mapping has similarly provided key insights into the networks underlying other behaviors in both vertebrates and invertebrates, such as feeding (Wu *et al.* 2003; Scott *et al.* 2009), sleep and circadian rhythms (Marcus *et al.* 2001; Im and Taghert 2010), offspring care (Insel 1990), and pair bonding (Young *et al.* 1997). In general, however, the labor-intensive nature of receptor mapping and the complexity of most hormonally governed neural networks has made them difficult to fully unravel.

A comparatively tractable neuroendocrine network that has been extensively characterized in insects governs the shedding of the exoskeleton at the time of molting, a process called ecdysis (Zitnan and Adams 2012; White and Ewer 2014). This process is initiated by two related peptides (ETH1 and ETH2) encoded by the *Ecdysis Triggering Hormone*

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Sequence data from this article have been deposited with the GenBank Data Libraries under accession no. NM_206533.2.
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the ETHR expression pattern and are essential to the ecdysis circuit. We also provide evidence that subsets of ETHR-expressing neurons that use the neurotransmitters acetylcholine and glutamate are functionally important. Suppression of the cholinergic subset potently blocks ecdysis at both the larval and pupal stages and may well include many neurons that also express peptides. The glutamatergic subset, however, is likely to be distinct from the cholinergic group based on the previously reported nonoverlapping expression of the cholinergic marker, ChaT, and the glutamatergic marker, VGlut (Diao *et al.* 2015). Interestingly, electrical suppression of a GABAergic subset of ETHR-expressing neurons does not result in overt ecdysis failure. It thus seems likely that inhibitory inputs previously shown to regulate the execution of different phases of the ecdysis sequence in *Manduca* and thought to also function in *Drosophila* (Baker *et al.* 1999; Zitnan and Adams 2000; Fuse and Truman 2002) do not derive from ETHR-expressing neurons, are not GABAergic, or are not strictly essential for ecdysis. We favor the last possibility and note that we have focused here only on gross ecdysis deficits. More subtle defects that affect behavioral coordination, execution, or timing and do not result in lethality will require closer analysis. Our preliminary results, however, suggest that many of the ETHR-expressing neurons identified in this study can be expected to play specific roles in ecdysis at some developmental stage.

The efficacy of Trojan exon-mediated receptor mapping

The Trojan exon methodology used here to identify, manipulate, and parse the patterns of ETHR expression represents a systematic and versatile strategy for mapping functional connectivity within hormone-mediated neural circuits. In the case of the ecdysis circuit, this strategy has not only facilitated analysis of the neural substrates of behavior and physiology, but has revealed unanticipated developmental differences in the importance of the two ETHR isoforms. In the fly as in other insects, the motor patterns that mediate ecdysis vary considerably across developmental stages to accommodate differences in body plan and environmental context. However, the changes that occur in the ecdysis circuit over development remain largely unknown. The tools developed here should provide the basis for a thorough-going investigation of this, and other, important issues.

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Literature Cited

Arakane, Y., B. Li, S. Muthukrishnan, R. W. Beeman, K. J. Kramer *et al.*, 2008 Functional analysis of four neuropeptides, EH, ETH, CCAP and bursicon, and their receptors in adult ecdysis behavior of the red flour beetle, *Tribolium castaneum*. Mech. Dev. 125: 984–995.

Bahadorani, S., P. Bahadorani, E. Marcon, D. W. Walker, and A. J. Hilliker, 2010 A Drosophila model of Menkes disease reveals a role for DmATP7 in copper absorption and neurodevelopment. Dis. Model. Mech. 3: 84–91.

Bainbridge, S. P., and M. Bownes, 1981 Staging the metamorphosis of *Drosophila melanogaster*. J. Embryol. Exp. Morphol. 66: 57–80.

Baines, R. A., J. P. Uhler, A. Thompson, S. T. Sweeney, and M. Bate, 2001 Altered electrical properties in Drosophila neurons developing without synaptic transmission. J. Neurosci. 21: 1523–1531.

Baker, J. D., S. L. McNabb, and J. W. Truman, 1999 The hormonal coordination of behavior and physiology at adult ecdysis in *Drosophila melanogaster*. J. Exp. Biol. 202: 3037–3048.

Cabrero, P., L. Richmond, M. Nitabach, S. A. Davies, and J. A. T. Dow, 2013 A biogenic amine and a neuropeptide act identically: tyramine signals through calcium in Drosophila tubule stellate cells. Proceedings of the Royal Society B-Biological Sciences, Vol. 280.

Chen, T. W., T. J. Wardill, Y. Sun, S. R. Pulver, S. L. Renninger *et al.*, 2013 Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 499: 295.

Clark, A. C., M. Del Campo, and J. Ewer, 2004 Neuroendocrine control of larval ecdysis behavior in *Drosophila*: complex regulation by partially redundant neuropeptides. J. Neurosci. 24: 4283–4292.

Diao, F., H. Ironfield, H. Luan, F. Diao, W. C. Shropshire *et al.*, 2015 Plug-and-play genetic access to Drosophila cell types using exchangeable exon cassettes. Cell Rep. 10: 1410–1421.

Ewer, J., S. C. Gammie, and J. W. Truman, 1997 Control of insect ecdysis by a positive-feedback endocrine system: roles of eclosion hormone and ecdysis triggering hormone. J. Exp. Biol. 200: 869–881.

Fuse, M., and J. W. Truman, 2002 Modulation of ecdysis in the moth Manduca sexta: the roles of the suboesophageal and thoracic ganglia. J. Exp. Biol. 205: 1047–1058.

Halberg, K. A., S. Terhzaz, P. Cabrero, S. A. Davies, and J. A. T. Dow, 2015 Tracing the evolutionary origins of insect renal function. Nat. Commun. DOI: 10.1038/ncomms7800.

Hewes, R. S., and P. H. Taghert, 2001 Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. Genome Res. 11: 1126–1142.

Im, S. H., and P. H. Taghert, 2010 PDF receptor expression reveals direct interactions between circadian oscillators in Drosophila. J. Comp. Neurol. 518: 1925–1945.

Insel, T. R., 1990 Regional changes in brain oxytocin receptors postpartum: time-course and relationship to maternal-behavior. J. Neuroendocrinol. 2: 539–545.

Iversen, A., G. Cazzamali, M. Williamson, F. Hauser, and C. J. P. Grimmelikhuijzen, 2002 Molecular identification of the first insect ecdysis triggering hormone receptors. Biochem. Biophys. Res. Commun. 299: 924–931.

Kim, D.-H., M.-R. Han, G. Lee, S. S. Lee, Y.-J. Kim *et al.*, 2015 Rescheduling behavioral subunits of a fixed action pattern

BROADENING THE IMAGING PHENOTYPE OF DYSFERLINOPATHY AT DIFFERENT DISEASE STAGES

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ABSTRACT: *Introduction:* MRI characterization of dysferlinopathy has been mostly limited to the lower limbs. We aimed to broaden the MRI description of dysferlinopathy and to correlate it with objective measures of motor dysfunction. *Methods:* Sequential whole-body axial MRI was performed in 27 patients with genetically confirmed dysferlinopathy classified according to disease duration. Spearman correlations of fatty infiltration scores versus Motor Function Measure (MFM) were calculated. *Results:* Significant fatty infiltration was symmetrically present in early stages mainly in the posterior compartments of legs and thighs, thigh adductors, pelvic girdle, and some paravertebral muscles and the subscapularis. Later, fatty infiltration involved leg and thigh anterior compartments, arms and forearms, paravertebral, and trunk muscles. MRI infiltration score correlated positively with disease duration and negatively with MFM scale. *Conclusions:* We expand MRI characterization of dysferlinopathy and provide evidence for use of MRI scoring combined with motor functional scales to assess the natural course of disease.

Muscle Nerve **000**:000–000, 2016

Limb girdle muscular dystrophy type 2B (LGMD2B, OMIM#253601), Miyoshi myopathy (MM, OMIM#254130), and distal myopathy with anterior tibialis onset (DMAT, OMIM#606768) are the main clinical phenotypes associated with dysferlin gene (*DYSF*, MIM*603009) mutations.[1–4] Previous imaging reports of dysferlinopathy by computed tomography[5–7] and magnetic resonance imaging (MRI)[7–13] have shown early and more severe involvement of the posterior compartments of thighs and legs, which is independent of clinical phenotype.[13] Additionally, early imaging alterations in asymptomatic patients[14] and unusual patterns of muscle involvement have been described.[12,15,16] With the exception of Kesper et al., 2009[17] and Tasca et al., 2014,[18] these studies were focused on the lower limbs, and only 1 correlated MRI findings with the level of motor impairment progression, measured by means of a manual muscle testing composite based on the Medical Research Council scale (MRC).[19]

Recently, using 3 different functional scales, we established a correlation between motor functional testing and disease duration in a group of patients with genetically confirmed dysferlinopathy.[19] To complete the analysis, we reviewed the pattern of muscle involvement by whole-body MRI in 27 patients at different disease stages and correlated the findings with the level of functional impairment assessed by the Motor Function Measure scale.[20]

Abbreviations: DDG, disease duration group; DMAT, Distal myopathy with anterior tibial onset; DYSF, human dysferlin gene; FOV, field of view; LGMD2A, Limb girdle muscular dystrophy type 2A; LGMD2B, Limb girdle muscular dystrophy type 2B; MRI, magnetic resonance imaging; MFM, Motor Function Measure Scale; MM, Miyoshi's myopathy; MRC, Medical Research Council; MRS, Modified Rankin Scale; STIR, short tau inversion recovery.
Additional supporting information may be found in the online version of this article.

Key words: clinical trials; dysferlinopathy; motor function measure; MRI; muscle disease; whole-body MRI
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MATERIALS AND METHODS

Patients. Between 2011 and 2014, a total of 27 dysferlinopathy patients underwent sequential whole-body MRI and were evaluated clinically at the Department of Neurology and Neurosurgery of the Hospital Clínico Universidad de Chile (HCUCH), Santiago, Chile. The Ethics Committee of HCUCH and the Chilean National Commission of Scientific Research and Technology (CONICYT) approved the study protocol, and all patients gave informed consent.

All patients were symptomatic, and diagnosis was confirmed by direct DNA sequencing. Patient Dysf#001 was reported previously.[21] The clinical and genetic data of the cohort have been published

Allosterism and Structure in Thermally Activated Transient Receptor Potential Channels

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Keywords

gating models, temperature sensor, thermodynamics, heat activation pathways, ligand binding, phosphatidylinositol 4,5-bisphosphate

Abstract

The molecular sensors that mediate temperature changes in living organisms are a large family of proteins known as thermosensitive transient receptor potential (TRP) ion channels. These membrane proteins are polymodal receptors that can be activated by cold or hot temperatures, depending on the channel subtype, voltage, and ligands. The stimuli sensors are allosterically coupled to a pore domain, increasing the probability of finding the channel in its ion conductive conformation. In this review we first discuss the allosteric coupling between the temperature and voltage sensor modules and the pore domain, and then discuss the thermodynamic foundations of thermo-TRP channel activation. We provide a structural overview of the molecular determinants of temperature sensing. We also posit an anisotropic thermal diffusion model that may explain the large temperature sensitivity of TRP channels. Additionally, we examine the effect of several ligands on TRP channel function and the evidence regarding their mechanisms of action.

model best fits most of the features of T-TRP ion channels. It splits the channel into several specialized modules that have at least one resting state and one activated state associated with a specific sensing modality and governed by the corresponding equilibrium constant. As expected from several subdomains that coexist in a single protein, these sensing modules are connected by their respective allosteric factors, which reflect the influence exerted by each domain on its neighbors. The sum of these interactions is reflected by the channel open probability, P_{oc}, an experimentally accessible parameter of the allosteric model. This procedure has explained how the TRPV1 and TRPM8 channels are gated by voltage and temperature. Other models, such as the classic two-state model and the heat capacity theory (26), have also been proposed but display major shortcomings, such as lack of experimental support. Given the functional diversity among the members of the T-TRP channel superfamily, we are still far from developing a unified gating model that explains all the peculiarities of channel function. More detailed and technically demanding experiments are required.

The advent of membrane protein crystallization (33) provided a structural framework in which to rationalize the experimental results obtained during previous decades. The near-atomic resolution (~4 Å) structures obtained by cryo-EM at the Julius lab (19, 78, 105) revealed for the first time the intimacies of TRP channels. Although several features of other voltage-gated ion channels are present, these structures have a more complex organization of subdomains that are largely involved in coupling different sources of energy that drive channel activation and pore opening. As the existence of a temperature sensor analog for the voltage sensor domain or the calcium-gating ring in Ca^{2+}-activated channels remains controversial, information concerning the precise location of each residue in the channel sequence allows us to evaluate a TRP channel's thermal sensitivity using theoretical and computational techniques. With these methods we introduced a novel hypothesis, the ATD model. This model proposed that more flexible or disordered regions are more likely to be the entry point for environmental heat fluxes, and that both the overall amino acid composition of the channels and the structural relationships between them dictate thermal flux across the protein. Thermal fluxes eventually follow a route leading to the channel activation gate, which regulates the C-O transition. Questions concerning the structural basis for temperature activation remain unsolved, and novel approaches, combining theoretical predictions and rigorous experimental testing, may shed some light on this controversial issue.

Ligand binding is the other well-known pathway for modulating TRP channel activity. Some of these substances, such as capsaicin, allicin, and menthol, are found in nature and others, such as PIP_2, are fundamental components of biological membranes. Given the increasing amount of evidence that links several TRP channels to pathologic conditions, a full understanding of how these substances activate or inhibit the channel is necessary. The cryo-EM structures of TRPV1 and TRPA1 channels in the absence or presence of channel modulators have provided scientists an excellent opportunity to rationally design novel TRP channel modulators to achieve increased binding affinity and specificity.

DISCLOSURE STATEMENT

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MINIREVIEW—A LATIN AMERICAN PERSPECTIVE ON ION CHANNELS

Structure-Driven Pharmacology of Transient Receptor Potential Channel Vanilloid 1

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ABSTRACT

The transient receptor potential vanilloid 1 (TRPV1) ion channel is a polymodal receptor that mediates the flux of cations across the membrane in response to several stimuli, including heat, voltage, and ligands. The best known agonist of TRPV1 channels is capsaicin, the pungent component of "hot" chili peppers. In addition, peptides found in the venom of poisonous animals, along with the lipids phosphatidylinositol 4,5-biphosphate, lysophosphatidic acid, and cholesterol, bind to TRPV1 with high affinity to modulate channel gating. Here, we discuss the functional evidence regarding ligand-dependent activation of TRPV1 channels in light of structural data recently obtained by cryoelectron microscopy. This review focuses on the mechanistic insights into ligand binding and allosteric gating of TRPV1 channels and the relevance of accurate polymodal receptor biophysical characterization for drug design in novel pain therapies.

Introduction

The pain sensation is triggered when the terminals of a specific subset of peripheral neurons called nociceptors are activated by noxious stimuli, such as irritant substances or heat. Specifically, the cationic nonselective transient receptor potential vanilloid 1 (TRPV1) ion channel detects these stimuli and induces the opening of the channel pore and a subsequent increase in membrane permeability (Caterina et al., 1997). The TRPV1 channel is a polymodal receptor originally shown to be activated by capsaicin, heat, protons (Tominaga et al., 1998), lipids (Hernández-García and Rosenbaum, 2014; Morales-Lázaro and Rosenbaum, 2015), and peptide toxins from some venomous animals (Bohlen et al., 2010; Hakim et al., 2015; Yang et al., 2015b). The TRPV1 channel has a tetrameric structure, with each subunit possessing six transmembrane domains, several ankyrin repeat domains (ARDs) at the N terminus, and a large intracellular C terminus containing a conserved amino acidic sequence called the "TRP box". (Liao et al., 2013). As with many other members of the transient receptor potential (TRP) ion channel superfamily, TRPV1 has been the focus of intense research because of the intriguing nature of its polymodal activation mechanism (Nilius et al., 2005; Latorre et al., 2007; Tominaga, 2007); however, until recently, experimental data lacked a structural framework to interpret functional findings.

A major breakthrough in the field of TRP channels came from David Julius's laboratory when the structure of the TRPV1 channel in different conformations was resolved using single-particle cryoelectron microscopy (cryo-EM) (Cao et al., 2013b; Liao et al., 2013). This picture showed that the TRPV1 channel shares many structural features with other ion channels of known structures, such as the voltage-gated Kv1.2 channel and the Kv1.2-2.1 paddle chimera (Long et al., 2005a, 2007). The TRPV1 channel structure supported previous studies proposing that the TRPV1 channel is assembled as a tetramer (Kedei et al., 2001; Kuzhikandathil et al., 2001) and confirmed functional

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ABBREVIATIONS: AMG9810, (2*E*)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-propenamide; APHC, analgesic peptide *Heteractis crispa*; APO, apoenzime; ARD, ankyrin repeat domain; CPZ, capsazepine; cryo-EM, single-particle cryoelectron microscopy; DkTx, double-knot toxin; DRG, dorsal root ganglia; HOLO, holoenzime; ICK, inhibitor cystine knot; LPA, lysophosphatidic acid; MD, molecular dynamics; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; rTRPV1, rat transient receptor potential vanilloid 1; RTX, resiniferatoxin; SDZ 249665, N-4-(2-aminoethoxy)-3-methoxybenzyl)-N'-(4-t-butylbenzyl)urea; TRP, transient receptor potential; TRPV1, transient receptor potential vanilloid 1; VaTx, vanillotoxin.

the mechanism for phosphatidylinositol 4,5-biphosphate-dependent gating is conserved in TRPV1 and distantly related ion channels, but additional experimental evidence is required to confirm this.

As stated above, based on structural and functional studies, it has been proposed that the S4 to S5 linker plays a pivotal role in coupling the channel-sensing modules with the pore domain in voltage-dependent potassium channels (Lu et al., 2002; Long et al., 2005b; Chowdhury et al., 2014). For TRPV1, structural data show that the S4 to S5 linker appears to be close to the "TRP domain" located at the proximal C-terminal region. In agreement with functional studies, it has been suggested that these domains may constitute the machinery that couples the channel-sensing modules to the activation gate (Brauchi et al., 2007; Susankova et al., 2007; Boukalova et al., 2010; Cao et al., 2013b; Taberner et al., 2014; Yang et al., 2015a). The binding site of $PI(4,5)P_2$ is therefore nontrivial because it seems that this location may be related to the channel activation gate, although the modulation of channel gating has not been exhaustively dissected as a function of $PI(4,5)P_2$ chemistry. However, this may eventually lead to the discovery of novel compounds from the de novo design of TRPV1 channel modulators.

Lysophosphatidic acid (LPA) plays a role in many cellular processes, including cell migration, apoptosis, cell differentiation, and angiogenesis (Oude Elferink et al., 2015). It has been proposed that LPA is the trigger for neuropathic pain through a signaling cascade involving the LPA receptor and the Rho-Rho kinase pathway (Inoue et al., 2004). As shown in rats, LPA potentiates TRPV1 activity in DRG neurons during bone cancer via an indirect mechanism involving protein kinase Cε (Pan et al., 2010) and this potentiation of TRPV1 channel activity occurs after the blockage of the signaling pathways associated with LPA. Given that the effect of LPA was markedly reduced using extracellular LPA applications, an intracellular LPA binding site was hypothesized to mediate these actions (Nieto-Posadas et al., 2011). Deletion of the channel region comprising residues 777–821 rendered the channel LPA insensitive, suggesting that the LPA binding site is located at the C terminus of the channel. Further charge neutralization of R701 and K710 showed that these residues, which had previously been proposed to stabilize the phosphatidylinositol 4,5-biphosphate binding pocket (Brauchi et al., 2007), are key components in LPA-dependent potentiation of TRPV1 channel activity (Nieto-Posadas et al., 2011; Morales-Lázaro and Rosenbaum, 2015).

Cholesterol, an abundant component of biologic membranes, is involved in membrane mechanical stability, fluidity, and subdomain organization (García-Sáez and Schwille, 2010). Cholesterol directly modulates the function of several ion channels, and at least three cholesterol-binding motifs have been described to date (Levitan et al., 2014). Depletion of cholesterol from membranes of DRG neurons via methyl-β-cyclodextrin application decreases both capsaicin-induced responses and TRPV1 immunoreactivity. These effects were specific for TRPV1 and not the purinergic receptor $P2X_3$ suggesting that cholesterol is involved in the stability of lipid rafts where TRPV1 channels are located (Liu et al., 2006). Consistent with this hypothesis, pharmacological disruption of lipid rafts in DRG neurons by the enzyme sphingomyelinase decreases the calcium influx in response to capsaicin (Szoke et al., 2010). To avoid trafficking effects that had previously been reported by Liu et al. (2006), Picazo-Juárez et al. (2011) assessed the effects of cholesterol levels on TRPV1 channel function in excised membrane patches. Data from excised membrane patches suggested that cholesterol specifically binds to rTRPV1 channels; an increase in cholesterol levels decreased capsaicin-evoked currents, whereas the increase in cholesterol's stereoisomer epicholesterol did not. The sensitivity of rTRPV1 channels to cholesterol was affected by mutations at isoleucine 585 in S5, a region wherein the sequence contains a cholesterol-binding motif between residues 579 and 586 (Picazo-Juárez et al., 2011). These findings suggest that cholesterol levels are critical for TRPV1 channel function in several ways and a decrease in its levels may produce structural disruption of membrane elements that are key for ion channel function/assembly. On the other hand, increases in cholesterol levels inhibit channel activity possibly by trapping the channel in its closed state (Picazo-Juárez et al., 2011).

Concluding Remarks

The TRPV1 channel is a polymodal receptor whose activation is driven by ligands, heat, voltage, and lipids (Nilius and Voets, 2004; Baez-Nieto et al., 2011). This channel has become an attractive target for developing novel pain inhibitors because of its role in nociceptive and inflammatory responses. The structure of the TRPV1 channel has been resolved by cryo-EM (Cao et al., 2013b; Liao et al., 2013), and this work has provided most of the structural details for protein–ligand docking algorithms that rapidly evaluate the binding of thousands of compounds from virtual libraries (Sousa et al., 2013). However, the search of novel TRPV1 modulators can be a hard task because of the polymodal nature of this ion channel, which becomes a double-edged sword. While dissection of channel activation modes and their allosteric crosstalk results is fascinating from a biophysical point of view, this represents a major challenge for pharmacologists who look for an unique, well defined response after ligand binding. This may represent a major shortcoming during the transit from in vitro assays to clinical trials. Several molecules acting as potent TRPV1 channel inhibitors displayed analgesic activity during in vivo assays but were also found to produce hyperthermia or impaired heat sensing, rendering them unsafe for human use (Lee et al., 2015). Thus, future work will require function-oriented pharmacology that includes the suppression of activation of the module that is sensitive to decreases in external pH, a widespread phenomenon during inflammatory processes (White et al., 2011). To achieve this goal, a detailed biophysical understanding of ion channel function will be necessary. Future efforts should also include a search for the structural determinants of each sensing module and aim to understand how these domains are functionally connected.

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Authorship Contributions

Performed data analysis: Caceres-Molina, Sepulveda.

Wrote or contributed to the writing of the manuscript: Díaz-Franulic, Gonzalez-Nilo, Latorre.

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Near-microsecond human aquaporin 4 gating dynamics in static and alternating external electric fields: Non-equilibrium molecular dynamics

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An extensive suite of non-equilibrium molecular-dynamics simulation has been performed for ~0.85-0.9 μs of human aquaporin 4 in the absence and presence of externally applied static and alternating electric fields applied along the channels (in both axial directions in the static case, taken as the laboratory z-axis). These external fields were of 0.0065 V/Å (r.m.s.) intensity (of the same order as physiological electrical potentials); alternating fields ranged in frequency from 2.45 to 500 GHz. In-pore gating dynamics was studied, particularly of the relative propensities for "open" and "closed" states of the conserved arginines in the arginine/aromatic area (itself governed in no small part by external-field response of the dipolar alignment of the histidine-201 residue in the selectivity filter). In such a manner, the intimate connection of field-response governing "two-state" histidine states was established statistically and mechanistically. Given the appreciable size of the energy barriers for histidine-201 alignment, we have also performed non-equilibrium metadynamics/local-elevation of static fields applied along both directions to construct the free-energy landscape thereof in terms of external-field direction, elucidating the importance of field direction on energetics. We conclude from direct measurement of deterministic molecular dynamics in conjunction with applied-field metadynamics that the *intrinsic* electric field within the channel points along the $+z$-axis, such that externally applied static fields in this direction serve to "open" the channel in the selectivity-filter and the asparagine-proline-alanine region. *Published by AIP Publishing.* [http://dx.doi.org/10.1063/1.4961072]

INTRODUCTION

Aquaporins (AQPs) constitute an extensive family of trans-membrane proteins forming channels which conduct selectively water, as well as other small uncharged molecules (such as glycerol). This selective permeation is as a result of osmotic pressure between both sides of the membrane, serving also to exclude very strictly the passage of ions and protons.[1,2] AQPs are in all known lifeforms and are essential for regulating precisely water content in organs and cells. In humans, their defective function is implicated in various pathological conditions, such as nephrogenic diabetes, insipidus, and congenital cataracts.[3] Since their original discovery by Agre *et al.*,[4] several hundred AQPs have been elucidated and characterised.[3,5] A more complete understanding of osmotically driven water permeabilities and fluxes in AQPs is essential for progress in medical research, to establish more confidently their function and their potential involvement in medical conditions. Bearing this in mind, water fluxes in AQPs are estimated relatively routinely, via reconstitution of channels in liposomes and monitoring changes in volume due to concentrations of an impermeable solute; it may also be possible to estimate diffusive permeability from isotope labelling.[1,2,4,6–8] To obtain single-channel permeabilities, knowing AQP density is essential, i.e., the liposome's precise lipid-to-protein composition—in most cases, a significant challenge.

Even knowing channel densities, obtaining an atomistic-level description of water-transport mechanisms in AQPs is not experimentally feasible, due primarily to the short, nanosecond timescales involved.[9,10] Given these relatively fast kinetics, and together with recent availability of atomic-resolution AQP structures,[11–13] molecular dynamics (MD) has become a very valuable tool for gaining theoretical insights into underlying mechanisms.[14–23] In particular, a permeation mechanism has been proposed in which the pore acts as a "two-stage filter"; a "selectivity filter" ("SF") (aromatic/arginine region) at the narrowest part of the channel acts as one, whilst a well-conserved asparagine-proline-alanine (NPA) motif (dubbed "CE") serves as the other, wherein a well-defined water dipolar rotation occurs during passage through the channels.[24] MD studies have considered the characteristics of proton blockage by AQPs,[25–30] the transport of other solutes,[31–34] the gating of aquaporins,[35–37] and aquaporin-mediated cell adhesion.[38]

In particular, Human Aquaporin 4 (h-AQP4) is abundantly expressed in blood-brain and brain-cerebrospinal fluid interfaces and is responsible for homeostasis of cerebral water; its function is related to neuropathological disorders

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microsecond NEMD, along with in-field metadynamics, particularly of the relative propensities for "open" and "closed" states of the conserved arginines in the arginine/aromatic constrictive area. In such a manner, the intimate connection of field-response governing "two-state" histidine states was established statistically and mechanistically. Direct measurement of deterministic MD in conjunction with applied-field metadynamics found that the *intrinsic* electric field within the pore points along the $+z$-axis, such that externally applied static fields in this direction served to "open" the channel in both the selectivity-filter and CE regions. This was found to be especially important for dipolar alignment of HIS-201 in the SF region, and also for HIS-95 in the CE region. In particular, the removal of the free-energy barrier for HIS-201-dihedral sampling of aggressively open configurations was determined to have a decisive, mechanistic impact in governing this process. In a sense, this study builds upon aspects of the very insightful and interesting earlier works of Hub *et al.*[24] and Alberga *et al.*,[37] by confirming electric-potential effects on the two-state/two-region paradigm and confirming the importance of the CE region and HIS-95 in regulating gating dynamics, whilst also tackling open questions left outstanding from our previous studies of Refs. 49 and 50, in the area of axial-field directionality and the interplay of intrinsic and external potentials of the same magnitude, with the $-z$-applied static field effectively "cancelling out" the zero-field intrinsic, electric field. Interestingly, the normal transmembrane potential ranges from −80 to −40 mV; thus, a field oriented towards the cell interior is generated. In this way, the tendency for the channel to be more closed under the effects static fields applied in the $-z$ direction (akin to normal physiological conditions) seems to be the "default setting" for h-AQP4. Also, although not considered in detail in this study, owing to the emphasis on the two-state/two-region model,[24] Alberga *et al.*[37] have highlighted the importance of CYS-178 and pore-mouth regions in general for water passage; we have considered electric-field effects on CYS-178 (and others) recently.[77]

In future, it would be interesting to probe via metadynamics, using dipolar orientation of key residues as a collective variable, e.g., HIS-95 and 201 (or, indeed, CYS-178)—the dihedral angle used here and in Ref. 50 is not a perfect proxy for the dipole in the case of HIS-201 (and other residues, more generally). Recent work is encouraging in this regard[78,79] in rendering this more feasible in the near- to medium-term. Another key intriguing, titillating, possibility relates to using mutation of key residues in aquaporins to regulate and control gating dynamics and water permeability.[80] MD may offer scope for rigorous *predictive* determination of such key mutation strategies for subsequent *in vitro* studies; external electric fields may offer a convenient operational-control strategy for such efforts.

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[1]S. Hohmann, S. Nielsen, and P. Agre, *Aquaporins* (Academic Press, San Diego, 2001).
[2]M. Borgnia, S. Nielsen, A. Engel, and P. Agre, Annu. Rev. Biochem. **68**, 425 (1999).
[3]L. S. King, D. Kozono, and P. Agre, Nat. Rev. Mol. Cell Biol. **5**, 687 (2004).
[4]G. M. Preston, P. Piazza-Carroll, W. B. Guggino, and P. Agre, Science **256**, 385 (1992).
[5]A. Engel and H. Stahlberg, Int. Rev. Cytol. **215**, 75 (2002).
[6]A. Finkelstein, *Water Movement Through Lipid Bilayers, Pores, and Plasma Membranes* (John Wiley & Sons, New York, 1987).
[7]G. Calamita, "The *Escherichia coli* aquaporin-z water channel," Mol. Microbiol. **37**, 254 (2000).
[8]M. J. Borgnia, D. Kozono, G. Calamita, P. C. Maloney, and P. Agre, J. Mol. Biol. **291**, 1169 (1999).
[9]Y. Fujiyoshi, K. Mitsuoka, B. L. de Groot, A. Philippsen, H. Grubmüller, P. Agre, and A. Engel, Curr. Opin. Struct. Biol. **12**, 509 (2002).
[10]R. M. Stroud, D. Savage, L. J. W. Miercke, J. K. Lee, S. Khademi, and W. Harries, FEBS Lett. **555**, 79 (2003).
[11]B. L. de Groot, A. Engel, and H. Grubmüller, FEBS Lett. **504**, 206 (2001).
[12]H. Sui, B.-G. Han, J. K. Lee, P. Walian, and B. K. Jap, Nature **414**, 872 (2001).
[13]D. Fu, A. Libson, L. J. Miercke, C. Weitzman, P. Nollert, J. Krucinski, and R. M. Stroud, Science **290**, 481 (2000).
[14]B. L. de Groot and H. Grubmüller, Science **294**, 2353 (2001).
[15]E. Tajkhorshid, P. Nollert, M. Ø. Jensen, L. J. W. Miercke, J. O'Connel, R. M. Stroud, and K. Schulten, Science **296**, 525 (2002).
[16]M. Ø. Jensen, E. Tajkhorshid, and K. Schulten, Biophys. J. **85**, 2884 (2003).
[17]F. Zhu, E. Tajkhorshid, and K. Schulten, FEBS Lett. **504**, 212 (2001).
[18]F. Zhu, E. Tajkhorshid, and K. Schulten, Biophys. J. **83**, 154 (2002).
[19]F. Zhu, E. Tajkhorshid, and K. Schulten, Biophys. J. **86**, 50 (2004).
[20]M. Hashido, M. Ikeguchi, and A. Kidera, FEBS Lett. **579**, 5549 (2005).
[21]M. Hashido, A. Kidera, and M. Ikeguchi, Biophys. J. **93**, 373 (2007).
[22]M. Ø. Jensen and O. G. Mouritsen, Biophys. J. **90**, 2270 (2006).
[23]B. G. Han, A. B. Guliaev, P. J. Walian, and B. K. Jap, J. Mol. Biol. **360**, 285 (2006).
[24]J. S. Hub, C. Aponte-Santamaría, H. Grubmüller, and B. L. de Groot, Biophys. J. **99**, L97 (2010).
[25]B. L. de Groot, T. Frigato, V. Helms, and H. Grubmuller, J. Mol. Biol. **333**, 279 (2003).
[26]N. Chakrabarti, E. Tajkhorshid, B. Roux, and R. Pomes, Structure **12**, 65 (2004).
[27]N. Chakrabarti, B. Roux, and R. Pomes, J. Mol. Biol. **343**, 493 (2004).
[28]B. Ilan, E. Tajkhorshid, K. Schulten, and G. A. Voth, Proteins **55**, 223 (2004).
[29]H. Chen, Y. Wu, and G. A. Voth, Biophys. J. **90**, L73 (2006).
[30]B. L. de Groot and H. Grubmüller, Curr. Opin. Struct. Biol. **15**, 176 (2005).
[31]M. Ø. Jensen, E. Tajkhorshid, and K. Schulten, Structure **9**, 1083 (2001).
[32]M. Ø. Jensen, S. Park, E. Tajkhorshid, and K. Schulten, Proc. Natl. Acad. Sci. U. S. A. **99**, 6731 (2002).
[33]J. S. Hub and B. L. de Groot, Biophys. J. **91**, 842 (2006).
[34]J. Henin, E. Tajkhorshid, K. Schulten, and C. Chipot, Biophys. J. **94**, 832 (2008).
[35]H. Khandelia, M. Ø. Jensen, and O. G. Mouritsen, J. Phys. Chem. B **113**, 5239 (2009).
[36]N. Smolin, B. Li, D. A. C. Beck, and V. Daggett, Biophys. J. **95**, 1089 (2008).
[37]D. Alberga, O. Nicolotti, G. Lattanzi, G. P. Nicchia, A. Frigeri, F. Pisani, V. Benfenati, and G. F. Mangiatordi, Biochim. Biophys. Acta **1838**, 3052–3060 (2014).
[38]M. Ø. Jensen, R. O. Dror, H. Xu, D. W. Borhani, I. T. Arkin, M. P. Eastwood, and D. E. Shaw, Proc. Natl. Acad. Sci. U. S. A. **105**, 14430 (2008).
[39]G. T. Manley, M. Fujimura, T. Ma, N. Noshita, F. Filiz, A. W. Bollen, P. Chan, and A. S. Verman, Nat. Med. **6**, 159 (2000).
[40]J. D. Ho, R. Yeh, A. Sandstrom, I. Chorny, W. E. C. Harries, R. A. Robbins, L. J. W. Miercke, and R. M. Stroud, Proc. Natl. Acad. Sci. U. S. A. **106**, 7437 (2009).

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Exploring the Membrane Potential of Simple Dual-Membrane Systems as Models for Gap-Junction Channels

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ABSTRACT The conductance of ion channels can be modulated by a transmembrane potential difference, due to alterations on ion-mobility and also by changes in the pore structure. Despite the vast knowledge regarding the influence of voltage on transport properties of ion channels, little attention has been paid to describe, with atomic detail, the modulation of ionic transport in gap-junction channels (GJCs). Hence, molecular dynamics simulations were performed to explore the conductance of simple dual-membrane systems that account for the very basic features of GJCs. In doing so, we studied the influence of different charge distributions in the channel surface on these idealized systems under external electric fields, paying attention to the behavior of the electrostatic potential, ion density, ion currents, and equilibrium properties. Our results demonstrate that the incorporation of a charge distribution akin GJCs decreased anionic currents, favoring the transport of cationic species. Moreover, a thermodynamic characterization of ionic transport in these systems demonstrate the existence of a kinetic barrier that hinders anionic currents, reinforcing the role played by the internal arrangement of charges in GJCs. Overall, our results provide insights at the atomic scale on the effects of charge distributions over ionic transport, constituting a step forward into a better understanding of GJCs.

INTRODUCTION

As a result of the specific permeabilities of ionic species across the cell membrane, charge density is generally different on both the cytosolic and the extracellular reservoir. This heterogeneity of charge distribution is produced by the action of ionic pumps and ion channels (1), generating an electrochemical gradient, i.e., a voltage difference, which is crucial to regulate a variety of intercellular communication processes such as the regulation of cell homeostasis and the conduction of the neuron action potential (2). In the case of single-membrane systems, voltage-dependent modulation of ionic transport via structural rearrangements of ion channels is well understood, being extensively described by several groups with atomistic resolution (3–6). It depends on the presence of charged residues that will react under the influence of an external electrostatic potential, generating conformational changes to allow or disrupt the passage of ionic species through the channels. These channels communicate both the intracellular and the extracellular compartments, and they can also connect two adjacent cells by forming a so-called gap-junction channel (GJC) (7,8). GJCs connect the cytoplasm of adjacent cells to provide a dual-membrane hydrophilic channel between cells that enables the passive diffusion of water, ions, and small molecules. In detail, GJCs are composed by two opposed hemichannels (HCs) comprising six connexin (Cx) monomers (9–11). Experimental data has shown that the transport properties of GJCs are modulated by potential differences (12,13), demonstrating the existence of a voltage-regulated gating mechanism (14,15). Thus, GJCs are sensitive to voltages applied in different orientations: 1) the trans-membrane voltage, V_m, i.e., the voltage difference between the cytoplasm and the extracellular space; and 2) the transjunctional voltage, V_j, i.e., the voltage difference between the cytoplasm of the two adjacent cells. Considering their distinctive conductances, at least two distinct voltage-gating mechanisms have been identified in connexin channels: the loop or slow gating linked to the cytoplasmatic loop and C-terminal loop, and the fast gating, linked to residues

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key element for ion selectivity, as suggested before (54,57,58).

The PMF calculations (Fig. 4) of sodium passage confirmed the previous results. For the CEC model, the PMF profile is relatively flat, with a global minimum at the channel center, favoring cation selectivity. On the other hand, the height of the PMF at the central region of the CEC_{inv} model, translates into a kinetic barrier, in full accordance with the cationic current reduction reinforcing the role played by negative internal charges on GJCs. The chloride PMFs further proved the influence of the intrinsic GJC charge distribution, with symmetrically reversed results with respect to the sodium profiles.

Overall, these somewhat simplified dual-membrane models offer a clear molecular picture with atomistic detail of more complex phenomena occurring in GJCs. Despite their simplicity, which does not encompass any structurally mediated voltage-gating mechanism, they offer a fertile landscape for the understanding of the role of charge distribution within GJCs and the resulting voltages when an external E-field is applied.

SUPPORTING MATERIAL

Supporting Materials and Methods, eleven figures, and one table are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(16)30281-8.

AUTHOR CONTRIBUTIONS

Y.E. and J.A.G. equally contributed to the article; Y.E., J.A.G., R.A.-S., R.Z., and T.P.-A. designed research; Y.E., R.A.-S., and T.H. performed molecular simulations; all authors discussed and analyzed the data; Y.E., J.A.G., and T.P.-A. wrote the main article; and R.A.-S., T.H., and R.Z. wrote and revised the article.

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REFERENCES

1. Hille, B. 2001. Ion Channels of Excitable Membranes. 3rd Ed. Sinauer Associates, Sunderland, MA.
2. Bezanilla, F. 2008. How membrane proteins sense voltage. *Nat. Rev. Mol. Cell Biol.* 9:323–332.
3. Groome, J. R. 2014. The voltage sensor module in sodium channels. *Handbook Exp. Pharmacol.* 221:7–31.
4. Khalili-Araghi, F., B. Ziervogel, …, B. Roux. 2013. Molecular dynamics simulations of membrane proteins under asymmetric ionic concentrations. *J. Gen. Physiol.* 142:465–475.
5. Villinger, S., R. Briones, …, M. Zweckstetter. 2010. Functional dynamics in the voltage-dependent anion channel. *Proc. Natl. Acad. Sci. USA.* 107:22546–22551.
6. Sotomayor, M., V. Vásquez, …, K. Schulten. 2007. Ion conduction through MscS as determined by electrophysiology and simulation. *Biophys. J.* 92:886–902.
7. Harris, A. L., and D. Locke. 2009. Connexins: a Guide. Humana Press, Totowa, NJ.
8. Meşe, G., V. Valiunas, …, T. W. White. 2008. Connexin26 deafness associated mutations show altered permeability to large cationic molecules. *Am. J. Physiol. Cell Physiol.* 295:C966–C974.
9. Yeager, M., and N. B. Gilula. 1992. Membrane topology and quaternary structure of cardiac gap junction ion channels. *J. Mol. Biol.* 223:929–948.
10. Yeager, M. 1998. Structure of cardiac gap junction intercellular channels. *J. Struct. Biol.* 121:231–245.
11. Saez, J. C., V. M. Berthoud, …, E. C. Beyer. 2003. Plasma membrane channels formed by connexins: their regulation and functions. *Physiol. Rev.* 83:1359–1400.
12. Ek-Vitorin, J. F., and J. M. Burt. 2013. Structural basis for the selective permeability of channels made of communicating junction proteins. *Biochim. Biophys. Acta.* 1828:51–68.
13. Oh, S., and T. A. Bargiello. 2015. Voltage regulation of connexin channel conductance. *Yonsei Med. J.* 56:1–15.
14. Harris, A. L., D. C. Spray, and M. V. Bennett. 1981. Kinetic properties of a voltage-dependent junctional conductance. *J. Gen. Physiol.* 77:95–117.
15. Spray, D. C., A. L. Harris, and M. V. Bennett. 1981. Equilibrium properties of a voltage-dependent junctional conductance. *J. Gen. Physiol.* 77:77–93.
16. Maeda, S., S. Nakagawa, …, T. Tsukihara. 2009. Structure of the connexin 26 gap junction channel at 3.5 Å resolution. *Nature.* 458:597–602.
17. Bukauskas, F. F., and V. K. Verselis. 2004. Gap junction channel gating. *Biochim. Biophys. Acta.* 1662:42–60.
18. Araya-Secchi, R., T. Perez-Acle, …, R. Zhou. 2014. Characterization of a novel water pocket inside the human Cx26 hemichannel structure. *Biophys. J.* 107:599–612.
19. Kwon, T., A. L. Harris, …, T. A. Bargiello. 2011. Molecular dynamics simulations of the Cx26 hemichannel: evaluation of structural models with Brownian dynamics. *J. Gen. Physiol.* 138:475–493.
20. Hub, J. S., C. Aponte-Santamaría, …, B. L. de Groot. 2010. Voltage-regulated water flux through aquaporin channels in silico. *Biophys. J.* 99:L97–L99.
21. Kutzner, C., H. Grubmüller, …, U. Zachariae. 2011. Computational electrophysiology: the molecular dynamics of ion channel permeation and selectivity in atomistic detail. *Biophys. J.* 101:809–817.
22. Li, J., X. Gong, …, R. Zhou. 2007. Electrostatic gating of a nanometer water channel. *Proc. Natl. Acad. Sci. USA.* 104:3687–3692.
23. Garate, J. A., N. English, and J. MacElroy. 2009. Carbon nanotube assisted water self-diffusion across lipid membranes in the absence and presence of electric fields. *Mol. Simul.* 35:3–12.
24. Garate, J. A., N. J. English, and J. M. D. MacElroy. 2009. Static and alternating electric field and distance-dependent effects on carbon nanotube-assisted water self-diffusion across lipid membranes. *J. Chem. Phys.* 131:114508.
25. Gumbart, J., F. Khalili-Araghi, …, B. Roux. 2012. Constant electric field simulations of the membrane potential illustrated with simple systems. *Biochim. Biophys. Acta.* 1818:294–302.

ANALES

COMO ELEGIR UN HORARIO PARA CHILE

*John Ewer Lothian**

Resumen: Nuestro reloj biológico determina cuando dormimos y cuando despertamos y es sincronizado a la hora local utilizando los ciclos planetarios de luz y oscuridad. Desfases entre nuestro horario interno y la hora del amanecer resultan en déficits crónicos de sueño ya que causan que el despertador interrumpa prematuramente nuestro sueño en un día de trabajo, afectando negativamente nuestro desempeño y nuestra salud. Puesto que los jóvenes se despiertan naturalmente más tarde son el segmento más vulnerable de la población. Los países deberían elegir un horario para el cual el amanecer ocurre cerca de la hora en que debe despertar la mayoría de la población en un día de trabajo, y mantenerlo durante todo el año. Para latitudes alejadas del ecuador ello significará que oscurecerá temprano durante el invierno; sin embargo retrasar el horario del amanecer para así tener más horas de luz en la tarde aumentará el déficit de sueño, con consecuencias todas negativas para la salud y el desempeño. En los últimos años Chile ha elegido horarios que se alejan del horario recomendable basado en una extensa literatura científica y clínica sobre el tema. Es hora que Chile adopte una estrategia racional para elegir un horario para el país.

Palabras claves: Reloj circadiano; reloj biológico; cronotipo; jetlag social; sueño.

Abstract: Our biological clock determines when we sleep and when we wake up, and is synchronized to local time using the planetary light-dark cycles. A lack of alignment between our internal clock and dawn causes chronic sleep deficits because the alarm clock prematurely cuts short our sleep during work days, which negatively impacts our health and performance. Since adolescents naturally wake up later, they are the most vulnerable segment of the population. Countries should choose a time zone for which dawn occurs close to the time when most people wake up during a work day; and it should be maintained without change during the entire year. For latitudes far from the Equator this will cause the sun to set early in the afternoon; however, choosing a time zone that delays dawn in order to have more hours of light in the afternoon will increase the sleep deficit, with consequences on health and performance that are all negative. In the last few years Chile has changed the country's time in ways that are not consistent with a large body of scientific and clinical literature. It is time for Chile to adopt a rational strategy to choose the country's time.

Keywords: Circadian clock; biological clock; chronotype; social jetlag; sleep.

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MINIREVIEW—A LATIN AMERICAN PERSPECTIVE ON ION CHANNELS

Pharmacological Modulation of Proton Channel Hv1 in Cancer Therapy: Future Perspectives.

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ABSTRACT

The pharmacological modulation of the immunosuppressive tumor microenvironment has emerged as a relevant component for cancer therapy. Several approaches aiming to deplete innate and adaptive suppressive populations, to circumvent the impairment in antigen presentation, and to ultimately increase the frequency of activated tumor-specific T cells are currently being explored. In this review, we address the potentiality of targeting the voltage-gated proton channel, Hv1, as a novel strategy to modulate the tumor microenvironment. The function of Hv1 in immune cells such as macrophages, neutrophils, dendritic cells, and T cells has been associated with the maintenance of NADPH oxidase activity and the generation of reactive oxygen species, which are required for the host defense against pathogens. We discuss evidence suggesting that the Hv1 proton channel could also be important for the function of these cells within the tumor microenvironment. Furthermore, as summarized here, tumor cells express Hv1 as a primary mechanism to extrude the increased amount of protons generated metabolically, thus maintaining physiologic values for the intracellular pH. Therefore, because this channel might be relevant for both tumor cells and immune cells supporting tumor growth, the pharmacological inhibition of Hv1 could be an innovative approach for cancer therapy. With that focus, we analyzed the available compounds that inhibit Hv1, highlighted the need to develop better drugs suitable for patients, and commented on the future perspectives of targeting Hv1 in the context of cancer therapy.

Introduction

Voltage-gated proton channel (Hv1) is a membrane protein with the capability to permeate protons through membranes with absolute specificity (DeCoursey, 2008). Hv1 channel is activated upon membrane depolarization in a time-, pH (Cherny et al., 1995; Musset and Decoursey, 2012)-, and temperature (DeCoursey and Cherny, 1998; Kuno et al., 2009)-dependent manner. The channel is composed of three functional domains: a voltage-sensing domain and the cytoplasmic N-terminal and C-terminal domains (Ramsey et al., 2006; Sasaki et al., 2006). The voltage-sensing domain of Hv1 channel comprises four transmembrane segments (S1–S4) and is equivalent to the one present in voltage-dependent K^+ channels (Ramsey et al., 2006; Sasaki et al., 2006) (Fig. 1, A and B). Unlike other voltage-gated channels, Hv1 lacks a pore domain (Ramsey et al., 2006; Sasaki et al., 2006) and proton permeation occurs through the voltage-sensing domain (Koch et al., 2008; Tombola et al., 2008; Lee et al., 2009). Hv1 channels assemble as homodimers through the interactions of the coiled-coil domains in the C-terminal region (Koch et al., 2008; Lee et al., 2008; Tombola et al., 2008; Fujiwara et al., 2012). Monomeric channels, obtained by deletion of the C-terminal domain, are also functional (Koch et al., 2008; Tombola et al., 2008). The N-terminal domain, which contains a phosphorylation site (T29 in human Hv1) that triggers an enhanced gating behavior (Morgan et al., 2007; Musset et al., 2010; Hondares et al., 2014), plays an important role in Hv1 regulation.

Interestingly, Hv1 is expressed in tumor cells, where this channel is involved in the maintenance of intracellular pH and regulates metastasis-related properties such as migration and

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ABBREVIATIONS: ClGBI, Cl-guanidinobenzimidazole; CTL, cytotoxic T lymphocytes; CTLA-4, cytotoxic T lymphocyte-associated protein 4; Erk, extracellular receptor-activated kinase; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; iNOS, nitric oxide synthase; LPS, lipopolysaccharide; MMP, metalloproteinase; NF-κB, nuclear factor kappa B; NOX, NADPH oxidase; PD-1, programmed cell death protein 1; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; TAMs, tumor-associated macrophages; TANs, tumor-associated neutrophils; TCR, T cell receptor; TGF-β, transforming growth factor β; TLR, toll-like receptor; TME, tumor microenvironment; TNF, tumor necrosis factor; Tregs, regulatory T cells; 2GBI, 2-guanidinobenzimidazole.

normal levels of phagosomal processing of tumor antigens (Fig. 3B), whereas the targeting of NOX2 would more likely cause an impairment in the cross-priming of antitumor CTLs. It cannot be completely disregarded, however, that Hv1 inhibition might affect cross-priming by inducing excessive antigen degradation. If this were the case, the negative effect of a therapy based on blocking Hv1 function could be solved by a combination with peptide vaccines, adoptive transference of dendritic cells already loaded with tumor antigens, or another feasible strategy that circumvents antigen processing.

TAMs and TANs are potent suppressors of T cell function within the TME. In these populations Hv1 channel might aid to sustain the production of high levels of ROS, which impairs T cell function through several mechanisms (Schmielau and Finn, 2001; Nagaraj et al., 2007; Molon et al., 2011) (Fig. 3A). Thus, the inhibition of Hv1 could lead to a diminished ability of TAMs and TANs to suppress antitumoral T cells and might impair the polarization of TAMs toward M2 phenotype, because ROS is required for macrophage differentiation into M2 functional state (Zhang et al., 2013) (Fig. 3B). Additionally, the migration of neutrophils toward the sites of inflammation is regulated by Hv1 channel (El Chemaly et al., 2010; Zhu et al., 2013), suggesting that in tumor-bearing hosts, the inhibition of Hv1 could lead to a reduced intratumoral infiltration of TANs (Fig. 3B). A diminished infiltration of TANs could be accompanied by a decreased recruitment of tumor-supporting Tregs and TAMs (Curiel et al., 2004; Fridlender and Albelda, 2012) (Fig. 3B).

A relevant issue that must be addressed in any therapy is potential toxicity. In addition to immune system-related cells and tissues, there is evidence of Hv1 expression (RNA and/or protein level) at different tissues, such as the brain (cerebral cortex, hippocampus, and lateral ventricle), endocrine tissues (thyroid and adrenal glands), muscles (heart, skeletal, and smooth), liver and gallbladder, gastrointestinal track, kidney and urinary bladder, testis and prostate, female tissues (endometrium, fallopian tube, ovary, and placenta), skin and adipose and soft tissue (tissue expression of HVCN1, The Human Protein Atlas. http://www.proteinatlas.org/ENSG00000122986-HVCN1/tissue; Uhlen et al., 2015). The functional role of Hv1 is still uncharacterized in most of these tissues, with some exceptions: in airway epithelium, Hv1 regulates the extracellular pH in airway surface liquid (Fischer et al., 2002; Cho et al., 2009; Fischer, 2012) and Hv1 plays multiple roles in human sperm (Babcock et al., 1983; Babcock and Pfeiffer, 1987; Lishko et al., 2010; Musset et al., 2012b). Then, Hv1 inhibition can potentially affect airway epithelium pH regulation and sperm capacitation (temporally hampering male fertility). The effects of Hv1 inhibition in other human tissues remain to be studied. Therefore, it is very important to determine the balance between the antitumoral effect and the associated toxicity when studying any drug targeting Hv1. Of note, Hv1-deficient mice develop some degree of autoimmunity associated with aging, but a life-threatening toxicity has not been described (Sasaki et al., 2013). Furthermore, these mice are able to clear bacterial infections in vivo (Ramsey et al., 2009). Similarly, Hv1-deficient rats lack a fatal/severe phenotype (Jin et al., 2014). The phenotypes of Hv1-deficient animal models cannot be directly extrapolated to the human scenario because of some differences regarding Hv1 expression among species (Lishko et al., 2010), and no human deficiency of Hv1 is known (DeCoursey, 2015). Nonetheless, these results suggest that the pharmacological inhibition of Hv1 channel could be feasible because of tolerable toxicity and potential relevant effect in both tumor cell biology and tumor-infiltrating immune cells. Moreover, a therapy directed to the Hv1 proton channel in cancer can be beneficiated with strategies that specifically deliver the drug in the TME, such as intratumoral inoculation or coupling with monoclonal antibodies specific for tumor antigens.

Because cancer needs a multifactorial strategy, it is desirable that Hv1-based therapy could be used together with the standard of care for this disease and with the novel developing therapies. From our point of view, the inhibition of Hv1 could be a strategy to target the TME that could be combined with cancer vaccines, monoclonal antibodies, immune checkpoint therapy, adoptive T cell transference, and low molecular weight inhibitors, etc. Although more direct experimental evidence needs to be obtained regarding the role of Hv1 in the TME, there is no doubt that it is a novel approach for cancer therapy that is worth exploring more seriously. With that in mind, a larger effort should be made in the design and development of efficient drugs targeting Hv1 channel that could be used in cancer patients.

Hv1 inhibition can also be beneficial for the treatment of other pathologies: Alzheimer's disease (Eder and DeCoursey, 2001), ischemic liver disease, atherosclerosis, Parkinson's disease (DeCoursey and Ligeti, 2005), ischemic stroke (Wu et al., 2012), and Crohn's disease (Haglund et al., 2013), but a complete analysis of this is beyond the scope of the current review.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript: Fernández, Pupo, Mena-Ulecia, and Gonzalez.

References

Abbas AK, Lichtman AH, and Pillai S (2014) *Cellular and Molecular Immunology*, 8th ed, Elsevier Saunders, Canada.

Aggarwal BB, Vijayalekshmi RV, and Sung B (2009) Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe. *Clin Cancer Res* **15**:425–430.

Alabi AA, Bahamonde MI, Jung HJ, Kim JI, and Swartz KJ (2007) Portability of paddle motif function and pharmacology in voltage sensors. *Nature* **450**: 370–375.

Anrather J, Racchumi G, and Iadecola C (2006) NF-kappaB regulates phagocytic NADPH oxidase by inducing the expression of gp91phox. *J Biol Chem* **281**: 5657–5667.

Antony S, Wu Y, Hewitt SM, Anver MR, Butcher D, Jiang G, Meitzler JL, Liu H, Juhasz A, Lu J, et al. (2013) Characterization of NADPH oxidase 5 expression in human tumors and tumor cell lines with a novel mouse monoclonal antibody. *Free Radic Biol Med* **65**:497–508.

Babcock DF and Pfeiffer DR (1987) Independent elevation of cytosolic [Ca2+] and pH of mammalian sperm by voltage-dependent and pH-sensitive mechanisms. *J Biol Chem* **262**:15041–15047.

Babcock DF, Rufo GA, Jr, and Lardy HA (1983) Potassium-dependent increases in cytosolic pH stimulate metabolism and motility of mammalian sperm. *Proc Natl Acad Sci USA* **80**:1327–1331.

Banchereau J and Steinman RM (1998) Dendritic cells and the control of immunity. *Nature* **392**:245–252.

Barese CN, Goebel WS, and Dinauer MC (2004) Gene therapy for chronic granulomatous disease. *Expert Opin Biol Ther* **4**:1423–1434.

Bauer CA, Kim EY, Marangoni F, Carrizosa E, Claudio NM, and Mempel TR (2014) Dynamic Treg interactions with intratumoral APCs promote local CTL dysfunction. *J Clin Invest* **124**:2425–2440.

Becker S, Wasser S, Hauses M, Hosele JP, Ott MG, Dinauer MC, Ganser A, Hoelzer D, Seger R, and Grez M (1998) Correction of respiratory burst activity in X-linked chronic granulomatous cells to therapeutically relevant levels after gene transfer into bone marrow CD34+ cells. *Hum Gene Ther* **9**:1561–1570.

Bellone G, Carbone A, Smirne C, Scirelli T, Buffolino A, Novarino A, Stacchini A, Bertetto O, Palestro G, Sorio C, et al. (2006) Cooperative induction of a tolerogenic dendritic cell phenotype by cytokines secreted by pancreatic carcinoma cells. *J Immunol* **177**:3448–3460.

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Regulation of cuticular hydrocarbon profile maturation by *Drosophila* tanning hormone, bursicon, and its interaction with desaturase activity

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ABSTRACT

Shortly after emergence the exoskeleton (cuticle) of adult insects is rapidly expanded, hardened (sclerotized), and pigmented (melanized). In parallel with this process, the oenocytes, which are large polyploid cells located below the abdominal epidermis, secrete onto the cuticle a cocktail of cuticular hydrocarbons (CHs) and waxes. These improve the waterproofing of the cuticle, and also provide important chemosensory and pheromonal cues linked with gender, age, and species differentiation. The hardening and pigmentation of the new cuticle are controlled by the neurohormone, bursicon, and its receptor, encoded by the DLGR2 receptor, *rickets* (*rk*); by contrast, little is known about the timecourse of changes in CH profile and about the role of bursicon in this process. Here we show in *Drosophila* that *rk* function is also required for the normal maturation of the fly's CH profile, with flies mutant for *rk* function showing dramatically elevated levels of CHs. Interestingly, this effect is mostly abrogated by mutations in the Δ9 desaturase encoded by the *desaturase1* gene, which introduces a first double bond into elongated fatty-acid chains, suggesting that *desaturase1* acts downstream of *rk*. In addition, flies mutant for *rk* showed changes in the absolute and relative levels of specific 7-monoenes (in males) and 7,11-dienes (in females). The fact that these differences in CH amounts were obtained using extractions of very different durations suggests that the particular CH profile of flies mutant for *rk* is not simply due to their unsclerotized cuticle but that bursicon may be involved in the process of CH biosynthesis itself.

1. Introduction

In insects, the cuticle (exoskeleton) undergoes dramatic changes during the first hours of adult life (Moussian, 2010; Moussian et al., 2015; Nicolaï et al., 2000). Immediately after emergence the new cuticle is expanded and is rapidly sclerotized through cross-linking of cuticular proteins and chitin, and is also pigmented through the deposition of melanin. In addition, a number of cuticular hydrocarbons (CHs) and waxes are secreted by subepidermal cells (oenocytes) and deposited on the cuticle (Qiu et al., 2012). These CHs aid in the waterproofing of the cuticle, and also provide important chemosensory and pheromonal cues linked with gender, age, and species differentiation.

Just as the cuticle changes rapidly following eclosion, so does the profile of CHs (Ferveur, 2005; Howard and Blomquist, 2005). For example, in species of the *D. melanogaster* subgroup, there is a striking change in CH profile around the first 24 h of adult life (immature *vs.* mature flies). Thus, whereas, immature flies of the two sibling species, *D. melanogaster* and *D. simulans*, produce long chain CHs (≥29carbons) with multiple double bonds and methyl branches (Pechine et al., 1988), mature adults produce CHs with shorter carbon chain length (23–29 carbons: Antony and Jallon, 1981, 1982). In addition, mature adult *D. melanogaster* —but not *D. simulans* —show a sexual dimorphism in CHs (Jallon, 1984), whereas, no such difference is detected in immature flies of either species (Pechine et al., 1988). As a reflection of this change in CH profile and of its significance, immature flies are intensely courted by mature males; the sexual activity towards immature flies is mostly induced by their high levels of polyunsaturated long chain CHs combined with their low levels of inhibitory CHs (such as

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found that *RK* mature mutant males and females carried very high amounts of CHs (∑CH). Interestingly, these increases were mostly associated with increased quantities of desaturated CHs. Nevertheless, the relatively constant proportion of desaturated CHs (compared to that of WT flies) indicates that both ∑CH and ∑Desat were increased to a similar degree. A closer look at individual CH components revealed that the absolute and relative levels of 7P (and to a lesser extent of 7,11ND) were increased in *RK* flies whereas the two other CHs (7T and 7,11HD) showed no variation (in fact they even showed a slight decrease) with respect to the levels for WT flies. In addition, comparisons between *DS* and *RK*;*DS* profiles, show that the effect of the *desat1* mutation largely neutralized that of the *rk* mutation. Indeed, mature flies mutant for both *desat1* and *rk* (*RK*;*DS*) did not produce high ∑CHs (as seen in *RK* flies) but showed a decreased ∑Desat and increased ∑Lin, (as occurs in *DS* mature flies).

How could we explain the dramatic increase of ∑CH seen in mature *RK* flies? One possibility is that the lack of normal hardening of the cuticle of *RK* flies provides a more absorbent cuticular matrix, resulting in an increased retention or deposition of CHs compared to control flies. Although this could explain the greatly increased amounts of ∑CH present in the cuticle of *RK* flies, it does not explain the divergent effect on 7T and 7,11HD, on one hand *vs.* 7P and 7,11ND, on the other (Figs. 3 and 4). Based on the classical biosynthesis pathway for CHs (Jallon, 1984), the increased production of 7T precursor (cis-17-tetracosenoic acid = C24:1) in *RK* males may have been too great to be to be fully converted into 7T (after the loss of the carboxyl carbon), such that the resulting excess in C24:1 was further processed to cause increased levels of 7P (after another elongation + decarboxylation step). A similar mechanism could explain the increased quantity of 7,11 ND (relative to 7,11 HD). This effect is suggested by our variable-duration extraction experiment. Since a very quick extraction was able to wash as much CHs as a very long one (Fig. 5), this suggests that the difference between RK mutant and control genotypes is not related to a change in the balance between the internal *vs* external CHs but rather to a variation in CH production, with consequences on the quality of the CH bouquet. This supports our hypothesis of the changed 7T:7P balance in males (and 7,11HD:7,11ND, in females). By comparison, the amount of cVA obtained increased with the duration of extraction, which can be explained by the fact that a large fraction of cVA remains in an internal organ of the fly (the ejaculatory bulb; Guiraudie-Capraz et al., 2007) whereas the amount of cVA externalized (and extracted after a brief wash) depends on social interaction of this fly (Farine et al., 2012).

Alternatively, the differential variation could be due to a physical process similar to the modification of the properties of the cuticle induced by a temperature shift where the same four CHs varied similarly in WT flies exposed to a temperature shift (20–25°) during the first hours of adult life (Savarit and Ferveur, 2002).

Very little information is currently available on CH accumulation and turnover during adult life. The adult maturation of CHs occurs under the control of *desat1* in large polyploid abdominal cells (oenocytes) between 12 and 36 h of adult age (Bousquet et al., 2012; Ferveur et al., 1997). In particular, *desat1* regulates the relative proportion of ∑Desat and ∑Lin. In mature flies, CH deposition on the cuticle apparently follows the circadian oscillation of *desat1* expression in oenocytes in relation with the social activity of flies (Krupp et al., 2008). However, social activity can also cause decreases in the levels of CHs. Indeed, flies exposed to conspecifics can rub each other's cuticle, and this abrasive process results in a decrease in the amounts of superficial CHs (Coyne et al., 1994; Marcillac and Ferveur, 2004).

In parallel to CH transformation during early adult life, it would be interesting to study the ontogenesis of other sex-related sensory cues such as the ability to produce a male acoustic signal (Moulin et al., 2001) or male and female ability to copulate (Arienti et al., 2010; Grosjean et al., 2007). Interestingly, the first 24 h of adult life is the critical period during which the sensory ability of *D. melanogaster* is permanently affected after exposure to sensory cues or food (Barth et al., 1997; Hirsch et al., 1995; Svetec et al., 2005; Flaven-Pouchon et al., 2014).

Despite the fact that the *rk* receptor and its ligand, bursicon, are involved in cuticle sclerotization and melanization, which are processes that occurs shortly after adult emergence, mutations in *rk* caused no detectable effect on the CH profile of immature adults. The absence of any obvious change in the CH profile of *rk* (as well as *desat1*) mutants could be explained if the effects induced by these genetic alterations during early adulthood were only visible hours or even days later. This "delayed-effect" hypothesis is supported by several studies that show that the manipulation of processes involved in CH biosynthesis induced early in a fly's life cause effects that are not detected until later, in mature adults (Bilen et al., 2013; Wicker and Jallon, 1995).

The role of *rk* and bursicon in adult maturation has been extensively examined with regard to their role in wing expansion behaviors, and in the processes of cuticle hardening and melanization. Here we show that *rk* function is also required for the normal maturation of the fly's CH profile. Furthermore the interaction between the *rk* and *desat1* mutations suggest that the changes in CH profile of *rk* mutants is not simply due to the physical changes of their cuticle, but could indicate that *rk* is also involved in CH synthesis or processing in the fly's oenocytes. It will be interesting to determine whether the action of *rk* on CHs involves the activation via phosphorylation of an enzyme that plays a key role in their synthesis, as occurs for tyrosine hydroxylase for cuticle maturation (Davis et al., 2007).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2016.10.007.

References

Andersen, S.O., 2010. Insect cuticular sclerotization: a review. Insect Biochem. Mol. Biol. 40, 166–178. http://dx.doi.org/10.1016/j.ibmb.2009.10.007.

Antony, C., Jallon, J., 1981. Evolution des hydrocarbures comportementalement actifs de Drosophila melanogaster au cours de la maturation sexuelle. CR Acad. Sci. Paris Ser. D 292, 239–242.

Antony, C., Jallon, J.M., 1982. The chemical basis for sex recognition in Drosophila melanogaster. J. Insect Physiol. 28, 873–880. http://dx.doi.org/10.1016/0022-1910(82)90101-9.

Arienti, M., Antony, C., Wicker-Thomas, C., Delbecque, J.P., Jallon, J.M., 2010. Ontogeny of Drosophila melanogaster female sex-appeal and cuticular hydrocarbons. Integr. Zool. 5, 272–282. http://dx.doi.org/10.1111/j.1749-4877.2010.00213.x.

Baker, J.D., Truman, J.W., 2002. Mutations in the Drosophila glycoprotein hormone receptor, rickets, eliminate neuropeptide-induced tanning and selectively block a stereotyped behavioral program. J. Exp. Biol. 205, 2555–2565.

Baker, K.D., Thummel, C.S., 2007. Diabetic larvae and obese flies-emerging studies of metabolism in Drosophila. Cell Metab. 6, 257–266. http://dx.doi.org/10.1016/j.cmet.2007.09.002.

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From dimers to collective dipoles: Structure and dynamics of methanol/ethanol partition by narrow carbon nanotubes

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Alcohol partitioning by narrow single-walled carbon nanotubes (SWCNTs) holds the promise for the development of novel nanodevices for diverse applications. Consequently, in this work, the partition of small alcohols by narrow tubes was kinetically and structurally quantified via molecular dynamics simulations. Alcohol partitioning is a fast process in the order of 10 ns for diluted solutions but the axial-diffusivity within SWCNT is greatly diminished being two to three orders of magnitude lower with respect to bulk conditions. Structurally, alcohols form a single-file conformation under confinement and more interestingly, they exhibit a pore-width dependent transition from dipole dimers to a single collective dipole, for both methanol and ethanol. Energetic analyses demonstrate that this transition is the result of a detailed balance between dispersion and electrostatics interactions, with the latter being more pronounced for collective dipoles. This transition fully modifies the reorientational dynamics of the loaded particles, generating stable collective dipoles that could find usage in signal-amplification devices. Overall, the results herein have shown distinct physico-chemical features of confined alcohols and are a further step towards the understanding and development of novel nanofluidics within SWCNTs. © *2016 AIP Publishing LLC.* [http://dx.doi.org/10.1063/1.4941331]

I. INTRODUCTION

Carbon derived particles such as Carbon nanotubes (CNTs)[1] have been proposed for application in many fields ranging from electronics to medicine.[2–5] One of those, the novel field of nanofluidics[6] which studies the fluxes of atoms and molecules across nanopores and nanochannels holds the potential for the development of efficient and cheap nanodevices.[2,7–13] Moreover, CNTs offer a simplistic model system allowing for the generation of analytical theories that aim towards the description of more complex structures, inherently present in nature, e.g., membrane channels.[14–17] Among many potential applications of nanofluidic devices, the partitioning/filtration of polar molecules, i.e., alcohols from the aqueous solution is of vital importance in industry, as these species are very hard to separate due to their hydrophilic nature.[18,19] Moreover, many water pollutants possess phenolic moieties with an ongoing concern on their effects on the environment and human health.[20] In fact, many efforts are currently being carried out to develop cheap alcohol nano-filtering devices. Overall, the use of carbon nanotubes increases the selectivity towards organic alcohols such as ethanol, butanol, and isopropyl alcohol.[13,21,22]

At the nanoscale level, the continuous description for hydrodynamics fails, i.e., Navier-Stokes equations, as the flux is dominated by the movement of discrete particles.[23] Since the first reports of molecular dynamics (MD) simulations of water penetration across single-walled carbon nanotubes (SWCNTs),[24,25] it became evident that MD offered the correct description of fluxes at the molecular level; the latter is supported by the vast amount of studies which have explored this phenomenon employing both equilibrium and non-equilibrium MD simulations[26,27] and experimental validation.[28–30] Regarding water, single-file diffusion across narrow (6,6) or (5,5) SWCNTs has been thoroughly reported in the literature.[26,27] Not withstanding the hydrophobic nature of SWCNTs, an interplay between dipolar interactions plus rotational and diffusional effects is present, rendering water partitioning both energetically and entropically driven processes.[27,31–35] Meanwhile, the interaction between CNTs and other solvents has been scarcely explored and only recently, reports of alcohol separation by CNTs have appeared in the literature.[19,36–43] Small alcohols such as ethanol and methanol share common features with water, having a polar OH group and presenting a dipole; therefore, it is not strange that carbon-nanotubes do partition alcohols from an aqueous phase given the common features shared with water, plus the additional hydrophobicity provided by the carbon backbone.

Consequently, in this work, MD simulations of SWCNT ((6,6), (7,7), and (8,8)) assisted partitioning of ethanol and methanol were performed; a fully characterization based on dynamical (rotational relaxation, diffusional, mean residence times) and structural properties (interaction energies, dipolar alignment, and displacement correlations) was carried out and compared with simulations of water and methane infiltration, taken as examples of the limiting conditions of small fully polar and hydrophobic molecules, respectively. Overall, our results allowed for a detailed description of the underlying molecular mechanisms involved in alcohol partitioning by narrow SWCNTs. In brief, small alcohols form a single-file structure in narrow pores in a very similar fashion to water.

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Quite interestingly, the dimeric to collective dipole transition is evidenced by the magnitudes of $\tau\vec{\mu}$ which are always smaller for the former case; indeed, for both the methanol-loaded and ethanol-loaded (7,7) and (8,8) pores, very few collective dipole flips were recorded during MD simulations, thus not allowing the calculations of $\tau\vec{\mu}$ or coll $\tau\vec{\mu}$ (see Fig. 11 of the supplementary material[47]). A further evidence of this strong dipolar alignment is reflected by the magnitude of the (single-file loaded) collective dipole vector, which for methanol are on average 7.7 ± 3.3 and 15 ± 1.1 D for (6,6) and (7,7) loaded tubes, respectively. Likewise the ethanol-loaded (6,6), (7,7), and (8,8) pores present values of 4.8 ± 1.8, 6.8 ± 2.7, and 23.1 ± 1.7 D, respectively. For completeness, the dipole for both ethanol and methanol in the current force field is of 1.91 D.

Last but not least, the collective configurations should present higher coll $\tau\vec{\mu}$ than $\tau\vec{\mu}$ (it is harder to flip a collective dipole than its individual components), this is the case for the (6,6) water-loaded tube. In fact, for water-loaded tubes, the tube-width increment renders coll $\tau\vec{\mu}$ smaller than $\tau\vec{\mu}$, an indication of the loss of correlations or collectiveness (see Table III). Regretfully, the impossibility to compute any τ for the methanol and ethanol loaded (7,7) and (8,8) pores precludes the same conclusions for alcohols; nevertheless, the behavior of the methanol-loaded (8,8) tube points towards this direction; despite it is not a rigorous single-file conformations, it contains two aligned dipoles (see Fig. 4). On the other hand, the local dipoles on the dimers are not necessarily aligned (see Fig. 4); thus, their values should be decoupled, and this seems to be case for all values shown in Table III.

IV. CONCLUSIONS

Methanol and ethanol partition by narrow SWCNTs has been thoroughly characterized via atomistic classical molecular dynamics simulations. For diluted solutions, the partitioning is very fast, normally in the order of 10 of ns, with selectivities generally proportional to the number of carbon atoms, as shown by others.[42] Structurally, all narrow tubes form single-files conformation, with a quite interesting passage from dipoles composed of two molecules to a single collective dipole that is pore-width dependent, we have termed the latter phenomena a dimeric to collective dipole transition. Energetic analyses evidenced that this transition is controlled by a fine balance between VdW interactions with the tube atoms and cooperative electrostatic interactions among the loaded particles. Mobility within the tubes is heavily hindered for alcohols with no substantial differences for either dimers or collective dipoles. Quite differently, the rotational dynamics presents a strong anisotropy between rotations parallel and perpendicular the dipole vector, both being substantially reduced for the collective systems.

The results presented in this work are a further step towards the understanding of nanofluidics, providing fundamental knowledge to support the design of nanoscale devices, in which a detailed atomistic characterization of nano-confinement effects is needed. We envision two types of applications: nano-filtering appliances for polar molecules and signal-amplification devices that can take advantage of the strong dipolar alignment; indeed, such constructs have been previously explored by other authors.[60–71]

Even though all evidence indicate that electrostatic interactions rising from nanotube polarization are rather weak,[65] the persistent dipoles formed by the collective arrangement of single-file alcohols, e.g., (7,7) methanol-loaded tube might lead to an instantaneous axial polarization of the tube, effect that is not accounted in our current model. We are currently implementing a polarizable carbon nanotube model that in conjunction with (an already developed) polarizable methanol force-field[68] will serve to assess polarization effects of confined alcohols within SWCNTs.

In future works, we plan to expand these studies by exploring the effects of tube functionalization, pressure driven fluxes and the response towards external electric fields. Moreover, we plan to fully describe the thermodynamical forces in alcohol partition by narrow pores, via rigorous free energy calculations.

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[1]S. Iijima, "Helical microtubules of graphitic carbon," Nature **354**, 56–58 (1991).
[2]B. J. Hinds, N. Chopra, T. Rantell, R. Andrews, V. Gavalas, and L. G. Bachas, "Aligned multiwalled carbon nanotube membranes," Science **303**, 62–65 (2004).
[3]J. H. Grossman and S. E. McNeil, "Nanotechnology in cancer medicine," Phys. Today **65**(8), 38 (2012).
[4]E. S. Snow, F. K. Perkins, E. J. Houser, S. C. Badescu, and T. L. Reinecke, "Chemical detection with a single-walled carbon nanotube capacitor," Science **307**, 1942–1945 (2005).
[5]E. Heister, V. Neves, C. Lamprecht, S. R. P. Silva, H. M. Coley, and J. McFadden, "Drug loading, dispersion stability, and therapeutic efficacy in targeted drug delivery with carbon nanotubes," Carbon **50**, 622–632 (2012).
[6]N.-T. N. Patrick Abgrall, *Nanofluidics* (Artech House, 2009).
[7]R. Singh, D. Pantarotto, L. Lacerda, G. Pastorin, C. Klumpp, M. Prato, A. Bianco, and K. Kostarelos, "Tissue biodistribution and blood clearance rates of intravenously administered carbon nanotube radiotracers," Proc. Natl. Acad. Sci. U. S. A. **103**, 3357–3362 (2006).
[8]A. Kalra, S. Garde, and G. Hummer, "Osmotic water transport through carbon nanotube membranes," Proc. Natl. Acad. Sci. U. S. A. **100**, 10175–10180 (2003).
[9]J.-A. Garate, N. J. English, and J. M. D. MacElroy, "Carbon nanotube assisted water self-diffusion across lipid membranes in the absence and presence of electric fields," Mol. Sim. **35**, 3–12 (2009).
[10]J. A. Garate, N. J. English, and J. M. D. MacElroy, "Static and alternating electric field and distance-dependent effects on carbon nanotube-assisted water self-diffusion across lipid membranes," J. Chem. Phys. **131**, 114508 (2009).
[11]J. S. Babu and S. P. Sathian, "The role of activation energy and reduced viscosity on the enhancement of water flow through carbon nanotubes," J. Chem. Phys. **134**, 194509 (2011).
[12]H. Verweij, M. C. Schillo, and J. Li, "Fast mass transport through carbon nanotube membranes," Small **3**, 1996–2004 (2007).

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REVIEW

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Connexinopathies: a structural and functional glimpse

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Abstract

Mutations in human connexin (Cx) genes have been related to diseases which we termed connexinopathies. Such hereditary disorders include non-syndromic or syndromic deafness (Cx26, Cx30), Charcot Marie Tooth disease (Cx32), oculodentodigital dysplasia and cardiopathies (Cx43), and cataracts (Cx46, Cx50). Despite the clinical phenotypes of connexinopathies have been well documented, their pathogenic molecular determinants remain elusive. The purpose of this work is to identify common/uncommon patterns in channels function among Cx mutations linked to human diseases. To this end, we compiled and discussed the effect of mutations associated to Cx26, Cx32, Cx43, and Cx50 over gap junction channels and hemichannels, highlighting the function of the structural channel domains in which mutations are located and their possible role affecting oligomerization, gating and permselectivity processes.

Keywords: Connexins, hemichannels, gap junction channels, structure and function, human genetic disease

Background

Connexin gap junction channels (GJCs) and hemichannels (HCs) are critical for cellular communication. GJCs allow the intercellular exchange of ions and small molecules (e.g., IP3, cAMP, cGMP, ATP) and diverse metabolites (e.g., sugars, amino acids, glutathione) (reviewed in [1]). The same molecules and ions can pass through HCs, but in this case to take part as autocrine and paracrine signals (reviewed by [2, 3]). Mutations in connexins (Cxs) genes are associated to genetic disorders such as skin abnormalities, cardiopathies, neurodegenerative and developmental diseases, cataracts, and most cases of hereditary deafness (reviewed by [4–6]).

Each HC is formed by the oligomerization of six Cxs subunits and the end-to-end docking of two HCs forms GJCs. The membrane topology of Cxs includes four transmembrane domains (designated as TM1-TM4) connected by two extracellular loops (ECL) and one intracellular loop (ICL). The amino terminus (NT) and the carboxyl terminus (CT) segments are cytoplasmic (Fig. 1a). Despite Cxs share high homology, there are important differences in the amino acid sequence of the ICL and CT. These segments contain motifs for regulatory kinases and cytoskeletal binding proteins [7, 8]. Oligomerization between suited isoforms also contributes to the assortment of Cx-based channels, for instances, heteromeric GJCs (HCs constituted by more than one Cxs type) and/or heterotypic channels (two homomeric HCs each made by a different Cxs type). These combinations may produce GJCs with particular functional and regulatory properties. Several works pointed out to TM3 in Cx32 [9–11] and Cx43 [12], and TM1 and NT segments in Cx26 [12, 13] as critical to regulate oligomerization of Cxs. In addition, a salt bridge between residues Glu-146 (TM3) and Arg-32 (TM1) in

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Conclusions

Most mutations causing connexinopathies generates total or partial loss of GJCs function. However, it is unclear if the severity of disease correlates with the level of GJCs loss of function. Mutations associated with loss of function GJCs are distributed along the entire protein sequence with no clear pattern of clustering at any segment, which suggest that GJC functionality is very sensitive to minor changes in Cxs protein, and that subtle changes in GJC functionality are sufficient to cause diseases. Less in known about the effect of mutations associated to connexinopathies on the functional state of HCs. The clearest correlation between gain of function HCs and disease has been found in most types of syndromic deafness associated to Cx26, in particular in KID syndrome. For others Cxs, few mutations are associated to gain of HCs function, however, we can not discard that this condition may be underestimated because most studies in the past have been more focused in GJCs than HCs. Therefore, it is yet difficult to make a general statement that represent all Cxs associated to connexinopathies. Nevertheless, it is clear that all mutations eliciting gain of HCs function are clustered in pore-associated domains like the NT and the TM1/ECL1, which are critical regions for gating and regulation.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
ADM, conceived the original idea. IEG, FP, and ADM co-wrote and co-edited the final version of the manuscript. AP, made the molecular models of Cxs subunits; GI, PM, DRG, CYM, JGC, CSR, BP, MAR and CG, co-designed and co-wrote the tables and contributed to the discussion. All authors read and approved the final manuscript.

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Declarations
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References

1. Laird DW. Syndromic and non-syndromic disease-linked Cx43 mutations. FEBS Lett. 2014;588(8):1339–48.
2. Sáez JC, Berthoud VM, Branes MC, Martínez AD, Beyer EC. Plasma membrane channels formed by connexins: their regulation and functions. Physiol Rev. 2003;83(4):1359–400.
3. Harris AL. Emerging issues of connexin channels: biophysics fills the gap. Q Rev Biophys. 2001;34(3):325–472.
4. Martínez AD, Acuña R, Figueroa V, Maripillan J, Nicholson B. Gap-junction channels dysfunction in deafness and hearing loss. Antioxid Redox Signal. 2009;11(2):309–22.
5. Delmar M, Makita N. Cardiac connexins, mutations and arrhythmias. Curr Opin Cardiol. 2012;27(3):236–41.
6. Beyer EC, Ebihara L, Berthoud VM. Connexin mutants and cataracts. Front Pharmacol. 2013;4:43.
7. Laird DW, Castillo M, Kasprzak L. Gap junction turnover, intracellular trafficking, and phosphorylation of connexin43 in brefeldin A-treated rat mammary tumor cells. J Cell Biol. 1995;131(5):1193–203.
8. Lampe PD, Lau AF. Regulation of gap junctions by phosphorylation of connexins. Arch Biochem Biophys. 2000;384(2):205–15.
9. Ahmad S, Martin PE, Evans WH. Assembly of gap junction channels: mechanism, effects of calmodulin antagonists and identification of connexin oligomerization determinants. Eur J Biochem. 2001;268(16):4544–52.
10. Maza J, Das Sarma J, Koval M. Defining a minimal motif required to prevent connexin oligomerization in the endoplasmic reticulum. J Biol Chem. 2008;283(2):1115–21.
11. Fleishman SJ, Unger VM, Yeager M, Ben-Tal N. A Calpha model for the transmembrane alpha helices of gap junction intercellular channels. Mol Cell. 2004;15(6):879–88.
12. Martínez AD, Maripillan J, Acuña R, Mínogue PJ, Berthoud VM, Beyer EC. Different domains are critical for oligomerization compatibility of different connexins. Biochem J. 2011;436(1):35–45.
13. García IE, Maripillán J, Jara O, Cerisoni R, Palacios-Muñoz A, Ramachandran J, Olivero P, Perez-Acle T, González C, Sáez JC, et al. Keratitis-Ichthyosis-Deafness Syndrome-Associated Cx26 Mutants Produce Nonfunctional Gap Junctions but Hyperactive Hemichannels When Co-Expressed With Wild-Type Cx43. J Invest Dermatol. 2015;135(5):1338–47.
14. Fleishman SJ, Sallay AD, Optiz E, Avraham KB, Ben-Tal N. The structural context of disease-causing mutations in gap junctions. J Biol Chem. 2006;281(38):28958–63.
15. Maeda S, Nakagawa S, Suga M, Yamashita E, Oshima A, Fujiyoshi Y, Tsukihara T. Structure of the connexin 26 gap junction channel at 3.5 A resolution. Nature. 2009;458(7238):597–602.
16. Lagree V, Brunschweig K, Lopez P, Gilula NB, Richard G, Falk MM. Specific amino-acid residues in the N-terminus and TM3 implicated in channel function and oligomerization compatibility of connexin43. J Cell Sci. 2003;116(Pt 15):3189–201.
17. Unger VM, Kumar NM, Gilula NB, Yeager M. Three-dimensional structure of a recombinant gap junction membrane channel. Science. 1999;283(5405):1176–80.
18. Skerrett IM, Aronowitz J, Shin JH, Cymes G, Kasperek E, Cao FL, Nicholson BJ. Identification of amino acid residues lining the pore of a gap junction channel. J Cell Biol. 2002;159(2):349–60.
19. Zhou XW, Pfahnl A, Werner R, Hudder A, Llanes A, Luebke A, Dahl G. Identification of a pore lining segment in gap junction hemichannels. Biophys J. 1997;72(5):1946–53.
20. Kronengold J, Trexler EB, Bukauskas FF, Bargiello TA, Verselis VK. Single-channel SCAM identifies pore-lining residues in the first extracellular loop and first transmembrane domains of Cx46 hemichannels. J Gen Physiol. 2003;122(4):389–405.
21. Tang Q, Dowd TL, Verselis VK, Bargiello TA. Conformational changes in a pore-forming region underlie voltage-dependent 'loop gating' of an unapposed connexin hemichannel. J Gen Physiol. 2009;133(6):555–70.
22. Kwon T, Harris AL, Rossi A, Bargiello TA. Molecular dynamics simulations of the Cx26 hemichannel: evaluation of structural models with Brownian dynamics. J Gen Physiol. 2011;138(5):475–93.
23. Purnick PE, Benjamin DC, Verselis VK, Bargiello TA, Dowd TL. Structure of the amino terminus of a gap junction protein. Arch Biochem Biophys. 2000;381(2):181–90.
24. Oshima A, Tani K, Hiroaki Y, Fujiyoshi Y, Sosinsky GE. Three-dimensional structure of a human connexin26 gap junction channel reveals a plug in the vestibule. Proc Natl Acad Sci U S A. 2007;104(24):10034–9.

REVIEW

From Hyperactive Connexin26 Hemichannels to Impairments in Epidermal Calcium Gradient and Permeability Barrier in the Keratitis-Ichthyosis-Deafness Syndrome

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The keratitis-ichthyosis-deafness (KID) syndrome is characterized by corneal, skin, and hearing abnormalities. KID has been linked to heterozygous dominant missense mutations in the GJB2 and GJB6 genes, encoding connexin26 and 30, respectively. In vitro evidence indicates that KID mutations lead to hyperactive (open) hemichannels, which in some cases is accompanied by abnormal function of gap junction channels. Transgenic mouse models expressing connexin26 KID mutations reproduce human phenotypes and present impaired epidermal calcium homeostasis and abnormal lipid composition of the stratum corneum affecting the water barrier. Here we have compiled relevant data regarding the KID syndrome and propose a mechanism for the epidermal aspects of the disease.

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CHARACTERISTIC PHENOTYPE OF THE KID SYNDROME

The keratitis-ichthyosis-deafness syndrome (KID) is a genetic disorder inherited in an autosomal dominant manner, with few cases of recessive inheritance (Burns, 1915; Caceres-Rios et al., 1996; Orozco-Covarrubias et al., 2008; Skinner et al., 1981). Hallmarks of the KID syndrome include eye problems, skin abnormalities, and severe hearing loss.

Keratitis is characterized by corneal inflammation and pain, increased light sensitivity (photophobia), corneal neovascularization, and conjunctivitis. In severe cases, patients may become blind (Orozco-Covarrubias et al., 2008). The main skin abnormalities included thickening of soles and palms (palmoplantar keratoderma [PPK]), furfuraceous, or dry/scaling (ichthyosis) in reddish skin patches (erythrokeratoderma), which can be randomly distributed on the body but concentrated at neck and armpits (Caceres-Rios et al., 1996). Patients also tend to develop carcinomas (Mazereeuw-Hautier et al., 2007). This complex corneal/skin disease is accompanied by profound hearing loss (Caceres-Rios et al., 1996; Richard et al., 2002). Fortunately, this severe syndrome is very rare, with approximately 100 cases reported affecting females slightly more often than males. Because the KID syndrome influences several organs, patients require multidisciplinary treatment. Unfortunately, in severe cases, the syndrome leads to death in infants because of massive skin problems and breathing disorders (Meigh et al., 2014; Sbidian et al., 2010). The application of skin-softening emollients on moist skin is helpful only in some milder cases of the KID syndrome.

GENETIC CAUSES

Although most KID syndrome cases arise from sporadic mutations, some cases of familial transmission support autosomal dominant inheritance (Grob et al., 1987; Kelly et al., 2008; Langer et al., 1990; Messmer et al., 2005; Nazzaro et al., 1990; Orozco-Covarrubias et al., 2008). The autosomal dominant form of KID has been linked to heterozygous missense mutations in the GJB2 gene, which codes for the connexin26 protein (Cx26; GJB2 13q11-12) (Richard et al., 2002; van Steensel et al., 2002) and in the GJB6 gene, which encodes the connexin30 protein (Cx30; GJB6 13q12). To date 14 different missense mutations (12 in GJB2; 2 in GJB6) have been identified in patients with KID (summarized in Table 1). Recently, a lethal form of KID was described, caused by reversion of the GJB2 nonsense mutation Y136X that has confined the effect of KID mutation G45E present in the same allele (Ogawa et al., 2014). The authors estimated that there are approximately 11,000 individuals in the Japanese population that carry both the Cx26G45E mutation and the confining Y136X mutation. These tandem mutations can cause hearing loss in an autosomal recessive

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Abbreviations: Cx, connexin; GJCs, gap junction channels: HCs, hemichannels; KID, keratitis-ichthyosis-deafness; PPK, palmoplantar keratoderma

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that these keratinocytes exhibited a gain of HC function (Mese et al., 2011).

COMPARISON OF KID MOUSE MODELS AND PATIENTS WITH KID

Despite differences in the molecular genetics of the mouse models Cx26S17F and Cx26G45E, one should also consider general differences between these mouse models and corresponding patients with KID carrying one of these mutations. In mice epidermis, Cx26 is only expressed in the highly differentiated stratum granulosum, whereas in humans Cx26 is also expressed in the proliferative stratum basale (Di et al., 2001; Kretz et al., 2004; Richard et al., 2002), where this Cx is coexpressed with Cx30 and Cx43. In the Cx26S17F mouse line, the mutation is expressed under control of the endogenous Cx26 promoter, allowing their expression only in stratum granulosum (Schütz et al., 2011). In contrast, in the Cx26G45E mouse line, there is an ectopic overexpression of the Cx26G45E protein in the stratum basale of the epidermis (Mese et al., 2011). Although the Cx26S17F mice may have the advantage of an endogenous regulation of the protein expression without overexpression, the Cx26G45E mice overexpress Cx26G45E in the same epidermal layer where it is expressed in humans.

In this context, it should be pointed out that all patients with KID were diagnosed with an inflammatory status of their skin. In most severe cases, this is associated with infection by opportunistic pathogens (Donnelly et al., 2012; Martin and van Steensel, 2015). The molecular details of these processes could also be studied with the mentioned KID mouse models. Recently, Levit et al. (2015) reported that HCs formed by expression of several Cx26 KID mutants in *Xenopus laevis* oocytes are inhibited by mefloquine ($IC_{50} \sim 16$ μM). It needs to be investigated whether specific inhibitory compounds for Cx HC can be used to repress the inflammatory and septic features of KID mutations in the skin of mouse models and eventually in patients with KID (Levit and White, 2015).

OPEN PROBLEMS AND FUTURE DIRECTIONS

What are the structural requirements for the functional paradox that some Cx26 KID mutants exhibit hyperactive HCs but abnormal function of GJCs?

Because HCs are the primary components of GJCs, the paradoxical functional property of some KID mutants (hyperactive HCs but impaired GJC function) suggests that HC docking induces important structural rearrangements. HCs and GJCs, although formed by the same Cxs, must have different regulatory properties in terms of gating and permeability. This needs to be further explored and correlated with refined structural investigations (Maeda et al., 2009).

Why do only certain Cx26 mutations lead to the KID syndrome?

As mentioned above, Cx26 KID mutations lead to an aberrant skin phenotype and profound hearing loss. Genodermatoses caused by other Cx26 mutations (i.e., PPK) or by mutations in Cx30 or Cx31, which lead to Clouston syndrome or erythrokeratoderma variabilis, respectively, usually exhibit milder phenotypes (Lamartine et al., 2000; Richard et al., 1998; Xu and Nicholson, 2013). In addition, most cases involving mutations of the Cx26 gene are nonsyndromic. To date, a unique feature of syndromic disease is the simultaneous formation of homomeric or heteromeric hyperactive HCs and abnormal GJC function (Table 1 and Figure 2). Moreover, a strong argument in favor of gain of function HCs as a primary reason for KID includes the observation that mutations Cx26G12V and Cx26A40G cause nonfunctional HCs and GJCs (García et al., 2015; Jara et al., 2012) and severe deafness without skin disease, contrasting the effects of KID mutations in same amino acid residues (G12R and A40V). It is not clear to which extent gain of functional HC and the functional impairment of GJCs in the epidermis (and possibly in cells of the inner ear) contribute to mechanisms leading to the KID syndrome.

What is the primary result of aberrant HC function in KID epidermis: the impairment of the Ca^{2+} gradient or the epidermal water barrier?

A decreased Ca^{2+} concentration surrounding cells of the stratum granulosum has been suggested to trigger the secretion of lamellar bodies (i.e., lipid containing vesicles from cells of the stratum granulosum) (Feingold, 2007). Because differentiation of keratinocytes depends on Ca^{2+} ions (Bikle et al., 2012), alterations in the epidermal Ca^{2+} gradient could affect the correct deposition of ceramides. The role of lipids in the formation and maintenance of cutaneous permeability barrier has been extensively reviewed (Feingold and Elias, 2014). Interestingly, a defective water barrier could also influence the establishment of the Ca^{2+} gradient (Elias et al., 2002). Hence in an alternative scenario, the Cx26S17F mutation may disrupt the water barrier because of loss of ceramide deposition, which in turn may alter the Ca^{2+} gradient. Currently, one cannot distinguish between these alternatives. Possibly both events may mutually affect each other. Therefore, further experiments need to clarify this problem.

Hyperkeratosis is a hallmark of the KID syndrome in patients and mouse models. As aforementioned, hyperactive HCs may cause the release of ATP and Ca^{2+} influx, leading to Ca^{2+} overload (Figure 2). Therefore, we hypothesize that the initiation of keratinocytic hyperproliferation might be triggered by disruption in Ca^{2+} homeostasis because of a preceding transitory epidermal barrier defect sufficient to trigger DNA synthesis and cell division.

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REFERENCES

Adachi N, Yoshida T, Nin F, Ogata G, Yamaguchi S, Suzuki T, et al. The mechanism underlying maintenance of the endocochlear potential by the K+ transport system in fibrocytes of the inner ear. J Physiol 2013;591:4459–72.

Adams MP, Mallet DG, Pettet GJ. Active regulation of the epidermal calcium profile. J Theor Biol 2012;301:112–21.

Alvarez A, del Castillo I, Pera A, Villamar M, Moreno-Pelayo MA, Moreno F, et al. De novo mutation in the gene encoding connexin-26 (GJB2) in a sporadic case of keratitis-ichthyosis-deafness (KID) syndrome. Am J Med Genet A 2003;117A:89–93.

Anselmi F, Hernandez VH, Crispino G, Seydel A, Ortolano S, Roper SD, et al. ATP release through connexin hemichannels and gap junction transfer of

A soluble activin receptor IIB fails to prevent muscle atrophy in a mouse model of spinal cord injury

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Running Title: Myostatin inhibition after SCI

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through upregulation of the expression of uncoupling proteins [47]. The reason for this is uncertain, but one possibility is that the energy demands of maintaining core temperature are elevated due greater heat losses per unit of body mass as a result of reduced body weight and, consequently, elevated body surface area relative to body weight. The tissue inflammation identified in mouse gastrocnemius muscle, as well as endocrine disturbances including hypogonadism and reduced growth hormone levels [4, 33], may also contribute,

Conclusions

RAP-031 stimulated gains in muscle mass in the forelimbs of mice at 56 days after a complete mid-thoracic transection of the spinal cord whereas forelimb muscles were reduced in size in mice with SCI that were not treated. It is not known whether arm muscle is lost over the first days to weeks after SCI in man, but it is likely that this is the case as individuals with SCI have multiple stressors in addition to their SCI, such as soft tissue trauma, infections, medications and bed rest, all of which exacerbate muscle atrophy. Our findings suggest that administration of an inhibitor of myostatin action early after an SCI, perhaps at the time rehabilitation is initiated, would minimize loss of muscle in regions with preserved neurological function and speed rehabilitation. The finding that RAP-031 had no effect on paralyzed muscle suggests that even when an intact lower motor neuron is present, physical activity is necessary for myostatin inhibitors to prevent atrophy of skeletal muscle. The findings also suggest that only inhibition of multiple upstream signals for atrophy will prevent the loss of skeletal muscle that occurs with paralysis and/or severe immobilization of other etiologies.

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Duox2 Regulates Pannexin1-mediated ATP Release in Primary Human Airway Epithelial Cells via changes in intracellular pH and not H_2O_2 production*

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*Running head: *Dual oxidase 2 regulates ATP release through pannexin 1*

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ABSTRACT

Human airway epithelial cells express pannexin 1 channels (Panx1) to release ATP that regulates mucociliary clearance. Airway inflammation causes mucociliary dysfunction. Exposure of primary human airway epithelial cell cultures to interferon gamma (IFN-γ) for 48 hours did not alter Panx1 protein expression but significantly decreased ATP release in response to hypotonic stress. The IFN-γ-induced functional downregulation of Panx1 was due to the upregulation of dual oxidase 2 (Duox2): Duox2 suppression by siRNA led to an increase in ATP release in control cells and the restoration of ATP release in cells treated with IFN-γ. Both effects were reduced by the pannexin inhibitor probenecid. Duox2 upregulation increases H_2O_2 and proton production stoichiometrically. H_2O_2 inhibited Panx1 function temporarily by formation of disulfide bonds at the thiol group of its terminal cysteine. Long-term exposure to H_2O_2, however, had no inhibitory effect. To assess the role of cellular acidification upon IFN-γ treatment, fully differentiated airway epithelial cells were exposed to ammonium chloride to alkalinize the cytosol. This led to a two fold increase in ATP release in cells treated with IFN-γ that was also inhibited by probenecid. Duox2 knock down also partially corrected IFN-γ-mediated acidification. The direct correlation between intracellular pH and Panx1 open probability was shown in oocytes. Thus, airway epithelial cells release less ATP in response to hypotonic stress in an inflammatory environment (IFN-γ exposure). Decreased Panx1 function is a response to cell acidification, mediated by IFN-γ-induced upregulation of Duox2, representing a novel mechanism for mucociliary dysfunction in inflammatory airway diseases.

ATP is a key regulator of innate pulmonary host defense by activating P2Y2 receptors, which promote chloride secretion by calcium activated Cl^- channels, inhibit Na^+ absorption by epithelial Na^+ channels, increase ciliary beat frequency, airway surface liquid volume and induce mucin release thereby activating mucociliary clearance (1-4).

While effects of ATP on airway epithelial cells have been widely studied, Panx1 channels have only been recently recognized to be involved in ATP release in these tissues (5,6). Mechanical stress was shown to be one of the prime stimuli to increase the ATP concentration on the airway surface to a concentration sufficient to activate P2Y2 receptors (7). This ATP release was neither dependent on the intracellular calcium concentration, excluding an exocytotic release mechanism, nor caused by the cystic fibrosis transmembrane conductance regulator (CFTR). In previous studies, we were the first to show that Panx1, an ortholog of the invertebrate innexin, is expressed at the apical membrane of airway epithelia, contributing to ATP release (5).

Pannexin proteins form pannexons, which are channels that open at resting membrane potential

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Reading Comprehension in Latin America: Difficulties and Possible Interventions

Séverin Lions, Marcela Peña

Abstract

Reading comprehension (RC) is below the international standard in many countries of Latin America (LA). Here we review factors that might be associated with failure in RC of the first language in LA. Then we present interventions reporting beneficial impact on RC in typically developing students from English-speaking countries and discuss their possible applicability in LA. We conclude that research-based pedagogical interventions are currently available to promote RC at school and may be suitable to implement in LA in order to improve RC.

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Effect of Terminal Groups of Dendrimers in the Complexation with Antisense Oligonucleotides and Cell Uptake

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Abstract

Poly(amidoamine) dendrimers are the most recognized class of dendrimer. Amino-terminated (PAMAM-NH_2) and hydroxyl-terminated (PAMAM-OH) dendrimers of generation 4 are widely used, since they are commercially available. Both have different properties, mainly based on their different overall charges at physiological pH. Currently, an important function of dendrimers as carriers of short single-stranded DNA has been applied. These molecules, known as antisense oligonucleotides (asODNs), are able to inhibit the expression of a target mRNA. Whereas PAMAM-NH_2 dendrimers have shown to be able to transfect plasmid DNA, PAMAM-OH dendrimers have not shown the same successful results. However, little is known about their interaction with shorter and more flexible molecules such as asODNs. Due to several initiatives, the use of these neutral dendrimers as a scaffold to introduce other functional groups has been proposed. Because of its low cytotoxicity, it is relevant to understand the molecular phenomena involving these types of dendrimers. In this work, we studied the behavior of an antisense oligonucleotide in presence of both types of dendrimers using molecular dynamics simulations, in order to elucidate if they are able to form stable complexes. In this manner, we demonstrated at atomic level that PAMAM-NH_2, unlike PAMAM-OH, could form a well-compacted complex with asODN, albeit PAMAM-OH can also establish stable interactions with the oligonucleotide. The biological activity of asODN in complex with PAMAM-NH_2 dendrimer was also shown. Finally, we revealed that in contact with PAMAM-OH, asODN remains outside the cells as TIRF microscopy results showed, due to its poor interaction with this dendrimer and cell membranes.

Background

Dendrimers, a class of hyperbranched polymer explored in nanomedicine applications, were first described by Tomalia [1] and Newkome [2]—separately—as core-shell structures, built in a layer-by-layer way, forming generations. This feature makes dendrimer a well-defined structure; thus, it is possible to control its surface functionality.

Dendrimers have promoted high interest in the field of biology and nanomedicine due to their multivalent and monodisperse properties, which favor reproducible applications in drug and gene delivery, as well as in chemotherapeutic research [3]. For example, different terminal groups of poly(amidoamine) (PAMAM) dendrimers have varying implications in the binding of several types of drugs, as our group has previously shown [4, 5]. Thus, the choice of certain terminal moieties is a critical task in the design of new dendrimers as carriers of therapeutic molecules.

In this sense, PAMAM-NH_2 dendrimer have been described first by Haensler and Szoka [6] and by Kukowska-Latallo et al. [7] as efficient carriers for nucleic acids, due to their positive charge at physiological pH [8]. Moreover, PAMAM dendrimers have been proposed as promissory RNAi nanocarriers [9]. On the other hand, by using ethidium bromide exclusion assays, neutral hydroxyl-terminated PAMAM (PAMAM-OH)

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release nucleic acids inside the cells representing a promissory carrier for DNA-based therapies.

Conclusions

An efficient nucleic acid carrier must form stable complexes with DNA or RNA and promote internalization into the cells, by means of a good affinity for the cell membranes. Here, we studied using TIRF microscopy and molecular dynamics method, the influence of terminal groups of a dendrimer, and essentially, the charge properties of each of them, in the ability to bind antisense oligonucleotides and later penetrate the cell membrane.

Previous articles have evidenced the inability of PAMAM-OH dendrimers to form a complex with plasmid DNA, using ethidium bromide exclusion assays [10, 12]. Here, we revealed that PAMAM-OH in fact can establish contacts with asODN, but it is unable to condense it. Moreover, most part of the asODN remained exposed to the solvent, in such a way that an intercalating dye such as ethidium bromide cannot be displaced by the dendrimer. This could explain the absence of DNA fluorescence quenching induced by PAMAM-OH as it was described in mentioned articles [43].

Furthermore, nucleic acids carriers must have positively charged groups to efficiently bind DNA, as it has been demonstrated in this article. As far as we are concerned, there are no previous articles have described the interaction, at atomic-level scale, between non-charged dendrimers and asODN.

Here, we demonstrated that PAMAM-NH_2 dendrimer forms a well-compacted complex with an asODN, promoting its cell uptake and its biological function. Specifically, in this case, we evaluated the inhibition of the expression of target protein Survivin compared when we used PAMAM-NH_2 as a transfection reagent. Differently, PAMAM-OH is not able to form a complex and efficiently protect asODN from solvent. From these evidences, we can argue why in presence of PAMAM-OH, asODN is unable to cross the cell membranes. First, contacts between a neutral dendrimer and the negatively charged oligonucleotide are not enough to condense the nanoparticle and avoid the repulsion of the membrane. Then, asODN remained outside the cell, as TIRF and fluorescent microscopy showed, because of its poor interaction with PAMAM-OH and cell membranes. In spite of that, neutral dendrimers could protect other classes of molecules, such as drug or peptides, and eventually, they could cross the cell membranes, probably with less efficiency than positively charged nanoparticles. Thus, these results support the idea that neutralizing some groups of PAMAM-NH_2, e.g., with hydroxyl groups, like in PAMAM-OH, could be a feasible approach to avoid the induction of cytotoxicity by highly charged dendrimers, conserving their properties as nucleic acid carriers. This study strongly supports the idea that, despite both are dendrimers of the same generation, their chemical composition might be crucial for membrane binding and cell penetration, and that a rational design of dendrimers, it means, an adequate modulation of the functional groups, could favor the development of safer and optimized carriers.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VMM designed the molecular dynamics simulations, the fluorescence quenching assay, obtained the graphs and images and wrote the manuscript. IAD ran the molecular dynamics simulations. JPP, SV and VR performed the Western Blot experiment. JPP, CO, VMM and RR did the TIRF and fluorescence microscopy. LV, JF and FC contributed to the analysis of the results and the revision of the manuscript. FDGN designed the analysis of molecular dynamics simulations and the general idea of the article. CO designed the experimental section, the general idea of the article and wrote the article. All authors read and approved the final manuscript.

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References

1. Tomalia DA, Baker H, Dewald J, Hall M, Kallos G, Martin S, Roeck J, Ryder J, Smith P (1985) A New Class of Polymers: Starburst-Dendritic Macromolecules. Polym J 17(1):117–132
2. Newkome GR, Yao Z, Baker GR, Gupta VK (1985) Micelles. Part 1. Cascade molecules: a new approach to micelles. A [27]-arborol. J Org Chem 50(11): 2003–2004
3. Challa T, Goud BA, Baskar S, Chandra Mouli G, Jukuri R (2011) Dendrimers: a novel polymer for drug delivery. Int J Pharm Sci Rev Res 9(o):1
4. Vergara-Jaque A, Comer J, Monsalve L, González-Nilo FD, Sandoval C (2013) Computationally Efficient Methodology for Atomic-Level Characterization of Dendrimer–Drug Complexes: A Comparison of Amine- and Acetyl-Terminated PAMAM. J Phys Chem B 117(22):6801–6813
5. Avila-Salas F, Sandoval C, Caballero J, Guiñez-Molinos S, Santos LS, Cachau RE, González-Nilo FD (2012) Study of interaction energies between the PAMAM dendrimer and nonsteroidal anti-inflammatory drug using a

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An ideal nucleic-acid transfection system should combine the physical and chemical characteristics of cationic lipids and linear polymers to decrease cytotoxicity and uptake limitations. Previous research described new types of carriers termed amphiphilic dendrimers (ADs), which are based on polyamidoamine dendrimers (PAMAM). These ADs display the cell membrane affinity advantage of lipids and preserve the high affinity for DNA possessed by cationic dendrimers. These lipid/dendrimer hybrids consist of a low-generation, hydrophilic dendron (G2, G1, or G0) bonded to a hydrophobic tail. The G2-18C AD was reported to be an efficient siRNA vector with significant gene silencing. However, shorter tail ADs (G2-15C and G2-13C) and lower generation (G0 and G1) dendrimers failed as transfection carriers. To date, the self-assembly phenomenon of this class of amphiphilic dendrimers has not been molecularly explored using molecular simulation methods. To gain insight into these systems, the present study used coarse-grained molecular dynamics simulations to describe how ADs are able to self-assemble into an aggregate, and, specifically, how tail length and generation play a key role in this event. Finally, explanations are given for the better efficiency of G2/18-C as gene carrier in terms of binding of siRNA. This knowledge could be relevant for the design of novel, safer ADs with well-optimized affinity for siRNA.

The development of safe and efficient carriers is one of the most important requirements for the clinical implementation of nucleic acid-based therapies. Of the non-viral delivery systems, cationic lipid and polymer members are the most popular[1]. Liposomes composed of cationic lipids are successful systems for DNA delivery. However, there are many concerns regarding *in vivo* use due to cytotoxicity, short half-lives, poor solubility, and instability[2]. On the other hand, polymers have an inherently complex structure and low efficiency as transfection reagents[3].

Polyamidoamine (PAMAM) dendrimers represent a class of hyperbranched polymers that are versatile vehicle candidates in nanomedicine, especially in the fields of diagnosis and cancer therapy[4]. PAMAM dendrimers consist of a central core with multiple emerging branches, which over classical polymers, the functions of which cannot be precisely controlled. Dendrimer terminal groups are also critical in binding several types of drugs[5,6] and in influencing dendrimer interactions with the cell membrane and cytoplasmic proteins[7].

Dendrimers present the following advantages compared to traditional transport molecules[8]: 1) multipurpose control over surface groups; 2) excellent cell uptake, which provides high drug bioavailability; 3) monodispersity and manageable size, which facilitates biomedical applications; 4) globular architecture that resembles a protein, which enables application without an immunoreaction[9]; and 5) high nucleic acid affinity and the ability to release drugs, which prevents complications during cancer therapy[10].

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Author Contributions

V.M.M. carried out the molecular dynamics simulations, performed the analysis and interpretation of the results and wrote the manuscript. I.A.D., M.B.C. and J.C. supported molecular dynamics simulations, the analysis of the results and design of the figures. J.C. made the molecular model of siRNA. F.D.G.N. and J.A.V.G. supervised the project and critically revised the manuscript. All authors have reviewed the manuscript.

Additional Information

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Article

Human Aquaporin 4 Gating Dynamics under Perpendicularly-Oriented Electric-Field Impulses: A Molecular Dynamics Study

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Abstract: Human aquaporin 4 has been studied using molecular dynamics (MD) simulations in the absence and presence of pulses of external static electric fields. The pulses were 10 ns in duration and 0.012–0.065 V/Å in intensity acting along both directions perpendicular to the pores. Water permeability and the dipolar response of all residues of interest (including the selectivity filter) within the pores have been studied. Results showed decreased levels of water osmotic permeability within aquaporin channels during orthogonally-oriented field impulses, although care must be taken with regard to statistical certainty. This can be explained observing enhanced "dipolar flipping" of certain key residues, especially serine 211, histidine 201, arginine 216, histidine 95 and cysteine 178. These residues are placed at the extracellular end of the pore (serine 211, histidine 201, and arginine 216) and at the cytoplasm end (histidine 95 and cysteine 178), with the key role in gating mechanism, hence influencing water permeability.

Keywords: water; permeability; molecular dynamics; electric field; aquaporin

1. Introduction

Aquaporins (AQPs) constitute an extensive family of trans-membrane proteins forming channels that conduct selectively water, as well as other small uncharged molecules (such as glycerol). This selective permeation is a result of osmotic pressure between both sides of the membrane, also serving to exclude very strictly the passage of ions and protons [1,2]. AQPs are in all known lifeforms and are essential for regulating precisely water content in organs and cells. In humans, their defective function is implicated in various pathological conditions, such as nephrogenicdiabetes, insipidus and congenital cataracts [3]. Since their original discovery by Agre et al. [4], several hundred AQPs have been elucidated and characterized [3,5]. In any event, a deeper and more complete understanding of osmotically-driven water permeabilities and fluxes in AQPs is both warranted and essential for progress in medical research to establish more confidently their function and gauge more adeptly their potential involvement in medical conditions. Bearing this goal in mind, water fluxes in AQPs are estimated relatively routinely via reconstitution of channels in liposomes and monitoring changes

The latter provides for the definition of the diffusion constant D_n, which follows from the mean square displacement (MSD) of $n(t)$:

$$D_n = \left\langle n^2(t) \right\rangle / 2t \quad (6)$$

measured in t^{-}. Lastly, p_f is computed by

$$p_f = v_w D_n \quad (7)$$

with v_w the average volume of a water molecule.

4. Conclusions

Water osmotic permeability and the dipolar response of all residues within h-AQP4 pores using MD in the absence and presence of pulses of external static electric fields were studied. The pulses were 10 ns in duration and 0.012–0.065 V/Å in intensity, acting along both directions perpendicular to pores. We found enhanced "dipolar flipping" of key residues, especially serine 211, histidine 95 and cysteine 178. The mouths of the pores were more amenable to more pronounced dipolar orientation and distortion, and this led to accelerated sampling of new dipolar states in more intense fields. This work has established the diverse behavior of the various residues lining the aquaporin channels, particularly at their mouths, in terms of influencing water permeability under the influence of external electric fields, and confirms the speculation of Alberga et al. [37] of the heterogeneity of residues' behavior in the case of some physiochemical perturbation—in this case, an external electric field. In view of the growing importance of electric fields in nanotechnology, medicine and industrial settings, this fundamental mechanistic understanding on such a prototypical transmembrane protein such as h-AQP4 is particularly timely.

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References

1. Hohmann, S.; Nielsen, S.; Agre, P. *Aquaporins*; Academic Press: San Diego, CA, USA, 2001.
2. Borgnia, M.; Nielsen, S.; Engel, A.; Agre, P. Cellular and molecular biology of the aquaporin water channels. *Annu. Rev. Biochem.* **1999**, *68*, 425–458. [CrossRef] [PubMed]
3. King, L.S.; Kozono, D.; Agre, P. From structure to disease: The evolving tale of aquaporin biology. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 687–698. [CrossRef] [PubMed]
4. Preston, G.M.; Piazza-Carroll, P.; Guggino, W.B.; Agre, P. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. *Science* **1992**, *256*, 385–387. [CrossRef] [PubMed]
5. Engel, A.; Stahlberg, H. Aquaglyceroporins: Channel proteins with a conserved core, multiple functions, and variable surfaces. *Int. Rev. Cytol.* **2002**, *215*, 75–104. [PubMed]
6. Finkelstein, A. *Water Movement through Lipid Bilayers, Pores, and Plasma Membranes*; John Wiley & Sons: New York, NY, USA, 1987.
7. Calamita, G. The Escherichia coli aquaporin-z water channel. *Mol. Microbiol.* **2000**, *37*, 254–262. [CrossRef] [PubMed]
8. Borgnia, M.J.; Kozono, D.; Calamita, G.; Maloney, P.C.; Agre, P. Functional reconstitution and characterization of AqpZ, the *E. coli* water channel protein. *J. Mol. Biol.* **1999**, *291*, 1169–1179. [CrossRef] [PubMed]

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RESEARCH ARTICLE

Graphlet Based Metrics for the Comparison of Gene Regulatory Networks

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Abstract

Understanding the control of gene expression remains one of the main challenges in the post-genomic era. Accordingly, a plethora of methods exists to identify variations in gene expression levels. These variations underlay almost all relevant biological phenomena, including disease and adaptation to environmental conditions. However, computational tools to identify how regulation changes are scarce. Regulation of gene expression is usually depicted in the form of a gene regulatory network (GRN). Structural changes in a GRN over time and conditions represent variations in the regulation of gene expression. Like other biological networks, GRNs are composed of basic building blocks called *graphlets*. As a consequence, two new metrics based on graphlets are proposed in this work: REConstruction Rate (REC) and REC Graphlet Degree (RGD). REC determines the rate of graphlet similarity between different states of a network and RGD identifies the subset of nodes with the highest topological variation. In other words, RGD discerns how th GRN was rewired. REC and RGD were used to compare the local structure of nodes in condition-specific GRNs obtained from gene expression data of *Escherichia coli*, forming biofilms and cultured in suspension. According to our results, most of the network local structure remains unaltered in the two compared conditions. Nevertheless, changes reported by RGD necessarily imply that a different cohort of regulators (i.e. transcription factors (TFs)) appear on the scene, shedding light on how the regulation of gene expression occurs when *E. coli* transits from suspension to biofilm. Consequently, we propose that both metrics REC and RGD should be adopted as a quantitative approach to conduct differential analyses of GRNs. A tool that implements both metrics is available as an on-line web server (http://dlab.cl/loto).

Introduction

Networks are everywhere [1]. They are used to represent complex data associations from different domains ranging from social interactions and technological developments up to

(EC); Indegree (ID); Outdegree (OD); Neighborhood Connectivity (NC); and Stress Centrality (SC). See below for a definition of the centralities used. This file also contains the definition of the centrality metrics employed and several figures showing the comparison of these centrality metrics versus RGD on the comparisons performed using the biofilm and suspension networks at 15 hours as reference.
(PDF)

S1 Text. Assessing REC and RGD on comparisons of random networks. This text explains the procedure followed to generate random GRNs of any given size and to randomize the *E. coli* gold standard. The file also shows the performance of the method with respect to network size and the values of REC and RGD on comparisons of randomized *E. coli* with the reference network.
(PDF)

S2 Text. Functional characterization of genes coding TFs with lowest RGD at 15 hours.
(PDF)

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Conceptualization: AJMM.

Data curation: AJMM.

Formal analysis: AJMM TPA CD.

Funding acquisition: AJMM DSH TPA.

Investigation: AJMM.

Methodology: AJMM.

Project administration: AJMM.

Resources: AJMM TPA.

Software: AJMM SCR.

Supervision: AJMM TPA.

Validation: AJMM.

Visualization: AJMM CD.

Writing – original draft: AJMM.

Writing – review & editing: AJMM DSH TPA CD SCR.

References

1. Newman M. Networks: An Introduction. New York, NY, USA: OUP Oxford; 2010.
2. Park J, Newman MEJ. Statistical mechanics of networks. Physical review E, Statistical, nonlinear, and soft matter physics. 2004 Dec; 70(6 Pt 2):066117. doi: 10.1103/PhysRevE.70.066117 PMID: 15697444

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11 Methods to Determine Formation of Heteromeric Hemichannels

Agustín D. Martínez, Oscar Jara, Ricardo Ceriani, Jaime Maripillán, Paula Mujica, and Isaac E. García

CONTENTS

11.1 INTRODUCTION

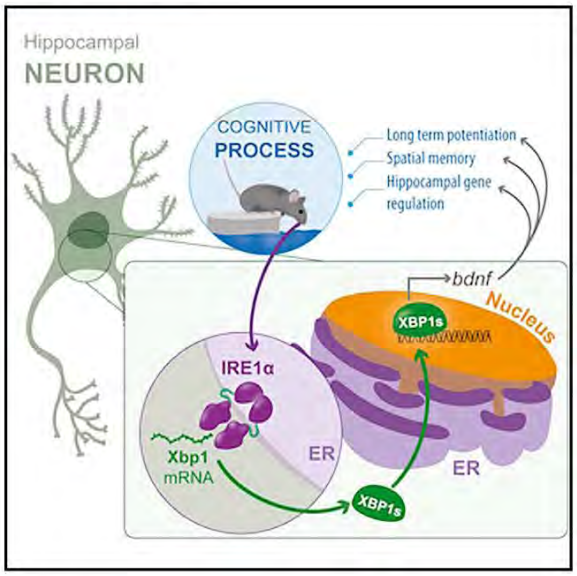
The oligomerization of proteins involves the sequential recognition between multiple compatible monomeric protein subunits. In the case of ion channels, this complex multistep process ends with a protein organization that confers specific structural and physicochemical characteristics, which make the proteins suitable for ion

Article

Cell Reports

Regulation of Memory Formation by the Transcription Factor XBP1

Graphical Abstract



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In Brief

Using gain- and loss-of-function approaches, Martinez et al. demonstrate that XBP1, a master regulator of the unfolded protein response (UPR), regulates learning and memory-related processes. This function of XBP1 in the nervous system involves the control of BDNF expression in the hippocampus.

Highlights

- Cognitive processes activate the IRE1 branch of the UPR pathway in the hippocampus
- The UPR transcription factor XBP1 controls learning and memory-related processes
- Enforced expression of XBP1s in the hippocampus improves spatial memory
- XBP1 controls synaptic plasticity-related genes, including the expression of BDNF

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REFERENCES

Acosta-Alvear, D., Zhou, Y., Blais, A., Tsikitis, M., Lents, N.H., Arias, C., Lennon, C.J., Kluger, Y., and Dynlacht, B.D. (2007). XBP1 controls diverse cell type- and condition-specific transcriptional regulatory networks. Mol. Cell *27*, 53–66.

Alberini, C.M. (2009). Transcription factors in long-term memory and synaptic plasticity. Physiol. Rev. *89*, 121–145.

Bekinschtein, P., Cammarota, M., and Medina, J.H. (2014). BDNF and memory processing. Neuropharmacology *76*, 677–683.

Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., and Silva, A.J. (1994). Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. Cell *79*, 59–68.

Chen, G., Chen, K.S., Knox, J., Inglis, J., Bernard, A., Martin, S.J., Justice, A., McConlogue, L., Games, D., Freedman, S.B., and Morris, R.G. (2000). A learning deficit related to age and beta-amyloid plaques in a mouse model of Alzheimer's disease. Nature *408*, 975–979.

Chen, X., Iliopoulos, D., Zhang, Q., Tang, Q., Greenblatt, M.B., Hatziapostolou, M., Lim, E., Tam, W.L., Ni, M., Chen, Y., et al. (2014). XBP1 promotes triple-negative breast cancer by controlling the HIF1α pathway. Nature *508*, 103–107.

Cheng, P.L., Song, A.H., Wong, Y.H., Wang, S., Zhang, X., and Poo, M.M. (2011). Self-amplifying autocrine actions of BDNF in axon development. Proc. Natl. Acad. Sci. USA *108*, 18430–18435.

Clark, R.E., Broadbent, N.J., and Squire, L.R. (2005). Hippocampus and remote spatial memory in rats. Hippocampus *15*, 260–272.

Clauss, I.M., Chu, M., Zhao, J.L., and Glimcher, L.H. (1996). The basic domain/leucine zipper protein hXBP-1 preferentially binds to and transactivates CRE-like sequences containing an ACGT core. Nucleic Acids Res. *24*, 1855–1864.

Cornejo, V.H., Pihán, P., Vidal, R.L., and Hetz, C. (2013). Role of the unfolded protein response in organ physiology: lessons from mouse models. IUBMB Life *65*, 962–975.

Costa-Mattioli, M., Gobert, D., Harding, H., Herdy, B., Azzi, M., Bruno, M., Bidinosti, M., Ben Mamou, C., Marcinkiewicz, E., Yoshida, M., et al. (2005). Translational control of hippocampal synaptic plasticity and memory by the eIF2alpha kinase GCN2. Nature *436*, 1166–1173.

Costa-Mattioli, M., Gobert, D., Stern, E., Gamache, K., Colina, R., Cuello, C., Sossin, W., Kaufman, R., Pelletier, J., Rosenblum, K., et al. (2007). eIF2alpha phosphorylation bidirectionally regulates the switch from short- to long-term synaptic plasticity and memory. Cell *129*, 195–206.

Costa-Mattioli, M., Sossin, W.S., Klann, E., and Sonenberg, N. (2009). Translational control of long-lasting synaptic plasticity and memory. Neuron *61*, 10–26.

Granger, A.J., and Nicoll, R.A. (2014). Expression mechanisms underlying long-term potentiation: a postsynaptic view, 10 years on. Philos. Trans. R. Soc. Lond. B Biol. Sci. *369*, 20130136.

Hayashi, A., Kasahara, T., Iwamoto, K., Ishiwata, M., Kametani, M., Kakiuchi, C., Furuichi, T., and Kato, T. (2007). The role of brain-derived neurotrophic factor (BDNF)-induced XBP1 splicing during brain development. J. Biol. Chem. *282*, 34525–34534.

Hayashi, A., Kasahara, T., Kametani, M., and Kato, T. (2008). Attenuated BDNF-induced upregulation of GABAergic markers in neurons lacking Xbp1. Biochem. Biophys. Res. Commun. *376*, 758–763.

Hayes, V.Y., Towner, M.D., and Isackson, P.J. (1997). Organization, sequence and functional analysis of a mouse BDNF promoter. Brain Res. Mol. Brain Res. *45*, 189–198.

Hetz, C. (2012). The unfolded protein response: controlling cell fate decisions under ER stress and beyond. Nat. Rev. Mol. Cell Biol. *13*, 89–102.

Hetz, C., and Mollereau, B. (2014). Disturbance of endoplasmic reticulum proteostasis in neurodegenerative diseases. Nat. Rev. Neurosci. *15*, 233–249.

Hetz, C., Lee, A.-H., Gonzalez-Romero, D., Thielen, P., Castilla, J., Soto, C., and Glimcher, L.H. (2008). Unfolded protein response transcription factor XBP-1 does not influence prion replication or pathogenesis. Proc. Natl. Acad. Sci. USA *105*, 757–762.

Hetz, C., Thielen, P., Matus, S., Nassif, M., Court, F., Kiffin, R., Martinez, G., Cuervo, A.M., Brown, R.H., and Glimcher, L.H. (2009). XBP-1 deficiency in the nervous system protects against amyotrophic lateral sclerosis by increasing autophagy. Genes Dev. *23*, 2294–2306.

Hetz, C., Chevet, E., and Harding, H.P. (2013). Targeting the unfolded protein response in disease. Nat. Rev. Drug Discov. *12*, 703–719.

Hetz, C., Chevet, E., and Oakes, S.A. (2015). Proteostasis control by the unfolded protein response. Nat. Cell Biol. *17*, 829–838.

Hu, Y., Park, K.K., Yang, L., Wei, X., Yang, Q., Cho, K.S., Thielen, P., Lee, A.H., Cartoni, R., Glimcher, L.H., et al. (2012). Differential effects of unfolded protein response pathways on axon injury-induced death of retinal ganglion cells. Neuron *73*, 445–452.

Impey, S., Mark, M., Villacres, E.C., Poser, S., Chavkin, C., and Storm, D.R. (1996). Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. Neuron *16*, 973–982.

Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (1996). Guide for the Care and Use of Laboratory Animals (National Academy Press).

Kakiuchi, C., Iwamoto, K., Ishiwata, M., Bundo, M., Kasahara, T., Kusumi, I., Tsujita, T., Okazaki, Y., Nanko, S., Kunugi, H., et al. (2003). Impaired feedback regulation of XBP1 as a genetic risk factor for bipolar disorder. Nat. Genet. *35*, 171–175.

Kakiuchi, C., Ishiwata, M., Umekage, T., Tochigi, M., Kohda, K., Sasaki, T., and Kato, T. (2004). Association of the XBP1-116C/G polymorphism with schizophrenia in the Japanese population. Psychiatry Clin. Neurosci. *58*, 438–440.

Kandel, E.R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. Science *294*, 1030–1038.

Kim, J.J., Rison, R.A., and Fanselow, M.S. (1993). Effects of amygdala, hippocampus, and periaqueductal gray lesions on short- and long-term contextual fear. Behav. Neurosci. *107*, 1093–1098.

Kogan, J.H., Frankland, P.W., Blendy, J.A., Coblentz, J., Marowitz, Z., Schütz, G., and Silva, A.J. (1997). Spaced training induces normal long-term memory in CREB mutant mice. Curr. Biol. *7*, 1–11.

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RESEARCH ARTICLE

Stereotyped responses of *Drosophila* peptidergic neuronal ensemble depend on downstream neuromodulators

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Abstract Neuropeptides play a key role in the regulation of behaviors and physiological responses including alertness, social recognition, and hunger, yet, their mechanism of action is poorly understood. Here, we focus on the endocrine control ecdysis behavior, which is used by arthropods to shed their cuticle at the end of every molt. Ecdysis is triggered by ETH (Ecdysis triggering hormone), and we show that the response of peptidergic neurons that produce CCAP (crustacean cardioactive peptide), which are key targets of ETH and control the onset of ecdysis behavior, depends fundamentally on the actions of neuropeptides produced by other direct targets of ETH and released in a broad paracrine manner within the CNS; by autocrine influences from the CCAP neurons themselves; and by inhibitory actions mediated by GABA. Our findings provide insights into how this critical insect behavior is controlled and general principles for understanding how neuropeptides organize neuronal activity and behaviors.
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Introduction

Understanding how ensembles of neurons produce behaviors is an important aim of neuroscience. The mapping of the neural circuits that underlie a behavior is considered a necessary first step toward this goal, and efforts to determine the 'connectome' of different parts of the nervous system have been present since the beginnings of modern neuroscience. They start with the classical inferred synaptic relationships in Cajal's anatomical analyses (*Ramón y Cajal, 1899*), through the detailed information on the wiring of some invertebrate circuits (e.g., *Carew et al., 1981*; *Comer and Robertson, 2001*; *King, 1976a*, *1976b*), culminating with the complete map of the *Caenorhabditis elegans* central nervous system (CNS) (*White et al., 1986*), and the wiring diagrams of the *Drosophila* optic lobes (*Takemura et al., 2013*) and the mammalian retina (*Helmstaedter et al., 2013*). Yet, research into the functioning of neuronal networks has revealed that a wiring diagram is usually not enough to understand what a neuronal network can do, although it does inform on its possible outcomes. One of the elements that adds tremendous multiplicity to the universe of possible outputs of a neural circuit is the action of neuromodulators, including biogenic amines and neuropeptides. In conjunction with classical transmitters, they can gate the input to a circuit or reconfigure its pattern of activity, thereby causing the same circuit to produce qualitatively different outputs (*Bargmann, 2012*; *Bargmann and Marder, 2013*; *Brezina, 2010*; *Leinwand and Chalasani, 2013*; *Marder, 2012*; *Nusbaum and Blitz, 2012*).

The influence of neuropeptides can be profound and underlies the expression of entire behavioral states, such as hunger and satiation (*Atasoy et al., 2012*; *Chambers et al., 2013*; *Gao and Horvath, 2007*), pair bonding and stress (*Lieberwirth and Wang, 2014*; *Neumann and Landgraf, 2012*), and arousal and attention (*Li et al., 2016*), and can involve many brain regions in addition to sensory and

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WM, JE, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; SD, Provided unpublished Ccap-LexA line, Contributed unpublished essential data or reagents; CW, Drafting or revising the article, Contributed unpublished essential data or reagents

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Supplementary files

- Supplementary file 1. Actual p values for statistical analyses for results reported in main ***Figures 2–9***, and for ***Figure 3—figure supplement 1***.

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References

Arakane Y, Li B, Muthukrishnan S, Beeman RW, Kramer KJ, Park Y. 2008. Functional analysis of four neuropeptides, EH, ETH, CCAP and bursicon, and their receptors in adult ecdysis behavior of the red flour beetle, Tribolium castaneum. *Mechanisms of Development* **125**:984–995. doi: 10.1016/j.mod.2008.09.002, PMID: 18835439

Atasoy D, Betley JN, Su HH, Sternson SM. 2012. Deconstruction of a neural circuit for hunger. *Nature* **488**:172–177. doi: 10.1038/nature11270, PMID: 22801496

Bainbridge SP, Bownes M. 1981. Staging the metamorphosis of Drosophila melanogaster. *Journal of Embryology and Experimental Morphology* **66**:57–80. PMID: 6802923

Baker JD, McNabb SL, Truman JW. 1999. The hormonal coordination of behavior and physiology at adult ecdysis in Drosophila melanogaster. *The Journal of Experimental Biology* **202**:3037–3048. PMID: 10518485

Bargmann CI, Marder E. 2013. From the connectome to brain function. *Nature Methods* **10**:483–490. doi: 10.1038/nmeth.2451, PMID: 23866325

Bargmann CI. 2012. Beyond the connectome: how neuromodulators shape neural circuits. *BioEssays* **34**:458–465. doi: 10.1002/bies.201100185, PMID: 22396302

Brezina V. 2010. Beyond the wiring diagram: signalling through complex neuromodulator networks. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**:2363–2374. doi: 10.1098/rstb.2010.0105

Cao G, Platisa J, Pieribone VA, Raccuglia D, Kunst M, Nitabach MN. 2013. Genetically targeted optical electrophysiology in intact neural circuits. *Cell* **154**:904–913. doi: 10.1016/j.cell.2013.07.027, PMID: 23932121

Carew TJ, Walters ET, Kandel ER. 1981. Classical conditioning in a simple withdrawal reflex in Aplysia californica. *Journal of Neuroscience* **1**:1426–1437. PMID: 7320755

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Effective pore size and radius of capture for K^+ ions in K-channels

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Reconciling protein functional data with crystal structure is arduous because rare conformations or crystallization artifacts occur. Here we present a tool to validate the dimensions of open pore structures of potassium-selective ion channels. We used freely available algorithms to calculate the molecular contour of the pore to determine the effective internal pore radius (r_E) in several K-channel crystal structures. r_E was operationally defined as the radius of the biggest sphere able to enter the pore from the cytosolic side. We obtained consistent r_E estimates for MthK and Kv1.2/2.1 structures, with $r_E = 5.3–5.9$ Å and $r_E = 4.5–5.2$ Å, respectively. We compared these structural estimates with functional assessments of the internal mouth radii of capture (r_C) for two electrophysiological counterparts, the large conductance calcium activated K-channel ($r_C = 2.2$ Å) and the Shaker Kv-channel ($r_C = 0.8$ Å), for MthK and Kv1.2/2.1 structures, respectively. Calculating the difference between r_E and r_C, produced consistent size radii of 3.1–3.7 Å and 3.6–4.4 Å for hydrated K^+ ions. These hydrated K^+ estimates harmonize with others obtained with diverse experimental and theoretical methods. Thus, these findings validate MthK and the Kv1.2/2.1 structures as templates for open BK and Kv-channels, respectively.

K-channel crystal structures reveal that they are machines optimized for efficient and selective K^+ ion transport. Thanks to the groundbreaking work from the Mackinnon lab, we know the structure of several K-channels in detail[1]. A great deal of effort has been invested in building a conceptual scaffold that makes functional sense of these K-channel structures. In the pore domain of K-channels, this scaffold works pretty well for understanding toxin binding and K^+ selectivity. This is partly because the external vestibule and the selectivity filters are experimentally more accessible, and also because of the low variance in atom's space coordinates among different K-channels across diverse crystallization conditions[2–6]. Thus, the structure of the external vestibule together with that of the selectivity filter enjoy a solid functional reputation. By contrast, the internal vestibule of the pore seems to be much less well-defined. On one hand, the dimensions of the cytosolic aspects of the pore structure differ from channel to channel in the various crystal structures[3,5,7,8]. On the other, the internal vestibule seems to be the flexible part of the protein where the voltage controlled gate is located. For example, the structures of KcsA (PDB:1K4C) and Slo2.2 (PDB:5A6E) seem to correspond to channels crystallized in the closed conformation because their pore's internal opening size is smaller than that of a hydrated K^+ [5,9]. Meanwhile, the structures of the bacterial MthK (PDB: 4HYO) and of the mammalian Kv1.2/2.1 paddle chimera (PDB: 2R9R) appear to be those of open channels, with the MthK internal vestibule being ~10 Å wider than that of Kv1.2/2.1[3,7,10–12].

The size difference in the pore internal vestibule in the structure of the MthK vs. the Kv1.2/2.1 chimera structure was somehow electrophysiologically corroborated in their functional counterparts, the mouse large conductance calcium and voltage gated K^+-channel (BK) and the *Drosophila* voltage gated Shaker K^+-channel, respectively. Cysteine substitution scanning accessibility experiments[12,13] in Shaker and BK channels, and also side-chain volume changes in residues located at the internal entrance of BK[14], suggested a pore several angstrom wider than Shaker's. Nevertheless in diffusional determinations of their radii of capture, BK is only ~1.4 Å wider (see below)[15,16]. To what extent do these differences represent structurally different K-channels, or do they just reflect diverse conformations? Are they the result of rarely visited conformational states or are they caused by crystallization artifacts? These questions are especially important when, based on sequence homology or functional properties such as single channel conductance and pharmacology, we use the atomic coordinates of one channel as a structural template for a distantly related one, as is the case when using the structure of MthK to model the BK channel.

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15. Brelidze, T. I. & Magleby, K. L. Probing the geometry of the inner vestibule of BK channels with sugars. *J Gen Physiol* **126,** 105–121 (2005).
16. Diaz-Franulic, I., Sepulveda, R. V., Navarro-Quezada, N., Gonzalez-Nilo, F. & Naranjo, D. Pore dimensions and the role of occupancy in unitary conductance of Shaker K channels. *J Gen Physiol* **146,** 133–146 (2015).
17. Ferry, J. D. Statistical Evaluation of Sieve Constants in Ultrafiltration. *J Gen Physiol* **20,** 95–104 (1936).
18. Andersen, O. S. Ion movement through gramicidin A channels. Studies on the diffusion-controlled association step. *Biophysical journal* **41,** 147–165 (1983).
19. Andersen, O. S. & Procopio, J. Ion movement through gramicidin A channels. On the importance of the aqueous diffusion resistance and ion-water interactions. *Acta physiologica Scandinavica* **481,** 27–35 (1980).
20. Langer, P. Diffusion-limited ion flow through pores. *Biochimica et biophysica acta* **455,** 493–509 (1976).
21. Richards, F. M. Areas, volumes, packing and protein structure. *Annual review of biophysics and bioengineering* **6,** 151–176 (1977).
22. Sanner, M. F., Olson, A. J. & Spehner, J. C. Reduced surface: an efficient way to compute molecular surfaces. *Biopolymers* **38,** 305–320 (1996).
23. Varshney, A., Brooks Jr, F. P. & Wright, W. V. Computing smooth molecular surfaces. *Computer Graphics and Applications, IEEE* **14,** 19–25%@ 0272–1716 (1994).
24. Ho, B. K. & Gruswitz, F. HOLLOW: generating accurate representations of channel and interior surfaces in molecular structures. *BMC structural biology* **8,** 49 (2008).
25. Enderby, J. E. Ion solvation via neutron scattering. *Chemical Society Reviews* **24,** 159–168 (1995).
26. Glezakou, V.-A., Chen, Y., Fulton, J., Schenter, G. & Dang, L. Electronic structure, statistical mechanical simulations, and EXAFS spectroscopy of aqueous potassium. *Theoretical Chemistry Accounts* **115,** 86–99 (2006).
27. Mancinelli, R., Botti, A., Bruni, F., Ricci, M. A. & Soper, A. K. Hydration of Sodium, Potassium, and Chloride Ions in Solution and the Concept of Structure Maker/Breaker. *The Journal of Physical Chemistry B* **111,** 13570–13577 (2007).
28. Bankura, A., Carnevale, V. & Klein, M. L. Hydration structure of salt solutions from ab initio molecular dynamics. *J Chem Phys* **138,** 014501 (2014).
29. Hille, B. *Ion channels of excitable membranes*. 3rd edn, (Sinauer, 2001).
30. Miller, C. See potassium run. *Nature* **414,** 23–24 (2001).
31. Kobertz, W. R. & Miller, C. K+ channels lacking the 'tetramerization' domain: implications for pore structure. *Nature structural biology* **6,** 1122–1125 (1999).
32. Budelli, G., Geng, Y., Butler, A., Magleby, K. L. & Salkoff, L. Properties of Slo1 K+ channels with and without the gating ring. *Proc Natl Acad Sci USA* **110,** 16657–16662 (2013).
33. Jensen, M. O. *et al.* Principles of conduction and hydrophobic gating in K+ channels. *Proc Natl Acad Sci USA* **107,** 5833–5838 (2010).
34. Treptow, W. & Tarek, M. Molecular restraints in the permeation pathway of ion channels. *Biophysical journal* **91,** L26–28 (2006).
35. Parsegian, A. Energy of an ion crossing a low dielectric membrane: solutions to four relevant electrostatic problems. *Nature* **221,** 844–846 (1969).
36. Lee, S. Y., Lee, A., Chen, J. & MacKinnon, R. Structure of the KvAP voltage-dependent K+ channel and its dependence on the lipid membrane. *Proc Natl Acad Sci USA* **102,** 15441–15446 (2005).
37. Jiang, Y. *et al.* X-ray structure of a voltage-dependent K+ channel. *Nature* **423,** 33–41 (2003).
38. Ye, S., Li, Y. & Jiang, Y. Novel insights into K+ selectivity from high-resolution structures of an open K+ channel pore. *Nature structural & molecular biology* **17,** 1019–1023 (2010).

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H.M. and I.D.-F. were involved in all aspects of the project. D.N. supervised the overall study, data analysis and writing. H.M., I.D.-F., F.G.-N. and D.N. contributed to manuscript preparation.

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Sustained Exocytosis after Action Potential-Like Stimulation at Low Frequencies in Mouse Chromaffin Cells Depends on a Dynamin-Dependent Fast Endocytotic Process

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Under basal conditions the action potential firing rate of adrenal chromaffin cells is lower than 0.5 Hz. The maintenance of the secretory response at such frequencies requires a continuous replenishment of releasable vesicles. However, the mechanism that allows such vesicle replenishment remains unclear. Here, using membrane capacitance measurements on mouse chromaffin cells, we studied the mechanism of replenishment of a group of vesicles released by a single action potential-like stimulus (AP_{ls}). The exocytosis triggered by AP_{ls} (ETAP) represents a fraction (40%) of the immediately releasable pool, a group of vesicles highly coupled to voltage dependent calcium channels. ETAP was replenished with a time constant of 0.73 ± 0.11 s, fast enough to maintain synchronous exocytosis at 0.2–0.5 Hz stimulation. Regarding the mechanism involved in rapid ETAP replenishment, we found that it depends on the ready releasable pool; indeed depletion of this vesicle pool significantly delays ETAP replenishment. On the other hand, ETAP replenishment also correlates with a dynamin-dependent fast endocytosis process (τ = 0.53 ± 0.01 s). In this regard, disruption of dynamin function markedly inhibits the fast endocytosis and delays ETAP replenishment, but also significantly decreases the synchronous exocytosis during repetitive AP_{ls} stimulation at low frequencies (0.2 and 0.5 Hz). Considering these findings, we propose a model in where both the transfer of vesicles from ready releasable pool and fast endocytosis allow rapid ETAP replenishment during low stimulation frequencies.

Keywords: membrane capacitance, endocytosis, secretion, Ca^{2+} current, immediately releasable pool, dynamin

Abbreviations: AntiDyn, anti-dynamin monoclonal antibody; APls, action potential like stimulus; C_m, membrane capacitance; ETAP, exocytosis triggered by action potential like stimulus; I_{Ca2+}, calcium currents; IRP, immediately releasable pool; RRP, ready releasable pool; VDCC, voltage dependent calcium channels.

results in rapid ETAP replenishment. On the other hand, inhibition of dynamin would interrupt the fission of partially fused vesicles, blocking fast endocytosis and delaying ETAP replenishment. Although the kiss-and-run mechanism is an attractive hypothesis, from our results, we cannot rule out the possibility that rapid endocytosis recovers membrane to an intermediate, non-releasable compartment, from where mature releasable vesicles are produced. In this direction, an alternative hypothesis was proposed by the groups of Takeshi Sakaba and Ling-Gang Wu in the calyx of Held (Hosoi et al., 2009; Wu and Wu, 2009; Wu et al., 2014). According with that hypothesis, after exocytosis, a fast endocytotic mechanism may facilitate vesicle replenishment by clearance of exocytotic materials from active zones, restoring the structure of the release sites. Simultaneously, the vesicles retrieved by fast endocytosis would recycle to a large recycling pool to prevent vesicle exhaustion, and from where mature releasable vesicles can be produced.

If the rapid replenishment of ETAP is coupled with fast endocytosis, the inhibition of vesicle fission process, by blocking dynamin, should affect the maintenance of synchronous exocytosis during trains of AP_{ls} applied at low frequencies. In agreement with this assumption, the application of the anti-dynamin antibody provoked a significant decrease in synchronous exocytosis during repetitive AP_{ls} stimulation at low frequencies (0.2 and 0.5 Hz, in **Figures 10B,C,** respectively, and **Figures 11A,B,** respectively).

In summary, we found that both the transfer of vesicles from RRP and fast endocytosis contribute to the replenishment of ETAP. It is possible that these two mechanisms represent steps of two independent pathways of vesicle replenishment. In agreement with this hypothesis, there was an additive effect on the inhibition of ETAP replenishment when these two processes were blocked together (**Figure 8Biii**, green diamonds). The residual refilling obtained in this condition might be explained by a not complete depletion of RRP with the double 100 ms depolarization protocol, or a slower replenishment process from upstream pools. However, we cannot rule out the possibility that fast endocytosis and transfer of vesicles from RRP are both single steps of the same path. Future investigations will be necessary to discriminate between these two alternatives.

In stress conditions chromaffin cells fire action potentials at high frequencies, promoting accumulation of cytosolic residual Ca^{2+} and massive exocytosis irrespective of the location of secretory vesicles respect to VDCC (Duan et al., 2003). Such high frequencies favors asynchronous over synchronous exocytosis (Zhou and Misler, 1995). On the opposite side, in rest conditions the firing frequency is low, Ca^{2+} does not accumulate between stimuli, and exocytosis would be limited to vesicles closely coupled to VDCC (Voets et al., 1999; Alvarez and Marengo, 2011). So, it is likely that the physiological importance of IRP resides in its highly efficient stimulus-exocytosis coupling, which allows secretion during action potentials at low rate (Oré and Artalejo, 2005; Cardenas and Marengo, 2016). If this is the case, IRP would need a rapid replenishment mechanism compatible with physiological basal action potential frequencies. The rapid replenishment of ETAP might be the solution of this problem. According to our results, secretory vesicles are replenished rapidly after ETAP depletion ($\tau < 1$ s), increasing the probability of release of the cell in response to a new AP_{ls}. In our experimental conditions the replenishment of ETAP is fast enough to account for an AP_{ls} frequency of 0.2–0.5 Hz, which is approximately the physiological basal frequency in chromaffin cells. However, in chromaffin cells *in situ*, physiological variables, such as hormones, cytokines, and other released transmitters, might modify this process. The understanding of the regulatory mechanisms and variables that control this fast vesicle replenishment process should be the subject of next investigations in the field.

AUTHOR CONTRIBUTIONS

JM-D: conducted animal surgeries, designed and performed experiments, interpreted results, performed statistical analysis and critically revised the manuscript. YÁ: conducted animal surgeries, designed and performed experiments, interpreted results and performed statistical analysis. MM, LB, and AB: conducted animal surgeries, performed experiments, interpreted results and performed statistical analysis. AG-J: conducted animal surgeries, designed and performed experiments, interpreted results, performed statistical analysis and critically revised the manuscript. AC: designed experiments, interpreted results, helped draft parts of the manuscript and critically revised the manuscript. FM: designed experiments, interpreted results, performed statistical analysis, conceived the study, and draft the manuscript. All authors red and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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ANALES

INTERACCIONES GENÉTICO-AMBIENTALES EN EL COMPORTAMIENTO HUMANO

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Resumen: La conducta animal involucra procesos críticos a nivel ecológico, los que tienen implicancias importantes en la evolución de las especies y sus interacciones con el medio ambiente. Para la selección adecuada de las conductas, tales como alimentarse, reproducirse y defenderse, entre otros, el sistema nervioso de los organismos está en una comunicación constante, recibiendo y procesando estímulos y determinando su proceder de acuerdo a dichas claves ambientales. Similares interacciones entre el ambiente y el organismo, en otros niveles, van seleccionando mecanismos de defensa en otras especies, con ejemplos notables de especialización. En el caso de mamíferos superiores, la manifestación de poderío físico entre individuos impacta en la jerarquía social, y sus consecuencias son aún observables en nuestra especie. La capacidad de empatizar con otros seres humanos o teoría de la mente, tiene consecuencias evidentes en cómo nos desenvolvemos a diario. Del mismo modo, el ambiente en que nos desenvolvemos a diario los seres humanos está sobrecargado de estímulos que van impactando nuestro comportamiento, tanto a nivel individual como colectivo. En este trabajo se revisará brevemente el concepto de interacción genético-ambiental.

Palabras claves: genética del comportamiento, biología humana, interacción genético-ambiental.

Abstract: Animal behavior involves critical processes at ecological level, which in turn have implications both on the evolution of species and their interactions with the environment. When properly selecting behaviors such as feeding, reproduction and defense, the individual's nervous system is in constant communication with the environment, receiving and processing stimuli in order to properly act based upon those environmental keys. For example, similar interactions with environment select exquisite defensive mechanisms. In the case of higher mammals, manifestation of physical power and dominance critically impact social hierarchy, consequences that are still observable among humans. Our capability to empathize with others (theory of mind) has obvious implications in the way we live daily. Similarly, the occidental lifestyle overloads our senses in a way that severely impact our individual and social behaviors. In this assay, I will briefly review the concept of gene-environment interactions.

Keywords: Behavioral genetics, Human genetics, Genetic-environmental interaction.

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EL CASO DE SERT, EL TRANSPORTADOR DE SEROTONINA

La serotonina (5-hidroxitriptamina, 5-HT) es un neuromodulador que participa en una plétora de funciones en el sistema nervioso, incluyendo regulación emocional, sueño, percepción, cognición y apetito. Dicha diversidad notable de roles puede explicarse, a grueso modo, por dos características clave del sistema 5-HTergico: a) su organización anatómica, donde sus cuerpos neuronales, agrupados en los núcleos del *rafe* del tronco encefálico, se proyectan a virtualmente todas las regiones del sistema nervioso, y b) la diversidad molecular y distribución celular diferencial de los catorce subtipos de receptores de 5-HT, expresados en el tejido nervioso y otros órganos (Moya, 2013).

Su acción a nivel de la sinapsis es controlada fuertemente por la proteína transportadora SERT, que regula a nivel espacial y temporal la acción de la serotonina sobre sus receptores específicos de membrana, ubicados tanto a nivel pre-sináptico como post-sináptico. Cambios en la actividad de SERT, por lo tanto, afectan los niveles de acción de este neurotransmisor. Dicha actividad puede afectarse tanto por cambios a nivel funcional (que modifiquen la tasa de transporte), como cambios en los niveles de expresión de la proteína (Murphy y Moya, 2011).

SERT es codificado por el gen *SLC6A4* localizado (en humanos) en el cromosoma 17, y está compuesto por catorce exones. La proteína contiene 630 aminoacidos con doce dominios transmembrana (Murphy, *et al.*, 2012).

En 1996, se publicó el primer artículo describiendo un polimorfismo en la región promotor de *SLC6A4*, denominada "región polimórfica asociada al transportador de serotonina" (*5-HT Transporter-Linked Polymorphic Region*, 5-HTTLPR). 5-HTTLPR posee dos variantes diferenciadas por la presencia (*long*, L) o ausencia (*short*, S) de un segmento de 44 pares de bases, ubicadas aproximadamente 1 kb rio arriba del sitio de inicio de la transcripción. Se demostró que los alelos S y L tienen actividad transcripcional diferente: comparado a L, el alelo S es menos eficaz (expresa menos transportador). En dicho trabajo, se encontró que el alelo S de 5-HTTLPR se asoció a neuroticismo, un rasgo de personalidad relacionado a ansiedad y depresión. Dicho trabajo fue una de los hitos fundamentales del inicio de la genética psiquiátrica.

En relación a las interacciones genético-ambientales, el grupo de Richie Poulton describió cómo la influencia de estresores en etapas tempranas del desarrollo humano interactuaba con el polimorfismo 5-HTTLPR en la probabilidad de padecer depresión en adultez. Específicamente, se encontró que en portadores con genotipo SS, la probabilidad de episodios de depresión mayor aumentaba de 0.30 en casos de ausencia de maltrato infantil a un valor superior a 0.60 en casos de maltrato severo. Por su parte, los portadores de genotipo LL no presentaron diferencias en la probabilidad de padecer episodios depresivos, independiente del nivel de maltrato infantil. Esta fue la primera demostración de una interacción GxE en el campo de la psiquiatría (Caspi, *et al.*, 2003).

CONCLUSIONES

El impacto genético sobre los rasgos comportamiento, incluyendo también la vulnerabilidad a trastornos neuropsiquiátricos, han sido claramente establecidas en humanos, así como en otras especies. Indudablemente, el factor ambiental es un componente muy importante, por lo que resulta crucial dilucidar si dichos factores operan, para un determinado rasgo de la conducta, de manera aditiva o efectivamente interactiva, como se ha discutido aquí. En la actualidad, el conocimiento de los procesos epigenéticos que controlan la expresión génica ha avanzado a pasos agigantados, ofreciendo una base mecanistica para, posiblemente, explicar cómo ocurren dichas interacciones. El acceso a tecnologías de secuenciación de bajo costo, además, ofrece un escenario impensado 20 años atrás, para pesquisar variantes genéticas que afecten la vulnerabilidad de los individuos a estresores ambientales. Dicha investigación debiese, sin duda, estar enfocada en proveer al sistema de salud metodologías precisas de pesquisa, y así ofrecer apoyo dirigido a quienes presenten una mayor susceptibilidad a condiciones de vulnerabilidad social, en un esfuerzo mancomunado a nivel país por mejorar las condiciones de crianza, promoción del apego y vinculación afectiva de la infancia.

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Research Article

Inhibition of DNA Methylation Impairs Synaptic Plasticity during an Early Time Window in Rats

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Although the importance of DNA methylation-dependent gene expression to neuronal plasticity is well established, the dynamics of methylation and demethylation during the induction and expression of synaptic plasticity have not been explored. Here, we combined electrophysiological, pharmacological, molecular, and immunohistochemical approaches to examine the contribution of DNA methylation and the phosphorylation of Methyl-CpG-binding protein 2 (MeCP2) to synaptic plasticity. We found that, at twenty minutes after theta burst stimulation (TBS), the DNA methylation inhibitor 5-aza-2-deoxycytidine (5AZA) impaired hippocampal long-term potentiation (LTP). Surprisingly, after two hours of TBS, when LTP had become a transcription-dependent process, 5AZA treatment had no effect. By comparing these results to those in naive slices, we found that, at two hours after TBS, an intergenic region of the RLN gene was hypomethylated and that the phosphorylation of residue S80 of MeCP2 was decreased, while the phosphorylation of residue S421 was increased. As expected, 5AZA affected only the methylation of the RLN gene and exerted no effect on MeCP2 phosphorylation patterns. In summary, our data suggest that tetanic stimulation induces critical changes in synaptic plasticity that affects both DNA methylation and the phosphorylation of MeCP2. These data also suggest that early alterations in DNA methylation are sufficient to impair the full expression of LTP.

1. Introduction

Precise control of gene expression is essential for proper neuronal function and the integrity of the central nervous system [1]. Although several concerted mechanisms work together to control gene transcription [2, 3], DNA methylation has drawn special interest as a cellular mechanism that is capable of adapting gene expression to environmental conditions [4]. Several studies have already established the importance of DNA methylation both during development [5] and in adult animals, with a particularly emphasis on its involvement in learning processes and long-term potentiation (LTP) [6, 7]. However, little is known regarding the mechanisms that regulate DNA methylation and demethylation. This is particularly important in the adult nervous system, where the regulation of transcription can be quite dynamic and require rigorous temporal control [8, 9].

In mammalian genomes, including that of humans, the addition of a methyl group occurs exclusively at a position 5 of the cytosine, located immediately before a guanosine (CpG). An interesting fact is that only neurons, virtually absent in other cell types [10], exhibit multiple CpH methylation sites, where H corresponds to another nucleotides, in a different context to the classical CpG dinucleotide [11]. Fetal brain exhibits very low levels of CpH, which gradually increase with age [12].

Since the first report that showed that neuronal depolarization resulted in the calcium-dependent phosphorylation of MeCP2 and its subsequent release from regulatory regions of genes such as Bdnf [24], remarkable progress has been made in exploring the roles of posttranslational modifications of MeCP2, some of which activate or inhibit transcription [21].

In particular, we studied the phosphorylation of S421, which is selectively expressed in neuronal tissues [3] and is modified by calcium influx and the subsequent activation of calcium/calmodulin-dependent protein kinase IV [44, 53].

Consistent with our data, a recent study showed that a hippocampal-dependent behavioral task increased the phosphorylation of S421 [25]. Although it was thought that the phosphorylation of S421 was related only to its selective detachment to DNA, more detailed genomic distribution analyses of phospho-S421 have revealed that, under both resting and stimulated conditions, MeCP2 is not released from the target sequences in the DNA. Therefore, the additional phosphorylation events that have been described for MeCP2 must necessarily also involve the regulation of DNA binding because neural activity modifies other residues on MeCP2.

Because it has been shown that the dephosphorylation of S80 does not necessarily coincide with the phosphorylation of S421 or vice versa [53], we studied the effect of the S80 residue, which is the most constitutively phosphorylated residue in resting neurons and is dephosphorylated by neuronal activity [44, 53]. In contrast to S421, we found that tetanic stimulation also activates unidentified calcium-dependent phosphatases that dephosphorylate the S80 residue and that this is a critical event during synaptic plasticity [53]. Functionally, the phosphorylation of S80 does not affect the overall subcellular localization of MeCP2, but it has a strong impact on the affinity of this protein for DNA [3, 53].

Finally, the data presented in this work raise a number of new questions that must be addressed in the future, and although the mechanisms by which the azanucleosides inhibit DNA methylation are not fully understood, its use in the future will continue [7, 35, 54] providing valuable information about DNA methylation in synaptic plasticity, learning, and memory.

Competing Interests

The authors have no conflict of interests or financial disclosures to declare.

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References

[1] A. L. Eagle, P. A. Gajewski, and A. J. Robison, "Role of hippocampal activity-induced transcription in memory consolidation," *Reviews in the Neurosciences*, 2016.

[2] E. Benito and A. Barco, "The neuronal activity-driven transcriptome," *Molecular Neurobiology*, vol. 51, no. 3, pp. 1071–1088, 2015.

[3] Z. Zhou, E. J. Hong, S. Cohen et al., "Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth, and spine maturation," *Neuron*, vol. 52, no. 2, pp. 255–269, 2006.

[4] H. M. Abdolmaleky, C. L. Smith, S. V. Faraone et al., "Methylomics in psychiatry: modulation of gene-environment interactions may be through DNA methylation," *American Journal of Medical Genetics B: Neuropsychiatric Genetics*, vol. 127, no. 1, pp. 51–59, 2004.

[5] A. Bird, "DNA methylation patterns and epigenetic memory," *Genes & Development*, vol. 16, no. 1, pp. 6–21, 2002.

[6] J. J. Day, D. Childs, M. C. Guzman-Karlsson et al., "DNA methylation regulates associative reward learning," *Nature Neuroscience*, vol. 16, no. 10, pp. 1445–1452, 2013.

[7] J. M. Levenson, T. L. Roth, F. D. Lubin et al., "Evidence that DNA (cytosine-5) methyltransferase regulates synaptic plasticity in the hippocampus," *The Journal of Biological Chemistry*, vol. 281, no. 23, pp. 15763–15773, 2006.

[8] J. J. Day, A. J. Kennedy, and J. D. Sweatt, "DNA methylation and its implications and accessibility for neuropsychiatric therapeutics," *Annual Review of Pharmacology and Toxicology*, vol. 55, pp. 591–611, 2015.

[9] C. Huidobro, A. F. Fernandez, and M. F. Fraga, "The role of genetics in the establishment and maintenance of the epigenome," *Cellular and Molecular Life Sciences*, vol. 70, no. 9, pp. 1543–1573, 2013.

[10] M. J. Ziller, F. Müller, J. Liao et al., "Genomic distribution and inter-sample variation of non-CpG methylation across human cell types," *PLoS Genetics*, vol. 7, no. 12, Article ID e1002389, 2011.

[11] J. U. Guo, Y. Su, J. H. Shin et al., "Distribution, recognition and regulation of non-CpG methylation in the adult mammalian brain," *Nature Neuroscience*, vol. 17, no. 2, pp. 215–222, 2014.

[12] W. Xie, M. D. Schultz, R. Lister et al., "Epigenomic analysis of multilineage differentiation of human embryonic stem cells," *Cell*, vol. 153, no. 5, pp. 1134–1148, 2013.

[13] A. Bird, M. Taggart, M. Frommer, O. J. Miller, and D. Macleod, "A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA," *Cell*, vol. 40, no. 1, pp. 91–99, 1985.

[14] R. Lister, M. Pelizzola, R. H. Dowen et al., "Human DNA methylomes at base resolution show widespread epigenomic differences," *Nature*, vol. 462, no. 7271, pp. 315–322, 2009.

[15] J. Turek-Plewa and P. P. Jagodziński, "The role of mammalian DNA methyltransferases in the regulation of gene expression," *Cellular and Molecular Biology Letters*, vol. 10, no. 4, pp. 631–647, 2005.

[16] M. G. Goll and T. H. Bestor, "Eukaryotic cytosine methyltransferases," *Annual Review of Biochemistry*, vol. 74, pp. 481–514, 2005.

[17] J. Feng, Y. Zhou, S. L. Campbell et al., "Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons," *Nature Neuroscience*, vol. 13, no. 4, pp. 423–430, 2010.

[18] S. S. Palii, B. O. Van Emburgh, U. T. Sankpal, K. D. Brown, and K. D. Robertson, "DNA methylation inhibitor 5-aza-2′-deoxycytidine induces reversible genome-wide DNA damage that is distinctly influenced by DNA methyltransferases 1 and 3B," *Molecular and Cellular Biology*, vol. 28, no. 2, pp. 752–771, 2008.

[19] O. Mortusewicz, L. Schermelleh, J. Walter, M. C. Cardoso, and H. Leonhardt, "Recruitment of DNA methyltransferase I

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Viewpoint

Pore size matters for potassium channel conductance

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Ion channels are membrane proteins that mediate efficient ion transport across the hydrophobic core of cell membranes, an unlikely process in their absence. K^+ channels discriminate K^+ over cations with similar radii with extraordinary selectivity and display a wide diversity of ion transport rates, covering differences of two orders of magnitude in unitary conductance. The pore domains of large- and small-conductance K^+ channels share a general architectural design comprising a conserved narrow selectivity filter, which forms intimate interactions with permeant ions, flanked by two wider vestibules toward the internal and external openings. In large-conductance K^+ channels, the inner vestibule is wide, whereas in small-conductance channels it is narrow. Here we raise the idea that the physical dimensions of the hydrophobic internal vestibule limit ion transport in K^+ channels, accounting for their diversity in unitary conductance.

The Journal of General Physiology

Introduction

That the charged nature of K^+ ions impairs their free movement across the plasma membrane derives from elementary physics. The calculation of the Born self-energy for K^+ within the low dielectric constant of the membrane shows the nonspontaneity of this process (Parsegian, 1969). Nevertheless, in K^+ channels, nature found a low-energy mechanism to move K^+ ions across the plasma membrane by developing proteins able to mimic its water coordination (Zhou et al., 2001). Thanks to these membrane proteins, K^+ is the most permeable ion in resting cells, and because K^+ is also the most abundant intracellular ion, the resting membrane potential in most living cells is close to the Nernst potential for K^+ (Hodgkin and Huxley, 1952). K^+ channels are probably an ancient protein family and are present in every living being (Armstrong, 2015). These membrane proteins belong to one of the biggest gene families, with ~90 representatives in the mammalian genome (Yu et al., 2005). Their physiological role is widespread: they guard the resting membrane potential, stabilize osmotic imbalance, set the excitability threshold in excitable membranes, and shape the neuronal action potential (Hille, 2001; Armstrong, 2015).

K^+ channels are endowed with an unsurpassed architectural mechanism that allows K^+ ions to permeate selectively across the cell membrane. However, they show a wide variability in unitary conductance (or ion transport rate), which spans approximately two orders of magnitude when measured under similar experimental conditions. In this viewpoint, we propose that the structural determinants for selectivity and conductance are segregated to two structures within the pore of K^+ channels: the selectivity filter and the internal vestibule, respectively. We raise the idea that the structure of the selectivity filter seems to be so dedicated to selective and efficient K^+ transport that it is unlikely to be the structural determinant of conductance diversity. On the contrary, the physical dimensions of the hydrophobic inner vestibule seem to be the factors that limit K^+ transport, accounting for the difference in unitary conductance among K^+ channels.

The structure of the K^+ permeation pathway

K^+ channels allow selective passage of K^+ ions, thermodynamically lured to flow against their own electrochemical gradient, to the exclusion of all other physiological cations. K^+ channels select for K^+ over Na^+ by almost 1,000-fold, a surprising task considering a difference of <0.5 Å between the ionic radii of these two cations (Hille, 1973). Nevertheless, because the difference in their hydration energies is ~16 kcal/mol, removal of the hydration water should be ~10^{10} harder for Na^+ (Robinson and Stokes, 2002). Thus, the simplest explanation for the high K^+ selectivity should involve, at least in part, the need for partial dehydration of the ion, excluding Na^+ because replacing its hydration waters is energetically costlier (Bezanilla and Armstrong, 1972). It has also been argued that the binding sites within the selectivity pore must be precisely shaped around a partially dehydrated K^+ so that it fits snugly (Mullins, 1959). The selectivity sequence of K^+ channels for alkali metal cations ($K^+ \approx Rb^+ > Cs^+ > Na^+ > Li^+$)

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in unitary conductance relevant or just collateral consequences of the protein–protein interaction controlling gating kinetics and inactivation? By dissociating kinetics from conductance phenotypes, these cases present an opportunity to understand how relevant the changes in the unitary conductance are to physiology.

Concluding remarks

K^+ channels are finely tuned to allow the selective passage of K^+ across the membrane at high rates. The channel selectivity filter is in charge of this task, dropping to near zero the energy for ion transfer from the bulk solution (Morais-Cabral et al., 2001). K^+ ions pass across the selectivity filter so efficiently that, even in the largest conductance channels, the physical dimensions of the internal vestibule limit channel conductance (Geng et al., 2011). Thus, we propose that the main difference between large- and small-conductance channels arises from the size of the entrance to the internal pore; large-conductance channels have wider vestibules than do smaller conductance ones. The effect of vestibule size on unitary conductance is clearly nonsteric because it is not proportional to the sectional area available for permeation. Inside narrow aqueous pores, embedded in low dielectric lipid membranes, ions require larger energies to become stabilized, limiting K^+ current (Parsegian, 1969). These equilibrium energy considerations would reduce the conductance gap from approximately two orders of magnitude to one third of the maximal transport rate, corresponding to ~1 kT in activation energy terms. The rest of the difference remains to be accounted for (Díaz-Franulic et al., 2015). Although Kv channels open just wide enough to let hydrated ions enter the permeation pathway, larger-conductance channels would require larger energies to open because of the hydrophobic nature of their inner walls. Thus, larger-conductance channels may host other activation gates as functional and structural data suggest (Zhou et al., 2011; Hite et al., 2015). Because structural determinants for unitary conductance and for electromechanical coupling colocalize toward the cytosolic end of S6, a mutual interference between pore occupancy and gating is expected. Therefore, S6 mutant Kv channelopathies require unitary conductance studies to fully understand their pathophysiology.

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REFERENCES

Aggarwal, S.K., and R. MacKinnon. 1996. Contribution of the S4 segment to gating charge in the *Shaker* K^+ channel. *Neuron.* 16:1169–1177. http://dx.doi.org/10.1016/S0896-6273(00)80145-9

Ahern, C.A., and R. Horn. 2004. Specificity of charge-carrying residues in the voltage sensor of potassium channels. *J. Gen. Physiol.* 123:205–216. http://dx.doi.org/10.1085/jgp.200308993

Alam, A., and Y. Jiang. 2009. High-resolution structure of the open NaK channel. *Nat. Struct. Mol. Biol.* 16:30–34. http://dx.doi.org/10.1038/nsmb.1531

Alcayaga, C., X. Cecchi, O. Alvarez, and R. Latorre. 1989. Streaming potential measurements in Ca2+-activated K+ channels from skeletal and smooth muscle. Coupling of ion and water fluxes. *Biophys. J.* 55:367–371. http://dx.doi.org/10.1016/S0006-3495(89)82814-0

Allen, T.W., O.S. Andersen, and B. Roux. 2004. On the importance of atomic fluctuations, protein flexibility, and solvent in ion permeation. *J. Gen. Physiol.* 124:679–690. http://dx.doi.org/10.1085/jgp.200409111

Andersen, O.S. 1983. Ion movement through gramicidin A channels. Studies on the diffusion-controlled association step. *Biophys. J.* 41:147–165. http://dx.doi.org/10.1016/S0006-3495(83)84416-6

Anishkin, A., and S. Sukharev. 2004. Water dynamics and dewetting transitions in the small mechanosensitive channel MscS. *Biophys. J.* 86:2883–2895. http://dx.doi.org/10.1016/S0006-3495(04)74340-4

Aqvist, J., and V. Luzhkov. 2000. Ion permeation mechanism of the potassium channel. *Nature.* 404:881–884. http://dx.doi.org/10.1038/35009114

Armstrong, C.M. 1971. Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. *J. Gen. Physiol.* 58:413–437. http://dx.doi.org/10.1085/jgp.58.4.413

Armstrong, C.M. 2015. Packaging life: the origin of ion-selective channels. *Biophys. J.* 109:173–177. http://dx.doi.org/10.1016/j.bpj.2015.06.012

Bankura, A., V. Carnevale, and M.L. Klein. 2013. Hydration structure of salt solutions from ab initio molecular dynamics. *J. Chem. Phys.* 138:014501. http://dx.doi.org/10.1063/1.4772761

Barel, O., S.A. Shalev, R. Ofir, A. Cohen, J. Zlotogora, Z. Shorer, G. Mazor, G. Finer, S. Khateeb, N. Zilberberg, and O.S. Birk. 2008. Maternally inherited Birk Barel mental retardation dysmorphism syndrome caused by a mutation in the genomically imprinted potassium channel KCNK9. *Am. J. Hum. Genet.* 83:193–199. http://dx.doi.org/10.1016/j.ajhg.2008.07.010

Batulan, Z., G.A. Haddad, and R. Blunck. 2010. An intersubunit interaction between S4-S5 linker and S6 is responsible for the slow off-gating component in Shaker K^+ channels. *J. Biol. Chem.* 285:14005–14019. http://dx.doi.org/10.1074/jbc.M109.097717

Beckstein, O., and M.S. Sansom. 2004. The influence of geometry, surface character, and flexibility on the permeation of ions and water through biological pores. *Phys. Biol.* 1:42–52. http://dx.doi.org/10.1088/1478-3967/1/1/005

Beckstein, O., P.C. Biggin, P. Bond, J.N. Bright, C. Domene, A. Grottesi, J. Holyoake, and M.S. Sansom. 2003. Ion channel gating: insights via molecular simulations. *FEBS Lett.* 555:85–90. http://dx.doi.org/10.1016/S0014-5793(03)01151-7

Bernèche, S., and B. Roux. 2000. Molecular dynamics of the KcsA K^+ channel in a bilayer membrane. *Biophys. J.* 78:2900–2917. http://dx.doi.org/10.1016/S0006-3495(00)76831-7

ANALES

RESEÑA BIBLIOGRÁFICA SOBRE LA ARAÑA DE RINCÓN (*LOXOSCELES LAETA*, FAM. SICARIIDAE): QUE SABEMOS Y QUE NECESITAMOS SABER

Jesús Olivares Dubart[*]

Resumen: En esta breve revisión, se abordan aspectos de la biología de la araña del rincón (*Loxosceles laeta*), con el fin de tener una visión general sobre lo que sabemos de esta especie y poder enfocar así los esfuerzos en estudiar lo que se desconoce.

Palabras claves: Araña del rincón, Loxosceles laeta, directrices de acción.

Abstract: In this brief review, we addressed aspects on the biology of Chilean recluse spider (*Loxosceles laeta*) in order to have an overview of how much is known about this species and thus focus efforts on studying what is unknown.

Keywords: Chilean recluse spider, Loxosceles laeta, guidelines for action.

INTRODUCCIÓN

Las arañas del rincón pertenecen a la Familia Sicariidae (sub-orden Labidognatha, Orden Araenae, clase Arachnida, Phylum Arthropoda), en la cual se incluyen dos géneros, *Loxosceles* y *Sicarius*, ambos presentes en nuestro país y con una amplia distribución en América, pero también presentes en otros continentes(Binford *et al*., 2008), destacándose en este sentido un origen Gondwanico de la familia, al contar ambos géneros con representantes en Sudamérica y África (Binford, et al., 2008; Gremski, *et al*., 2014). El género *Loxosceles*, por su parte, cuenta con 114 especies descritas alrededor del mundo (World Spider Catalog, 2016) y en Chile por lo menos se han descrito y confirmado la presencia de tres especies: *L. laeta*, *L. rufa* y *L. Coquimbo*, además de sugerirse la posible presencia de *L. rufescens* y *L. rufipes*, lo que a la fecha no se ha confirmado (Tacaure-Rios, 2011).

ANATOMÍA

L. laeta presenta coloración variable que va desde negro hasta el marrón, con un tamaño máximo en los machos de hasta 6 cm si se consideran las patas, con una contextura menos robusta que las hembras, presentan 6 ojos distribuidos en pares que no se conectan entre sí formando una U (uno anterior y dos pares laterales, siendo esta una de las características principales en el reconocimiento de la especie), por detrás de los cuales se observa una figura que recuerda un violín invertido con la base hacia los ojos (Chaim, *et al*., 2011).

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jan por meses sin alimentarse, al ser transportadas accidentalmente por humanos. Además, es muy común observar series de exosqueletos de distintos tamaños junto a una araña adulta de este género, lo que se puede interpretar como que gran parte de su vida la pasan en un mismo lugar.

MORDEDURA Y VENENO

Dadas sus preferencias térmicas, que por un lado contribuyen a determinar su distribución geográfica, la araña de rincón también presenta un aumento de actividad en primavera y verano, lo que genera un aumento de los accidentes que involucran mordeduras por esta especie, recibiendo el cuadro clínico provocado el nombre de Loxoscelismo, envenenamiento asociado a una serie de diversos síntomas clínicos (Chaim, et al., 2011; Canals, *et al.*, 2015b).

Tras una mordedura, frecuentemente se siente dolor y por lo menos un enrojecimiento de la región afectada directamente por la mordedura, a veces con un halo de color morado alrededor de este enrojecimiento inicial, pudiendo llegar a producir una ampolla y una necrosis local, lo que se conoce como cuadro cutáneo, que en caso de complicarse (lo que es muy poco frecuente) y dependiendo de muchos factores, podría generar una necrosis más extensa, o incluso, debido a una cascada de reacciones inflamatorias y la difusión del veneno, podría extenderse el daño a otros órganos lo que se conoce como cuadro visceral, causando entre otros síntomas fiebre, debilidad, vómitos, prurito, falla renal e incluso la ruptura de glóbulos rojos de la sangre (hemólisis) y falla multisistémica (Chaim, *et al.*, 2011).

El veneno producido por esta especie, es una mezcla compleja de toxinas, entre las que se pueden destacar la fosfolipasa D (anteriormente conocida como esfingomielinasa D, la cual presenta acción necrotizante), nucleosidos sulfatados (que tienen efectos de parálisis y fatales en insectos), proteasas (como las astacinas, encargadas de digerir proteínas de superficie), hialuronidasa, factores liberadores de histamina (también conocidas como TCTPs), metaloproteasas, nucleotidasas, colagenasa, esterasas, fosfatasa ácida y alcalina (con un rol en la quimiotaxis, necrosis y agregación plaquetaria) y pequeños péptidos neurotóxicos, aunque a pesar de ser muy conocidos los componentes y sus efectos generales, no se conoce aún en detalle los mecanismos moleculares por medio de los cuales llevan a cabo sus efectos.

Con el fin de contrarrestar los efectos del veneno, tras la confirmación en un centro asistencial de una mordedura por esta especie, se lleva a cabo un tratamiento consistente muchas veces en la aplicación de un suero antiloxoscélico monovalente, importado regularmente en nuestro país para mantener su disponibilidad, sin embargo, un estudio realizado en nuestro país, sugiere que no hay evidencias suficientes que avalen la efectividad del tratamiento con este suero y su efecto podría limitarse a prevenir la aparición de lesiones dermonecróticas o limitar su extensión, resaltando la idea de que es necesario educar a la población respecto a la efectividad real, con el fin de no generar falsas expectativas (Araujo, 2004).

De lo anterior se desprenden las siguientes conclusiones:

1.- No se conoce exactamente la diversidad de especies del género *Loxosceles* presentes en el territorio chileno, sin embargo, se acepta actualmente que son tres las especies identificadas: *L. laeta*, *L. surca* y *L. coquimbo*.

2.- A pesar que se conoce bastante sobre la anatomía de la araña de rincón, no existe información sobre su fisiología sensorial o de la anatomía de su sistema nervioso.

3.- Existe solo un posible predador natural descrito, la especie *Scytodes globula*, sin embargo, su eficacia como tal no es tan relevante por si solo en el control de su potencial presa.

4.- En condiciones favorables, *L. laeta* puede presentar una gran longevidad y dado su bajo metabolismo, puede soportar periodos de hambruna y desecación extensos, sin embargo, no se entiende completamente los mecanismos que subyacen.

5.- No se conoce completamente el mecanismo de acción de las toxinas que componen el veneno y no existe un tratamiento eficaz contra sus efectos.

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Neuron

Report

CaMKII Phosphorylation of TARPγ-8 Is a Mediator of LTP and Learning and Memory

Highlights

- CaMKIIα phosphorylates TARPγ-8 directly at S277 and S281
- TARPγ-8 phosphorylation at CaMKIIα sites is enhanced during chemical LTP
- CaMKIIα enhances AMPAR-mediated transmission via TARPγ-8 phosphorylation sites
- CaMKIIα phosphorylation of TARPγ-8 is required for LTP and learning and memory

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In Brief

Park et al. report hippocampus-enriched TARPγ-8 as a critical CaMKIIα substrate for LTP and learning and memory. LTP increases TARPγ-8 phosphorylation, and this phosphorylation is required for CaMKII-dependent increase of AMPAR-mediated transmission, LTP, and fear conditioning.

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phosphorylation that drive synaptic insertion of AMPARs have emerged (Herring and Nicoll, 2016b). Our findings implicate TARPγ-8 as a key substrate of NMDAR-dependent LTP in the hippocampus. We found that CaMKIIα activity-dependent increase in AMPAR-mediated transmission was significantly impaired when the CaMKIIα phosphorylation sites of TARPγ-8 were disrupted, supporting the notion that TARPγ-8 is a CaMKIIα substrate for AMPAR delivery to synapses during LTP. TARPγ-8 phosphorylation may increase the stability or capture of AMPARs in the PSD. AMPARs interact with PSD-95-like MAGUKs through the PDZ binding motif of TARPs and by this means are stabilized at synapses under basal transmission (Nicoll et al., 2006). An increase in TARP interaction with PSD-95 during LTP was proposed based on the occlusion of LTP by PSD-95 overexpression (Ehrlich et al., 2007; Stein et al., 2003) and the requirement of TARP PDZ binding for CaMKII-mediated diffusional trap of AMPARs (Opazo et al., 2010). However, despite substantial LTP reduction in γ-8 KO mice (Rouach et al., 2005), deletion of the PDZ binding domain (γ-8Δ4) in γ-8Δ4 KI mice showed normal LTP (Sumioka et al., 2011). Furthermore, PSD-95 KO mice showed enhanced LTP (Béïque et al., 2006; Carlisle et al., 2008a; Migaud et al., 1998) with reduction in basal transmission (Béïque et al., 2006; Carlisle et al., 2008a), suggesting that the PDZ binding of TARPγ-8 to PSD-95 is not necessary for LTP. Stabilization or capture of AMPARs during LTP may be mediated by PDZ-independent mechanisms. Phosphorylation-related electrostatic changes of the TARPγ-2 cytoplasmic domain can affect TARP interaction with negatively charged plasma membrane lipids, and dissociation of the TARPγ-2 cytoplasmic domain from lipids upon its phosphorylation is required for its interaction with PSD-95 (Hafner et al., 2015; Sumioka et al., 2010). While TARPγ-8 dissociation from the plasma membrane could be sufficient to trigger AMPAR/TARP complex driving into synapses through lateral diffusion, delivery from an intracellular pool could also contribute (Ahmad et al., 2012; Jurado et al., 2013; Park et al., 2004).

We observed a residual LTP (~40%) in γ-8^{Cm} KI mice that carry disruption of two CaMKIIα phosphorylation sites in TARPγ-8 (i.e., S277 and S281) (Figures 2A–2C). Likewise, a previous study also showed some residual LTP (~26%) in γ-8 KO mice (Rouach et al., 2005). While the 14% difference between γ-8 ^{Cm} KI and γ-8 KO mice could be due to compensation by remaining intact sites, such as S275 (Figure 1C), these results also suggest TARPγ-8-independent mechanisms of LTP. An obvious redundant LTP target is other type I TARP isoforms (γ-2/3/4) that shared phosphorylation sites with γ-8. However, using gene-targeting mice we found unique roles of TARPγ-8 as an LTP substrate among other type I TARPs (Figures 2 and S2). Given that phosphorylation sites are not conserved in the type II TARPγ-5/7 (Kato et al., 2008; Tomita et al., 2005), our results support an additional TARP-independent mechanism underlying the residual LTP observed in TARPγ-8 mutant mice.

There are probably multiple CaMKIIα substrates that are important for the induction and/or expression of LTP. Indeed, recent evidence suggests that, besides components of native AMPAR complex (Granger et al., 2013), SynGAP and Kalirin/Trio family proteins are other potential CaMKII substrates (Araki et al., 2015; Herring and Nicoll, 2016a). Multiple CaMKII phosphorylation sites were identified in both SynGAP (Araki et al., 2015; Carlisle et al., 2008b; Oh et al., 2004) and Kalirin/Tiam1/Trio family proteins (Fleming et al., 1999; Tolias et al., 2005; Xie et al., 2007). Molecular replacement of WT protein with SynGAP mutant proteins carrying mutations in CaMKII phosphorylation sites showed impairments in structural plasticity and chemical LTP in primary neurons (Araki et al., 2015) and in LTP in organotypic slice cultures manipulated with Trio/Kalirin (Herring and Nicoll, 2016a). Although multiple CaMKII substrates for LTP may exist, the present study demonstrates that disruption of only TARPγ-8 phosphorylation impairs LTP and performance in a fear conditioning task. The selective effect of our TARPγ-8 manipulation on plasticity, but not basal transmission, is reminiscent of previous observations in which CaMKIIα was knocked out or its autophosphorylation site (T286A) was mutated (Giese et al., 1998; Silva et al., 1992). Our finding that disrupting major CaMKIIα phosphorylation sites of TARPγ-8 phenocopies CaMKIIα-deficient mice strongly supports the notion that an NMDAR activation/CaMKIIα activation/TARPγ-8 phosphorylation sequence is essential for normal plasticity. Finally, our behavioral results show that TARPγ-8-dependent LTP is particularly important for setting the threshold for fear learning, thus providing additional evidence for LTP as a crucial cellular mechanism underlying learning and memory.

EXPERIMENTAL PROCEDURES

Full Experimental Procedures are available in the Supplemental Experimental Procedures. All data are given as mean ± SEM. Statistical significance between means was calculated using unpaired Student's t test or one-way ANOVA with post hoc Tukey's test. All animal handling was in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Yale University and the Albert Einstein College of Medicine.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.09.002.

AUTHOR CONTRIBUTIONS

S.T. conceived the project. S.T., J.P., and P.E.C. wrote the manuscript. J.P. performed all biochemical and histochemical studies. S.T., M.M.-T., and K.S.K. generated gene-targeting mice and antibodies. P.E.C. supervised and A.E.C. and S.L. designed and performed all electrophysiological recordings. M.R.P. and Y.S.M. supervised and J.P. performed behavior analysis. All authors contributed to the final version of the manuscript.

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Overexpressed Down Syndrome Cell Adhesion Molecule (DSCAM) Deregulates P21-Activated Kinase (PAK) Activity in an In Vitro Neuronal Model of Down Syndrome: Consequences on Cell Process Formation and Extension

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Abstract In humans, Down syndrome (DS) is caused by the presence of an extra copy of autosome 21. The most striking finding in DS patients is intellectual disability and the onset of Alzheimer's disease (AD)-like neuropathology in adulthood. Gene overdose is most likely to underlie both developmental impairments, as well as altered neuronal function in DS. Lately, the disruption of cellular signaling and regulatory pathways has been implicated in DS pathophysiology, and many of such pathways may represent common targets for diverse DS-related genes, which could in turn represent attractive therapeutical targets. In this regard, one DS-related gene Down Syndrome Cell Adhesion Molecule (DSCAM), has important functions in neuronal proliferation, maturation, and synaptogenesis. p21-associated kinases (PAKs) appear as a most interesting possibility for study, as DSCAM is known to regulate the PAKs pathway. Hence, in DS, overexpressed DSCAM could deregulate PAKs activity and affect signaling pathways that regulate synaptic plasticity such as dendritic spine dynamics and axon guidance and growth. In the present work, we used an immortalized cell line derived from the cerebral cortex of an animal model of DS such as the trisomy 16 (Ts16) fetal mouse (named CTb), and a similar cell line established from a normal littermate (named CNh), to study the effect of DSCAM in the PAKs pathway. The present study shows that DSCAM is overexpressed in CTb cells by approximately twofold, compared to CNh cells. Congruently, PAK1, as well as its downstream effectors LIMK and cofilin, stay phosphorylated for longer periods after DSCAM activation in the CTb cells, leading to an altered actin dynamics, expressed as an increased basal F/G ratio and reduced neurite growth, in the trisomic condition. The present work presents the correlation between DSCAM gene overexpression and a dysregulation of the PAK pathway, resulting in altered morphological parameters of neuronal plasticity in the trisomic cell line, namely decreased number and length of processes.

Keywords P21-activated kinases · Trisomy 21 · Down syndrome · DSCAM

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Introduction

Down syndrome (DS) is caused by the presence of an extra copy of autosome 21 (Epstein 1986a, b), and it represents the aneuploidy that most frequently survives gestation. Currently, DS has an incidence of 1/700–1000 live births (Gardiner 2014), and has risen steadily over the last two decades. DS results in various anomalies such as muscular hypotonia, cardiovascular malformations, immunodeficiencies, and increased incidence of leukemia. At present, many of these ailments can be treated to various extents. Yet, the most striking features, which are mental retardation and an early onset of neuropathology similar to that of Alzheimer's disease (AD) (Head et al. 2016; Pulsifer 1996; Oster-Granite 1986), have yet no solution in sight. Further, as the life expectancy of DS patients has increased, the potential effects of the aforementioned AD-related

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peptide C31 could be elevated, further contributing to the increased activity of the PAK pathway. More studies are needed to confirm if this alternative route could also be affecting the dynamics of the actin cytoskeleton and consequently neurite guidance and growth. On the other hand, netrin is known to activate Deleted in Colon Carcinoma (DCC), which also mediates netrin effects on axon outgrowth (Keino-Masu et al. 1996). Yet, DCC is mapped to autosome 18 in both humans and mice, hence a gene dosage hypersensitivity related solely to DCC is unlikely in our trisomic cell line. However, we acknowledge that definite proof of the association of DSCAM with the hypersensitivity of the PAK pathway is needed, and in pursuit of this goal, we are currently restudying these mechanisms after knocking down DSCAM in CTb cells with siRNAs, in order to reduce expression of this protein to levels comparable to those of CNh. Interestingly, DCC and DSCAM interact in a collaborative fashion to regulate actin and microtubule dynamics, via a mechanism related to binding with TUBB3, a very dynamic β-tubulin isoform in neurons (Huang et al. 2015). It is tempting to speculate that such mechanisms may be potentiating the effects herein described on the PAK pathway, a possibility that certainly merits exploration.

Conclusions

Down Syndrome being a disease based on excess gene dosage, a gene therapy approach is largely impractical due to the great number of genes involved. Further, the multiple interactions between gene products that arise in the condition introduce further complications in gene targeting therapy. Hence, the identification of regulatory circuits where several DS-related gene products converge to destabilize their function appears as a most desirable approach. In this regard, the PAK pathway presents several potential and specific therapeutical targets. Further, PAK inhibitors do exist (IPA-3, FRAX486) which could constitute lead compounds to generate pharmacological agents that could revert the imbalance herein reported, and correct a critical neuronal function such as process extension.

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References

Acuña M, Pérez-Nuñez R, Noriega J, Cárdenas AM, Bacigalupo J, Delgado R, Arriagada C, Segura-Aguilar J, Caviedes R, Caviedes P (2012) Altered voltage dependent calcium currents in a neuronal cell line derived from the cerebral cortex of a trisomy 16 fetal mouse, an animal model of Down syndrome. Neurotox Res 22(1):59–68

Allen D, Martin J, Arriagada C, Cárdenas AM, Rapoport SI, Caviedes R, Caviedes P (2000) Impaired cholinergic function in cell lines derived from the cerebral cortex of normal and trisomy 16 mice. Eur J Neurosci 12(9):3259–3264

Arber S, Barbayannis FA, Hanser H, Schneider C, Stanyon CA, Bernard O, Caroni P (1998) Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature 393(6687):805–809

Arias-Romero LE, Chernoff J (2008) A tale of two Paks. Biol Cell 100(2):97–108

Arron JR, Winslow MM, Polleri A, Chang CP, Wu H, Gao X, Neilson JR, Chen L, Heit JJ, Kim SK, Yamasaki N, Miyakawa T, Francke U, Graef IA, Crabtree GR (2006) NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21. Nature 7093:595–600

Ault B, Caviedes P, Rapoport SI (1989) Neurophysiological abnormalities in cultured dorsal root ganglion neurons from the trisomy 16 mouse fetus, a model for Down Syndrome. Brain Res 485:165–170

Bagrodia S, Dérijard B, Davis RJ, Cerione RA (1995) Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. J Biol Chem 270(47): 27995–27998

Bahn S, Mimmack M, Ryan M, Caldwell MA, Jauniaux E, Starkey M, Syndersen CN, Emson P (2002) Neuronal target genes of the neuron-restrictive silencer factor in neurospheres derived from fetuses with Down's syndrome: a gene expression study. Lancet 359(9303):310–315

Belichenko NP, Belichenko PV, Kleschevnikov AM, Salehi A, Reeves RH, Mobley WC (2009) The "Down syndrome critical region" is sufficient in the mouse model to confer behavioral, neurophysiological, and synaptic phenotypes characteristic of Down syndrome. J Neurosci 29(18):5938–5948

Bokoch GM (2003) Biology of the p21-activated kinases. Annu Rev Biochem 72:743–781

Cárdenas AM, Rodríguez MP, Cortés MP, Alvarez RM, Wei W, Rapoport SI, Shimahara T, Caviedes R, Caviedes P (1999) Intracellular calcium signals in immortal cell lines derived from the cerebral cortex of normal and trisomy 16 fetal mice, an animal model of human trisomy 21 (Down syndrome). NeuroReport 10(2):363–369

Caviedes P, Ault B, Rapoport SI (1990) The role of altered sodium currents in the action potential abnormalities of cultured dorsal root ganglion neurons from trisomy 21 (Down syndrome) human fetuses. Brain Res 510:229–236

Caviedes P, Caviedes R, Rapoport SI (2006) Altered calcium currents in cultured sensory neurons of normal and trisomy 16 mouse fetuses, an animal model for human trisomy 21 (Down syndrome). Biol Res 39:471–481

Chen BE, Kondo M, Garnier A, Watson FL, Püettmann-Holgado R, Lamar DR, Schmucker D (2006) The molecular diversity of Dscam is functionally required for neuronal wiring specificity in Drosophila. Cell 125(3):607-620

Chong C, Tan L, Lim L, Manser E (2001) The mechanism of PAK activation. Autophosphorylation events in both regulatory and kinase domains control activity. J Biol Chem 276(20): 17347–17353

Cook M, Bolkan BJ, Kretzschmar D (2014) Increased actin polymerization and stabilization interferes with neuronal function and survival in the AMPKγ mutant Loechrig. PLoS One 9(2):E89847

Delabar JM, Theophile D, Rahmani Z, Chettouh Z, Blouin JL, Prieur M, Noel B, Sinet PM (1993) Molecular mapping of twenty-four

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Charged residues at the first transmembrane region contribute to the voltage dependence of connexins slow gate.

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Running title: Molecular determinants of slow gating in connexins

Keywords: Connexin, Hemichannel, Ion Channel, Electrophysiology, Transmembrane Domain, Cx26, Cx42, Cx46, Cx50 voltage gating, hemichannels, kinetic model

Abstract

Connexins (Cxs) are a family of membrane-spanning proteins that form gap junction channels and hemichannels. Connexin-based channels exhibit two distinct voltage-dependent gating mechanisms termed *slow* and *fast* gating. Residues located at the C-terminus of the first transmembrane segment (TM-1) are important structural component of the slow gate. Here, we determined the role of the charged residues at the end of TM-1 in voltage sensing in Cx26, Cx46 and Cx50. Conductance/voltage curves obtained from tail currents together with kinetics analysis reveal that the fast and slow gates of Cx26 involves the movement of 2 and 4 charges across the electric field, respectively. Primary sequence alignment of different Cxs shows the presence of well-conserved glutamate residues in the C-terminus of TM-1, only Cx26 contains a lysine in that position (lysine-41). Neutralization of lysine-41 in Cx26, increases the voltage dependence of the slow gate. Swapping of lysine-41 with glutamate-42 maintains the voltage dependence. In Cx46, neutralization of negative charges or addition of positive charge in the Cx26 equivalent region reduced the slow gate voltage dependence. In Cx50 the addition of a glutamate in the same region decreased the voltage dependence and the neutralization of a negative charge increased it. These results indicate that the charges at the end of TM-1 are part of the slow gate voltage sensor in Cxs. The fact that Cx42, which has no charge in this region, still presents voltage dependent slow gating suggests that charges still unidentified also contribute to the slow gate voltage sensitivity.

Introduction

Cxs are a family of membrane-spanning proteins involved in intercellular and paracrine/autocrine cellular communication. There are 21 human Cx genes that are widely expressed in virtually all human tissues (1). The topology of Cxs involves four trans-membrane (TM) segments connected through two extracellular loops (EL) and one intracellular loop (IL), and the amino- and carboxy-terminals are facing the cytoplasm

Voltage gating process on the pore

In this work we support the idea that Cxs sense the voltage drop across the pore of the channel through charges present in the NT and at the end of the TM-1 segment.

The mechanism of voltage gating in the pore is not unique to Cxs. The KcsA channel, which is a bacterial K^+ channel that contains only a pore domain comprised of two transmembrane segments (51), show a voltage-dependent gating of around 0.7 e_0 (52). This voltage dependence is eliminated when glutamate-71, which is a residue at the selectivity filter, is neutralized (52). Nevertheless, this glutamate does not seem to be exposed to the solvent like residues that mediate voltage dependence in Cxs. On the other hand, several voltage sensor domains can act as ion conducting pores. This is the case for proton currents generated when a basic residue in the S4 segment (R371) of the *Shaker* K^+ channel is mutated to histidine (53), or in the voltage gated Hv1 proton channel (54). In both cases, the activation of the voltage sensor and the opening of the ion conducting pathway are merged. This might also be the case for Cx channels.

Conclusion

Our work has shown that residues at the end of the TM-1 contribute to the voltage dependence of the slow gating mechanism of Cx channels. However, the exact contribution in each Cx cannot be defined *a priori*. Other residues involved in the voltage dependence of the slow gate are yet to be described.

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Conflict of interest

The authors declare that they have no conflicts of interest regarding the contents of this article.

Author contributions

BP, RL and CG, conceived and coordinated the study, and designed the experiments. BP, AP, IEG performed and analyzed the experimental data. BP, CG, RL AP and IEG interpreted the experimental data and formulated the kinetic model. All authors co-wrote and approved the final version of the manuscript.

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Linoleic acid permeabilizes gastric epithelial cells by increasing connexin 43 levels in the cell membrane via a GPR40- and Akt-dependent mechanism

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ABSTRACT

Linoleic acid (LA) is known to activate G-protein coupled receptors and connexin hemichannels (Cx HCs) but possible interlinks between these two responses remain unexplored. Here, we evaluated the mechanism of action of LA on the membrane permeability mediated by Cx HCs in MKN28 cells. These cells were found to express connexins, GPR40, GPR120, and CD36 receptors. The Cx HC activity of these cells increased after 5 min of treatment with LA or GW9508, an agonist of GPR40/GPR120; or exposure to extracellular divalent cation–free solution (DCFS), known to increase the open probability of Cx HCs, yields an immediate increase in Cx HC activity of similar intensity and additive with LA-induced change. Treatment with a CD36 blocker or transfection with siRNA-GPR120 maintains the LA-induced Cx HC activity. However, cells transfected with siRNA-GPR40 did not show LA-induced Cx HC activity but activity was increased upon exposure to DCFS, confirming the presence of activatable Cx HCs in the cell membrane. Treatment with AKTi (Akt inhibitor) abrogated the LA-induced Cx HC activity. In HeLa cells transfected with Cx43 (HeLa-Cx43), LA induced phosphorylation of surface Cx43 at serine 373 (S373), site for Akt phosphorylation. HeLa-Cx43 but not HeLa-Cx43 cells with a S373A mutation showed a LA-induced Cx HC activity directly related to an increase in cell surface Cx43 levels. Thus, the increase in membrane permeability induced by LA is mediated by an intracellular signaling pathway activated by GPR40 that leads to an increase in membrane levels of Cx43 phosphorylated at serine 373 via Akt.

1. Introduction

Connexin (Cx) proteins are encoded by a gene family composed by 21 and 20 members in human and mouse, respectively [1,2], and they are assembled into hexamers to form hemichannels (HCs) or connexons, and dodecamers to form gap junction channels (GJCs) connecting two cells. HCs can act as relatively non-selective channels and are found at the cell surface of most vertebrate cells [1,3–5]. HCs are often involved in paracrine and autocrine cellular signaling, being membrane pathways for releasing signaling molecules (e.g., ATP, NAD^+ and glutamate) to the extracellular space [3]. Different tissues express different Cxs depending on their developmental and physiologic state, and they are named alphanumerically with the prefix Cx followed by the molecular mass of the human family member in kilodaltons (e.g., Cx43) [1,6].

Experimental evidence suggests that functional HCs and GJCs are involved in physiological and pathophysiological cell responses. In the gastrointestinal system, they might participate as paracellular permeability pathway in intestinal epithelial cells [7] or intestinal innate immune defense [8]. Moreover, in tanycytes and skeletal muscle, Cx43 HCs or pannexin 1 channels (Panx1 Chs), respectively, allow diffusional entry of molecules such as glucose [9,10], so they could be thought of as membrane pathways for cellular uptake of nutrients. At least 7 different Cx variants have been described in the human gastrointestinal system [11], with the three Cxs found in gastric cells being connexin 26 (Cx26), Cx32 and Cx43 [12]. The functional roles of these Cxs are poorly understood but one proposed by Guttman et al. [13], postulated that Cx43 HCs present in the intestine are involved in transport of water, at least under pathological conditions such as in diarrhea caused by bacterial infections. Also, the expression of Cx26 is associated with intestinal

Abbreviations: Cx, connexin; DCFS, divalent cation-free solution; Etd^+, ethidium; FFA, free fatty acid; GPCR, G-protein coupled receptor; GPR40, G-protein receptor 40; HC, hemichannel; LA, linoleic acid.

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effects of LA have been associated mainly with inflammatory conditions [35,55]. Since the cells expressing Cx43-S373A do not respond to metabolic inhibition, a proinflammatory condition that mimics hypoxia-reoxygenation [37], most likely the physiological or pathophysiological outcome of LA on cells via Cx HCs will depend on the intensity and duration of the stimulus; high concentrations or long term action of an effective LA concentration could lead to Ca^{2+} overload and thus degenerative mechanisms that activate an inflammatory response.

Our results lead us to propose that LA increases the Cx43 HC activity through activation of a signaling pathway that involves the membrane receptor GPR40 and AKT kinase activity. Then, the phosphorylation of Cx43 in serine 373 by AKT promotes an increase in the number of Cx43 HC at the cell surface, which increases the open probability of Cx43 HCs via a mechanism that is independent of extracellular $[Ca^{2+}]$.

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Transparency document

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

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References

[1] P. Bedner, C. Steinhäuser, M. Theis, Functional redundancy and compensation among members of gap junction protein families? Biochim. Biophys. Acta 1818 (2012) 1971–1984, http://dx.doi.org/10.1016/j.bbamem.2011.10.016.
[2] G. Söhl, K. Willecke, Gap junctions and the connexin protein family, Cardiovasc. Res. 62 (2004) 228–232.
[3] J.C. Sáez, M.A. Retamal, D. Basilio, F.F. Bukauskas, M.V. Bennett, Connexin-based gap junction hemichannels: gating mechanisms, Biochim. Biophys. Acta 1711 (2005) 215–224.
[4] E. De Vuyst, E. Decrock, M. De Bock, H. Yamasaki, C.C. Naus, W.H. Evans, L. Leybaert, Connexin hemichannels and gap junction channels are differentially influenced by lipopolysaccharide and basic fibroblast growth factor, *Mol. Biol. Cell* 18 (2007) 34–46.
[5] M.A. Retamal, N. Froger, N. Palacios-Prado, P. Ezan, P.J. Sáez, J.C. Sáez, C. Giaume, Cx43 hemichannels and gap junction channels in astrocytes are regulated oppositely by proinflammatory cytokines released from activated microglia, J. Neurosci. 27 (2007) 13781–13792.
[6] M. Rackauskas, V. Neverauskas, V.A. Skeberdis, Diversity and properties of connexin gap junction channels, Medicina(Kaunas) 46 (2010) 1–12.
[7] H. Morita, T. Katsuno, A. Hoshimoto, N. Hirano, Y. Saito, Y. Suzuki, Connexin 26-mediated gap junctional intercellular communication suppresses paracellular permeability of human intestinal epithelial cell monolayers, Exp. Cell Res. 298 (2004) 1–8.
[8] B. Ey, A. Eyking, G. Gerken, D.K. Podolsky, E. Cario, TLR2 mediates gap junctional intercellular communication through connexin-43 in intestinal epithelial barrier injury, J. Biol. Chem. 284 (2009) 22332–22343, http://dx.doi.org/10.1074/jbc.M901619200.
[9] J.A. Orellana, P.J. Sáez, C. Cortés-Campos, R.J. Elizondo, K.F. Shoji, S. Contreras-Duarte, V. Figueroa, V. Velarde, J.X. Jiang, F. Nualart, J.C. Sáez, M.A. García, Glucose increases intracellular free Ca(2+) in tanycytes via ATP released through connexin 43 hemichannels, *Glia* 60 (2012) 53–68, http://dx.doi.org/10.1002/glia.21246.
[10] M.A. Riquelme, L.A. Cea, J.L. Vega, M.P. Boric, H. Monyer, M.V. Bennett, M. Frank, K. Willecke, J.C. Sáez, The ATP required for potentiation of skeletal muscle contraction is released via pannexin hemichannels, *Neuropharmacology* 75 (2013) 594–603, http://dx.doi.org/10.1016/j.neuropharm.2013.03.022.
[11] M. Maes, B. Cogliati, S. Crespo-Yanguas, J. Willebrords, M. Vinken, Roles of connexins and pannexins in digestive homeostasis, Cell. Mol. Life Sci. 72 (2015) 2809–2821, http://dx.doi.org/10.1007/s00018-015-1961-8.
[12] M. Maes, S. Crespo-Yanguas, J. Willebrords, B. Cogliati, M. Vinken, Connexin and pannexin signaling in gastrointestinal and liver disease, Transl. Res. S1931-5244 (15) (2015) 00172–00173, http://dx.doi.org/10.1016/j.trsl.2015.05.005.
[13] J.A. Guttman, A.E. Lin, Y. Li, J. Bechberger, C.C. Naus, A.W. Vogl, B.B. Finlay, Gap junction hemichannels contribute to the generation of diarrhoea during infectious enteric disease, Gut 59 (2010) 218–226, http://dx.doi.org/10.1136/gut.2008.170464.
[14] X. Liu, T. Furuya, D. Li, J. Xu, X. Cao, Q. Li, J. Xu, Z. Xu, K. Sasaki, X. Liu, Connexin 26 expression correlates with less aggressive phenotype of intestinal type-gastric carcinomas, Int. J. Mol. Med. 25 (2010) 709–716.
[15] Y. Wang, L.H. Huang, C.X. Xu, J. Xiao, L. Zhou, D. Cao, X.M. Liu, Y. Qi, Connexin 32 and 43 promoter methylation in helicobacter pylori-associated gastric tumorigenesis, World J. Gastroenterol. 20 (2014) 11770–11779, http://dx.doi.org/10.3748/wjg.v20.i33.11770.
[16] T. Reinehr, C.L. Roth, The gut sensor as regulator of body weight, Endocrine 49 (2015) 35–50, http://dx.doi.org/10.1007/s12020-014-0518-1.
[17] S. Aikawa, T. Hashimoto, K. Kano, J. Aoki, Lysophosphatidic acid as a lipid mediator with multiple biological actions, J. Biochem. 157 (2015) 81–89, http://dx.doi.org/10.1093/jb/mvu077.
[18] J.F. Glatz, Lipids and lipid binding proteins: a perfect match, Prostaglandins Leukot. Essent. Fat. Acids 93 (2015) 45–49, http://dx.doi.org/10.1016/j.plefa.2014.07.011.
[19] T. Hara, A. Hirasawa, A. Ichimura, I. Kimura, G. Tsujimoto, Free fatty acid receptors FFAR1 and GPR120 as novel therapeutic targets for metabolic disorders, *J. Pharm. Sci.* 100 (2011) 3594–3601, http://dx.doi.org/10.1002/jps.22639.
[20] G. Milligan, T. Ulven, H. Murdoch, B.D. Hudson, G-protein-coupled receptors for free fatty acids: nutritional and therapeutic targets, Br. J. Nutr. 111 (2014) S3–S7 doi: 10.1017/S0007114513002249.
[21] M.A. Retamal, F. Evangelista-Martínez, C.G. León-Paravic, G.A. Altenberg, L. Reuss, Biphasic effect of linoleic acid on connexin 46 hemichannels, Pflugers Arch. 461 (2011) 635–643, http://dx.doi.org/10.1007/s00424-011-0936-3.
[22] B. Kiec-Wilk, U. Czech, K. Janczarska, A. Knapp, J. Goralska, U. Cialowicz, M.T. Malecki, A. Dembinska-Kiec, Connexin 43 and metabolic effect of fatty acids in stressed endothelial cells, Genes Nutr. 7 (2012) 257–263, http://dx.doi.org/10.1007/s12263-011-0247-5.
[23] A. Marandykina, N. Palacios-Prado, L. Rimkutė, V.A. Skeberdis, F.F. Bukauskas, Regulation of connexin36 gap junction channels by n-alkanols and arachidonic acid, J. Physiol. 591 (2013) 2087–2101, http://dx.doi.org/10.1113/jphysiol.2013.250910.
[24] V. Figueroa, P.J. Sáez, J.D. Salas, D. Salas, O. Jara, A.D. Martínez, J.C. Sáez, M.A. Retamal, Linoleic acid induces opening of connexin26 hemichannels through a PI3K/Akt/Ca(2+)-dependent pathway, Biochim. Biophys. Acta 1828 (2013) 1169–1179, http://dx.doi.org/10.1016/j.bbamem.2012.12.006.
[25] Y. Itoh, Y. Kawamata, M. Harada, M. Kobayashi, R. Fujii, S. Fukusumi, K. Ogi, M. Hosoya, Y. Tanaka, H. Uejima, H. Tanaka, M. Maruyama, R. Satoh, S. Okubo, H. Kizawa, H. Komatsu, F. Matsumura, Y. Noguchi, T. Shinohara, S. Hinuma, Y. Fujisawa, M. Fujino, Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40, Nature 422 (2003) 173–176.
[26] A. Hirasawa, K. Tsumaya, T. Awaji, S. Katsuma, T. Adachi, M. Yamada, Y. Sugimoto, S. Miyazaki, G. Tsujimoto, Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120, Nat. Med. 11 (2005) 90–94.
[27] A. Ichimura, A. Hirasawa, T. Hara, G. Tsujimoto, Free fatty acid receptors act as nutrient sensors to regulate energy homeostasis, Prostaglandins Other Lipid Mediat. 89 (2009) 82–88, http://dx.doi.org/10.1016/j.prostaglandins.2009.05.003.
[28] A. Hirasawa, T. Hara, S. Katsuma, T. Adachi, G. Tsujimoto, Free fatty acid receptors and drug discovery, Biol. Pharm. Bull. 31 (2008) 1847–1851.
[29] C. Martin, M. Chevrot, H. Poirier, P. Passilly-Degrace, I. Niot, P. Besnard, CD36 as a lipid sensor, Physiol Behav. 30;105 (1) (2011) 36–42, http://dx.doi.org/10.1016/j.physbeh.2011.02.029.
[30] D. Naville, A. Duchampt, M. Vigier, D. Oursel, R. Lessire, H. Poirier, I. Niot, M. Bégeot, P. Besnard, G. Mithieux, Link between intestinal CD36 ligand binding and satiety induced by a high protein diet in mice, PLoS ONE 7 (1) (2012), e30686, http://dx.doi.org/10.1371/journal.pone.0030686.
[31] M.H. Ozdener, S. Subramaniam, S. Sundaresan, O. Sery, T. Hashimoto, Y. Asakawa, P. Besnard, N.A. Abumrad, N.A. Khan, CD36- and GPR120-mediated Ca2+ signaling in human taste bud cells mediates differential responses to fatty acids and is altered in obese mice, Gastroenterology 146 (2014) 995–1005.
[32] S.M. Innis, Human milk: maternal dietary lipids and infant development, Proc. Nutr. Soc. 66 (2007) 397–404.
[33] G. Schmitz, J. Ecker, The opposing effects of n−3 and n−6 fatty acids, Prog. Lipid Res. 47 (2008) 147–155, http://dx.doi.org/10.1016/j.plipres.2007.12.004.
[34] G. Barceló-Coblijn, E.J. Murphy, Alpha-linolenic acid and its conversion to longer chain n−3 fatty acids: benefits for human health and a role in maintaining tissue n−3 fatty acid levels, Prog. Lipid Res. 48 (2009) 355–374, http://dx.doi.org/10.1016/j.plipres.2009.07.002.
[35] Z. Yang, S.L. Huffman, Modelling linoleic acid and α-linolenic acid requirements for infants and young children in developing countries, Matern. Child. Nutr. 9 (Suppl. 1) (2013) 72–77, http://dx.doi.org/10.1111/j.1740-8709.2012.00448.x.
[36] C.A. Dunn, P.D. Lampe, Injury-triggered akt phosphorylation of Cx43: a ZO-1-driven molecular switch that regulates gap junction size, J. Cell Sci. 127 (2014) 455–464, http://dx.doi.org/10.1242/jcs.142497.
[37] D. Salas, C. Puebla, P.D. Lampe, S. Lavandero, J.C. Sáez, Role of Akt and Ca2+ on cell permeabilization via connexin43 hemichannels induced by metabolic inhibition, Biochim. Biophys. Acta 1852 (2015) 1268–1277, http://dx.doi.org/10.1016/j.bbadis.2015.03.004.
[38] L.E. Wroblewski, L. Shen, S. Ogden, J. Romero-Gallo, L.A. Lapierre, D.A. Israel, J.R. Turner, R.M. Peek Jr., Helicobacter pylori dysregulation of gastric epithelial tight junctions by urease-mediated myosin II activation, Gastroenterology 136 (2009) 236–246, http://dx.doi.org/10.1053/j.gastro.2008.10.011.
[39] H. Li, T.F. Liu, A. Lazrak, C. Peracchia, G.S. Goldberg, P.D. Lampe, R.G. Johnson, Properties and regulation of gap junctional hemichannels in the plasma membranes of cultured cells, J. Cell Biol. 134 (1996) 1019–1030.
[40] J.M. Gómez-Hernández, M. de Miguel, B. Larrosa, D. González, L.C. Barrio, Molecular basis of calcium regulation in connexin-32 hemichannels, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 16030–16035.

PNAS PLUS

NEUROSCIENCE

Molecular mechanism of Zn^{2+} inhibition of a voltage-gated proton channel

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Voltage-gated proton (Hv1) channels are involved in many physiological processes, such as pH homeostasis and the innate immune response. Zn^{2+} is an important physiological inhibitor of Hv1. Sperm cells are quiescent in the male reproductive system due to Zn^{2+} inhibition of Hv1 channels, but become active once introduced into the low-Zn^{2+}-concentration environment of the female reproductive tract. How Zn^{2+} inhibits Hv1 is not completely understood. In this study, we use the voltage clamp fluorometry technique to identify the molecular mechanism of Zn^{2+} inhibition of Hv1. We find that Zn^{2+} binds to both the activated closed and resting closed states of the Hv1 channel, thereby inhibiting both voltage sensor motion and gate opening. Mutations of some Hv1 residues affect only Zn^{2+} inhibition of the voltage sensor motion, whereas mutations of other residues also affect Zn^{2+} inhibition of gate opening. These effects are similar in monomeric and dimeric Hv1 channels, suggesting that the Zn^{2+}-binding sites are localized within each subunit of the dimeric Hv1. We propose that Zn^{2+} binding has two major effects on Hv1: (*i*) at low concentrations, Zn^{2+} binds to one site and prevents the opening conformational change of the pore of Hv1, thereby inhibiting proton conduction; and (*ii*) at high concentrations, Zn^{2+}, in addition, binds to a second site and inhibits the outward movement of the voltage sensor of Hv1. Elucidating the molecular mechanism of how Zn^{2+} inhibits Hv1 will further our understanding of Hv1 function and might provide valuable information for future drug development for Hv1 channels.

Hv1 | voltage-gated proton channel | Zn^{2+} | inhibition | molecular model

Voltage-gated proton (Hv1) channels are depolarization-activated channels that are highly selective for protons. Hv1 channel currents were first identified in snail neurons where they are thought to be important for acid extrusion to maintain physiological intracellular pH (1). In the immune system, proton currents through Hv1 channels compensate for the electrogenic currents and reduce the intracellular acidification caused by the activity of NADPH oxidase in human neutrophil (2–5) and lung airway epithelial cells (6). Hv1 channels in human microglia play an important role in NADPH oxidase-mediated brain damage in ischemic stroke (7). Hv1 channels are also found abundantly in human sperm cells, and the activation of Hv1 channels alkalizes the sperm cells during spermatozoa activation (8).

Hv1 channels belong to the superfamily of voltage-gated cation channels. However, in contrast to other voltage-gated cation channels, such as voltage-gated potassium (Kv) channels, which have six transmembrane (TM) segments per subunit and form tetramers, Hv1 has only four TM segments per subunit and forms dimers. The four TM segments in an Hv1 subunit are homologous to the first four TM segments of a Kv subunit that form the voltage-sensing domain in Kv channels. In contrast to Kv channels, which have a common pore formed by the assembly of the last two TM segments from all four subunits, each subunit of Hv1 contains a pore (9, 10).

Zn^{2+} is the most potent physiological inhibitor of Hv1 channels (11). Zn^{2+} is an essential mineral that is naturally present in our bodies. Zn^{2+} deficiency can cause severe mental retardation and immune dysfunction (12, 13), whereas excess Zn^{2+} accumulation leads to neurotoxicity and neurodegeneration (14). In addition to affecting a myriad of Zn^{2+}-binding enzymes, Zn^{2+} inhibits Hv1, voltage-gated sodium (Nav), potassium (Kv), and calcium (Cav) channels (15–20). Sperm cells are quiescent in the male reproductive tract where high concentrations of Zn^{2+} inhibit Hv1. This helps the maturation of sperm cells in the male reproductive system. Sperm cells become active once introduced into the low-Zn^{2+} concentrations in the female reproductive tract by the removal of Zn^{2+} inhibition of Hv1 channels. Active Hv1 channels alkalize the cytosol, which activates CatSperm calcium channels and initiates sperm motility (8). Despite the importance of the inhibitory effect of Zn^{2+} on Hv1 channels, the molecular mechanism of how Zn^{2+} inhibits Hv1 is not completely understood.

The pH dependence of the Zn^{2+} inhibition of Hv1 suggests that Zn^{2+} is coordinated by several residues with a pK_a similar to histidine residues (11). The Zn^{2+} sensitivity is profoundly attenuated when two extracellular histidines are mutated to alanines in human Hv1 channels (21). Musset et al. proposed that two Zn^{2+} ions are bound at the interface of two Hv1 subunits in the dimeric Hv1 channel and that each Zn^{2+} is coordinated by two histidines, one from each of the two subunits (22). Recently, an X-ray crystal structure of the voltage-gated proton channel was reported with a Zn^{2+} bound to a single subunit (23). Zn^{2+} is coordinated by several charged acidic residues of Hv1, in addition to the two previously proposed histidines. Therefore, the location of the Zn^{2+}-binding site is still under debate. Here we compare the Zn^{2+} effect on both wild-type and mutant Hv1 channels in dimeric and monomeric forms to clarify the location of Zn^{2+} binding, the mechanism of Zn^{2+} inhibition, and the contribution of different amino acid residues to Zn^{2+} binding.

Significance

Zn^{2+} inhibition of voltage-gated proton (Hv1) channels has important physiological roles, such as quiescence of sperm in the male reproductive system. Here, we show that Zn^{2+} binds to different states of Hv1, and we propose a possible mechanism for Zn^{2+} inhibition of Hv1. Several residues are found to be involved in Zn^{2+} binding, and we provide detailed information about how these residues contribute to the functional effect of Zn^{2+} binding. This study provides valuable information for future drug development for Hv1 channels.

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within each subunit. A previous study suggested that Zn^{2+} binds at the interface between two Hv1 subunits because mutations in only one of the two Hv1 subunits in a linked Hv1 dimer had specific effects on the Zn^{2+} inhibition of the Hv1 currents (22). However, the two subunits in the dimeric Hv1 activate cooperatively (10, 24, 26). Therefore, Zn^{2+} binding in one subunit of the Hv1 dimer could affect cooperative channel opening in both subunits. Conversely, if Zn^{2+} binding is impaired in one subunit, this might impact Zn^{2+} binding in the other subunit, as has been seen with other Hv1 blockers (33), or the functional effect of Zn^{2+} binding in the other subunit on the cooperative channel opening transition. More studies are clearly needed to understand Zn^{2+} inhibition and cooperative opening. The Zn^{2+} inhibitory effect in the monomer is slightly lower than in the dimer, especially for the inhibition of S4 movement (cf. Figs. 1 and 6). One possible explanation for this is that the dimerization alters slightly the structure of each subunit, so that there is a slightly higher affinity of Zn^{2+} in the dimeric Hv1 than in the monomeric Hv1.

In conclusion, our study showed that Zn^{2+} is coordinated mainly by residues within each subunit of *Ci*-Hv1. The negatively charged residues D160, E167, D233, and H188 in the middle and at the extracellular end of S1 and S3 are important for Zn^{2+} binding to site 2 in the resting closed state and prevent outward S4 movement upon membrane depolarization. H188, D233, and E167, in addition, seem to contribute to Zn^{2+} binding to site 1 that prevents channel opening. We have shown here that Zn^{2+} can inhibit proton currents by binding to either the activated closed or resting closed state of the Hv1 channel, providing several conformational states that can be used as platforms for designing inhibitors for Hv1.

Methods

Mutagenesis and Expression of *Ci*-Hv1 Channels. We performed site-directed mutagenesis, in vitro transcription of cRNA, and injection of cRNA encoding the Ci-VSOP (here called *Ci*-Hv1) into *Xenopus laevis* oocytes as described previously (26). The ΔNΔC *Ci*-Hv1 was constructed with a stop codon at Val270 and initiator methionine replacing Glu129 (26).

Two-Electrode Voltage Clamp and VCF Recordings. We performed VCF experiments as described previously (26). Briefly, we labeled oocytes for 30 min with 100 μM Alexa-488 maleimide (Molecular Probes) in Na^+ Ringer's solution. Fluorescence was monitored through a FITC filter cube: exciter, HQ480/40; dichroic, Q505LP; and emitter, HQ535/50. Fluorescence intensities were low-pass-filtered at 200–500 Hz and digitized at 1 kHz. We performed two-electrode voltage clamp (TEVC) recordings as described earlier. Solutions for TEVC contained 88 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 100 mM Hepes (pH = 7.4). We injected oocytes with 50 nL of 1 M Hepes (pH = 7.0) to minimize pH changes due to the proton currents. This results in ~100 mM Hepes in the cytosol. We also added 100 mM Hepes (pH = 7.4) to the external solutions for these recordings. Currents were leak subtracted off-line, assuming ohmic leak and using currents from potentials between −80 and −40 mV. Solutions were applied with a gravity-driven perfusion system with a solution exchange time of <2 s.

BCECF Measurements. We incubated the oocytes with 50 μM BCECF dye (Life Technologies) in ND96 solutions for 30 min and washed before recording. Fluorescence was monitored through a FITC filter cube: exciter, HQ480/40; dichroic, Q505LP; and emitter, HQ535/50. Fluorescence intensities were low-pass-filtered at 200–500 Hz and digitized at 1 kHz.

MD Simulations and Potential of Mean Force Computations. The simulation system was constructed initially using CHARMM-GUI protocol and CHARMM27 (34, 35) for proteins with NBFIX (36) and CHARMM36 (37) for lipids. A crystal structure of hHv1 (PDB: 3WKV) was embedded into lipid bilayer 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) constructed with a protein-membrane builder from the CHARMM-GUI project (38). The homology model of *Ci*-Hv1 was developed previously (27, 28). To ensure correct hydration of the permeation pathway, we used the GCMC protocol for water insertion on two protein structures. The details of the protocol were similar to that of Deng and Roux (39, 40). The permeation pathway or water insertion-deletion region for the GCMC simulations was defined as a box with boundaries of 30 Å × 30 Å × 60 Å. The two constructed protein-lipid systems were equilibrated for 50 *ns* with additive force fields. The average representative structures were used to create fully polarizable systems with a Drude force field developed by the groups of MacKerell and Roux (41–43). The positions of auxiliary Drude particles attached only to heavy atoms were propagated via an extended Lagrangian formalism through the assignment of a small mass (0.4 amu) at low temperature (1 K) using a separate thermostat. The Velocity-Verlet (VV2) integrator and the Langevin thermostat were used for all simulations involving polarizable models (44). The 1D PMF computations with umbrella sampling were carried out by applying a total of 80 windows (Zn^{2+} was positioned between 0 Å and 20 Å from the center of the membrane, with a force constant of 25 kcal/mol/Å^2) along a reaction coordinate normal to the membrane (*z* axis). We used Zn^{2+} parameters published by Riahi et al. (45) Each PMF window was pre-equilibrated for 250 ps before a production run of 1 *ns*. The data were analyzed using weighted histogram analysis method (WHAM) to yield a converged PMF (tolerance was set to 0.0001 kcal/mol). 2D PMF computations were performed for the resting closed-state model of *Ci*-Hv1 developed previously (27, 28). To unravel energetics of two Zn^{2+}-binding/permeation, we used multidimensional umbrella sampling methods, a powerful computational technique used with a considerable success in the past for various ion channels (46). Umbrella sampling simulations were performed with harmonic biasing potentials with a force constant of 20 kcal/(mol·Å^2) along the *z* axis. The sampling windows for 2D PMF computations were spaced every 0.5 Å from 0 to 10 Å for the first Zn^{2+} ion and from 0 to 19 Å from the center of the lipid bilayer for the second Zn^{2+} ion. During the simulations used to calculate the single-ion and two-ion PMFs, other ions were excluded from the sphere of radius of 20 Å defining the tentative blockade pathway, using a repulsive flat-bottom spherical harmonic restraint with force constant 5 kcal/mol. To limit the lateral displacement of the Zn^{2+} ion, and thus ensure good sampling in a well-defined region of configurational space, we used a flat-bottom cylindrical restraint with radius 12 Å (relative to the center of mass of the monomer) and force constant 10 kcal/mol/Å (47). The 2D PMF setup resulted in 760 windows total. The simulation time per window was set to 1 *ns* with total simulation time for the 2D PMF map of ~0.76 μs. The energy surfaces were rebuilt with WHAM (48, 49). The tolerance for WHAM was set to 0.001.

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1. Thomas RC, Meech RW (1982) Hydrogen ion currents and intracellular pH in depolarized voltage-clamped snail neurones. *Nature* 299(5886):826–828.
2. Henderson LM, Chappell JB, Jones OT (1987) The superoxide-generating NADPH oxidase of human neutrophils is electrogenic and associated with an H+ channel. *Biochem J* 246(2):325–329.
3. Henderson LM, Chappell JB, Jones OT (1988) Internal pH changes associated with the activity of NADPH oxidase of human neutrophils. Further evidence for the presence of an H+ conducting channel. *Biochem J* 251(2):563–567.
4. Henderson LM, Chappell JB, Jones OT (1988) Superoxide generation by the electrogenic NADPH oxidase of human neutrophils is limited by the movement of a compensating charge. *Biochem J* 255(1):285–290.
5. DeCoursey TE, Morgan D, Cherny VV (2003) The voltage dependence of NADPH oxidase reveals why phagocytes need proton channels. *Nature* 422(6931):531–534.
6. DeCoursey TE (1991) Hydrogen ion currents in rat alveolar epithelial cells. *Biophys J* 60(5):1243–1253.
7. Wu LJ, et al. (2012) The voltage-gated proton channel Hv1 enhances brain damage from ischemic stroke. *Nat Neurosci* 15(4):565–573.
8. Lishko PV, Botchkina IL, Fedorenko A, Kirichok Y (2010) Acid extrusion from human spermatozoa is mediated by flagellar voltage-gated proton channel. *Cell* 140(3):327–337.
9. Koch HP, et al. (2008) Multimeric nature of voltage-gated proton channels. *Proc Natl Acad Sci USA* 105(26):9111–9116.
10. Tombola F, Ulbrich MH, Isacoff EY (2008) The voltage-gated proton channel Hv1 has two pores, each controlled by one voltage sensor. *Neuron* 58(4):546–556.
11. Cherny VV, DeCoursey TE (1999) pH-dependent inhibition of voltage-gated H(+) currents in rat alveolar epithelial cells by Zn(2+) and other divalent cations. *J Gen Physiol* 114(6):819–838.

frontiers in Physiology

HYPOTHESIS AND THEORY

Extracellular Cysteine in Connexins: Role as Redox Sensors

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Connexin-based channels comprise hemichannels and gap junction channels. The opening of hemichannels allow for the flux of ions and molecules from the extracellular space into the cell and vice versa. Similarly, the opening of gap junction channels permits the diffusional exchange of ions and molecules between the cytoplasm and contacting cells. The controlled opening of hemichannels has been associated with several physiological cellular processes; thereby unregulated hemichannel activity may induce loss of cellular homeostasis and cell death. Hemichannel activity can be regulated through several mechanisms, such as phosphorylation, divalent cations and changes in membrane potential. Additionally, it was recently postulated that redox molecules could modify hemichannels properties *in vitro*. However, the molecular mechanism by which redox molecules interact with hemichannels is poorly understood. In this ware we discuss the current knowledge on connexin redox regulation and we propose the hypothesis that extracellular cysteines could be important for sensing changes in redox potential. Future studies on this topic will offer new insight into hemichannel function, thereby expanding the understanding of the contribution of hemichannels to disease progression.

Keywords: connexins, hemichannels, redox potential, gap junction channels, post-translational modifications, pressure transmitters

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INTRODUCTION

Connexins are a large family of transmembrane proteins involved in cellular communication in humans, there are 21 genes encoding for connexins (Beyer and Willecke, 2011). These proteins are named based on their predicted molecular weight, connexin23 (Cx23, ~23 kDa) being the smallest, and Cx62 (62 kDa) the largest (reviewed in Sáez et al., 2003). Despite the different amino acid sequences among connexin isoforms, they share similar plasma membrane topology, which include the N- and C-termini (NT and CT respectively) being oriented toward the cytoplasm, four transmembrane domains (TM1-TM4), one intracellular loop (IL), and two extracellular loops (EL1 and EL2) (**Figure 1**; Bennett et al., 1991; Yeager and Nicholson, 1996). Oligomerization of six connexin subunits forms a channel known as hemichannel. Growing evidence support the role of hemichannels as a route to paracrine and signaling molecules that participate in paracrine and autocrine signaling in normal and pathological conditions.

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REFERENCES

Banerjee, R. (2012). Redox outside the box: linking extracellular redox remodeling with intracellular redox metabolism. *J. Biol. Chem.* 287, 4397–4402. doi: 10.1074/jbc.R111.287995

Bao, X., Chen, Y., Reuss, L., and Altenberg, G. A. (2004). Functional expression in Xenopus oocytes of gap-junctional hemichannels formed by a cysteine-less connexin 43. *J. Biol. Chem.* 279, 9689–9692. doi: 10.1074/jbc.M311438200

Chandrasekhar, A., and Bera, A. K. (2012). Hemichannels: permeants and their effect on development, physiology and death. *Cell Biochem. Funct.* 30, 89–100. doi: 10.1002/cbf.2794

Cantin, A. M., Larivée, P., and Bégin, R. O. (1990). Extracellular glutathione suppresses human lung fibroblast proliferation. *Am. J. Respir. Cell. Mol. Biol.* 3, 79–85. doi: 10.1165/ajrcmb/3.1.79

Castrop, H., Schweda, F., Mizel, D., Huang, Y., Briggs, J., Kurtz, A., et al. (2004). Permissive role of nitric oxide in macula densa control of renin secretion. *Am. J. Physiol. Renal. Physiol.* 286, F848–F857. doi: 10.1152/ajprenal.00272.2003

Cheung, G., Chever, O., and Rouach, N. (2014). Connexons and pannexons: newcomers in neurophysiology. *Front. Cell. Neurosci.* 8:348. doi: 10.3389/fncel.2014.00348

Chever, O., Lee, C. Y., and Rouach, N. (2014). Astroglial connexin43 hemichannels tune basal excitatory synaptic transmission. *J. Neurosci.* 34, 11228–11232. doi: 10.1523/JNEUROSCI.0015-14.2014

Circu, M. L., and Aw, T. Y. (2012). Glutathione and modulation of cell apoptosis. *Biochim. Biophys. Acta.* 1823, 1767–1777. doi: 10.1016/j.bbamcr.2012.06.019

Contreras, J. E., Sáez, J. C., Bukauskas, F. F., and Bennett, M. V. (2003). Gating and regulation of connexin 43 (Cx43) hemichannels. *Proc. Natl. Acad. Sci. U.S.A.* 100, 11388–11393. doi: 10.1073/pnas.1434298100

Dahl, G., Levine, E., Rabadan-Diehl, C., and Werner, R. (1991). Cell/cell channel formation involves disulfide exchange. *Eur. J. Biochem.* 197, 141–144. doi: 10.1111/j.1432-1033.1991.tb15892.x

Del Rio, R., Quintanilla, R. A., Orellana, J. A., and Retamal, M. A. (2015). Neuron-glia crosstalk in the autonomic nervous system and its possible role in the progression of metabolic syndrome: a new hypothesis. *Front. Physiol.* 6:350. doi: 10.3389/fphys.2015.00350

Falkowska, A., Gutowska, I., Goschorska, M., Nowacki, P., Chlubek, D., and Baranowska-Bosiacka, I. (2015). Energy metabolism of the brain, including the cooperation between astrocytes and neurons, especially in the context of glycogen metabolism. *Int. J. Mol. Sci.* 16, 25959–25981. doi: 10.3390/ijms161125939

Figueroa, X. F., Lillo, M. A., Gaete, P. S., Riquelme, M. A., and Sáez, J. C. (2013). Diffusion of nitric oxide across cell membranes of the vascular wall requires specific connexin-based channels. *Neuropharmacology* 75, 471–478. doi: 10.1016/j.neuropharm.2013.02.022

Foote, C. I., Zhou, L., Zhu, X., and Nicholson, B. J. (1998). The pattern of disulfide linkages in the extracellular loop regions of connexin 32 suggests a model for the docking interface of gap junctions. *J. Cell. Biol.* 140, 1187–1197. doi: 10.1083/jcb.140.5.1187

Good, M. E., Ek-Vitorin, J. F., and Burt, J. M. (2014). Structural determinants and proliferative consequences of connexin 37 hemichannel function in insulinoma cells. *J. Biol. Chem.* 289, 30379–30386. doi: 10.1074/jbc.M114.583054

Griffiths, H. R., Dias, I. H., Willetts, R. S., and Devitt, A. (2014). Redox regulation of protein damage in plasma. *Redox Biol.* 2, 430–435. doi: 10.1016/j.redox.2014.01.010

Hanner, F., Sorensen, C. M., Holstein-Rathlou, N. H., and Peti-Peterdi, J. (2010). Connexins and the kidney. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 298, R1143–R1155. doi: 10.1152/ajpregu.00808.2009

Hawkes, W. C., and Alkan, Z. (2010). Regulation of redox signaling by selenoproteins. *Biol. Trace. Elem. Res.* 134, 235–251. doi: 10.1007/s12011-010-8656-7

Iovine, M. K., Gumpert, A. M., Falk, M. M., and Mendelson, T. C. (2008). Cx23, a connexin with only four extracellular-loop cysteines, forms functional gap junction channels and hemichannels. *FEBS Lett.* 582, 165–170. doi: 10.1016/j.febslet.2007.11.079

Kar, R., Batra, N., Riquelme, M. A., and Jiang, J. X. (2012). Biological role of connexin intercellular channels and hemichannels. *Arch. Biochem. Biophys.* 524, 2–15. doi: 10.1016/j.abb.2012.03.008

Koistinaho, J., Swanson, R. A., de Vente, J., and Sagar, S. M. (1993). NADPH-diaphorase (nitric oxide synthase)-reactive amacrine cells of rabbit retina: putative target cells and stimulation by light. *Neuroscience* 57, 587–597. doi: 10.1016/0306-4522(93)90008-4

Kronengold, J., Srinivas, M., and Verselis, V. K. (2012). The N-terminal half of the connexin protein contains the core elements of the pore and voltage gates. *J. Membr. Biol.* 245, 453–463. doi: 10.1007/s00232-012-9457-z

Lane, D. J., and Lawen, A. (2009). Ascorbate and plasma membrane electron transport–enzymes vs efflux. *Free Radic. Biol. Med.* 47, 485–495. doi: 10.1016/j.freeradbiomed.2009.06.003

Lash, L. H., Putt, D. A., and Zalups, R. K. (1998). Role of extracellular thiols in accumulation and distribution of inorganic mercury in rat renal proximal and distal tubular cells. *J. Pharmacol. Exp. Ther.* 285, 1039–1050.

Le Gal, L., Alonso, F., Mazzolai, L., Meda, P., and Haefliger, J. A. (2015). Interplay between connexin40 and nitric oxide signaling during hypertension. *Hypertension* 65, 910–915. doi: 10.1161/HYPERTENSIONAHA.114.04775

León-Paravic, C. G., Figueroa, V. A., Guzmán, D. J., Valderrama, C. F., Vallejos, A. A., Fiori, M. C., et al. (2014). Carbon monoxide (CO) is a novel inhibitor of connexin hemichannels. *J. Biol. Chem.* 289, 36150–36157. doi: 10.1074/jbc.m114.602243

Maeda, S., Nakagawa, S., Suga, M., Yamashita, E., Oshima, A., Fujiyoshi, Y., et al. (2009). Structure of the connexin 26 gap junction channel at 3.5 A resolution. *Nature* 2458, 597–602. doi: 10.1038/nature07869

Mannu, G. S. (2014). Retinal phototransduction. *Neurosciences (Riyadh).* 19, 275–280.

Meens, M. J., Alonso, F., Le Gal, L., Kwak, B. R., and Haefliger, J. A. (2015). Endothelial Connexin37 and Connexin40 participate in basal but not agonist-induced NO release. *Cell Commun. Signal.* 13, 34. doi: 10.1186/s12964-015-0110-1

Matsuo, Y., and Yodoi, J. (2013). Extracellular thioredoxin: a therapeutic tool to combat inflammation. *Cytokine Growth Factor Rev.* 24, 345–353. doi: 10.1016/j.cytogfr.2013.01.001

Mehta, B., Begum, G., Joshi, N. B., and Joshi, P. G. (2008). Nitric oxide-mediated modulation of synaptic activity by astrocytic P2Y receptors. *J. Gen. Physiol.* 132, 339–349. doi: 10.1085/jgp.200810043

Miyachi, E., Murakami, M., and Nakaki, T. (1990). Arginine blocks gap junctions between retinal horizontal cells. *Neuroreport* 1, 107–110. doi: 10.1097/00001756-199010000-00006

Orellana, J. A., Shoji, K. F., Abudara, V., Ezan, P., Amigou, E., Sáez, P. J., et al. (2011). Amyloid β-induced death in neurons involves glial and neuronal hemichannels. *J. Neurosci.* 31, 4962–4977. doi: 10.1523/JNEUROSCI.6417-10.2011

Orellana, J. A., and Stehberg, J. (2014). Hemichannels: new roles in astroglial function. *Front. Physiol.* 5:193. doi: 10.3389/fphys.2014.00193

Oshima, A., Tani, K., Hiroaki, Y., Fujiyoshi, Y., and Sosinsky, G. E. (2007). Three-dimensional structure of a human connexin26 gap junction channel reveals a plug in the vestibule. *Proc. Natl. Acad. Sci. U.S.A.* 104, 10034–10039. doi: 10.1073/pnas.0703704104

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BMC Cell Biology

EDITORIAL Open Access

Proceedings of the International Gap Junction Conference 2015

Juan C. Sáez[1,2]

From International Gap Junction Conference 2015
Valparaíso, Chile. 28 March - 2 April 2015

From March 28 through April 2, 2015, the scientific community performing research on connexin-, pannexin- and innexin-based channels (gap junction channels and hemichannels) attended the International Gap Junction Conference in Valparaíso, Chile (Fig. 1). The Conference counted with the participation of 147 attendees from 19 different countries. Several topics were presented and discussed in platform and poster sessions, including novel insights into the structure, cell biology, regulation and pharmacology of the channels as well as different mechanisms by which these channels contribute to diverse pathologies. The keynote speakers covered specific topics. Dr. Eric Beyer gave a recount of the changes that took place in the field after the cloning of the first connexin cDNAs. Dr. Tomás Pérez-Acle presented novel findings into the structure-function relationships of different connexin channels. Dr. Eduardo Macagno presented a historical perspective of innexins and emphasized how the identity of the subset of innexins expressed in a neuron determines its morphology and connectivity during development. Dr. Alberto Pereda talked about the structural complexity and dynamic regulation of electrical synapses. Dr. Akio Suzumura presented the latest progress of his group on the use of connexin hemichannel blockers to prevent neuronal degeneration in several neurodegenerative diseases. The participation of undergraduate and graduate students as well as post-doctoral fellows played a significant role in the success of the meeting. Several of them were recognized for their scientific accomplishments and received honorable mentions and prizes. One of the highlights of the Conference was the development of specific modulators of channel and hemichannel activities. These modulators may be useful to further understand the role these proteins play in different physiological and pathological conditions. Some of them have been successfully tested for the treatment of certain pathological conditions in mice and have the potential of being used for the treatment of different human diseases in the future. The main topics discussed at this International Conference have been reviewed by specialists in the field and will be published in two special issues in BMC Cell Biology.

The organizers would like to give special recognition to Drs. Michael Bennett from USA and Klaus Willecke from Germany for their major contributions to the field, and their critical questions and opinions. The scientific community also thanks Dr. Klaus Willecke for making all the mice with targeted deletion or replacement of connexins available to the community. We also acknowledge Mr. Juan Carlos García and his team from the Centro Interdisciplinario de Neurociencias de Valparaíso (CINV) for their invaluable support and assistance with the logistics of the meeting. In addition, we are indebted

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Competing interests
The author declares that he has no competing interests.

Declarations
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Biological Research

REVIEW Open Access

Genetic variants associated with neurodegenerative Alzheimer disease in natural models

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Abstract

The use of transgenic models for the study of neurodegenerative diseases has made valuable contributions to the field. However, some important limitations, including protein overexpression and general systemic compensation for the missing genes, has caused researchers to seek natural models that show the main biomarkers of neurodegenerative diseases during aging. Here we review some of these models—most of them rodents, focusing especially on the genetic variations in biomarkers for Alzheimer diseases, in order to explain their relationships with variants associated with the occurrence of the disease in humans.

Keywords: Degus, Genome, APP, APOE

Background

A valuably strategy for the study of neurodegenerative diseases like Alzheimer's disease (AD), has been the use of transgenic mice bearing a particular human allele, to evaluate its pathogenic potential [43]. Unfortunately, most transgenic models don't recapitulate the full spectrum of a particular disease and require protein overexpression. Although new knock-in mouse models promise to show a more realistic and faithful progress of human diseases [85], nevertheless the short life of mice still prevents an accurate association between age and sporadic diseases. Therefore, a promising alternative approach is the search for non-transgenic models (NTM), in which the main hallmarks of a pathological phenotype appear naturally during aging [15]. More recently, the extraordinary advent and growth in genomic information has lead to the availability of complete genomes from a large number of different species. The latter offer a unique opportunity to investigate the involvement of particular genes in different diseases, in NTM. So it is possible today to ask, What might be the genetic basis of a neuropathology? What would be the importance of inherited or risk genes for the start and/or progress of AD pathology?

Here we review NTM of neurodegeneration and use published genomes to compare the sequences of specific gene variants in relation to idiopathic or sporadic form of AD (SAD). The latter in order to identify in NTM of AD gene sequences (tau, APOE, APP, PSEN, Aβ) corresponding to gene variants for causing AD in human.

Genes implicated in familial Alzheimer's disease

AD in its familial (early onset) or sporadic (late onset) form is characterized by the occurrence of a series of critical biomarkers. Among these the main indicators of neural degeneration, including synaptic failure and cognitive decline [88] are the accumulation of phosphorylated tau protein, which form neurofibrillary tangles (NFT), and the overexpression of amyloid precursor protein (APP), which leads to the accumulation of Amiloid-β (Aβ) peptide in senile plaques,

APP is an integral membrane protein present in the brain [50] and has been related to diverse functions including cell adhesion, growth factor and signaling associated with synaptogenesis and synaptic plasticity [98]. The proteolytic processing of APP releases potentially neurotoxic species, e.g., the Aβ peptide, which is considered one of the key pathogenic events in AD. The

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References

1. Andziak B, O'Connor TP, Qi W, DeWaal EM, Pierce A, Chaudhuri AR, Van Remmen H, Buffenstein R. High oxidative damage levels in the longest-living rodent, the naked mole-rat. Aging Cell. 2006;5:463–71.
2. Ardiles AO, Ewer J, Acosta ML, Kirkwood A, Martinez AD, Ebensperger LA, Bozinovic F, Lee TM, Palacios AG. Octodon degus (Molina 1782): a model in comparative biology and biomedicine. Cold Spring Harb Protoc. 2013;2013(4):312–8.
3. Ardiles AO, Tapia-Rojas CC, Mandal M, Alexandre F, Kirkwood A, Inestrosa NC, Palacios AG. Postsynaptic dysfunction is associated with spatial and object recognition memory loss in a natural model of Alzheimer's disease. Proc Natl Acad Sci U S A. 2012;109(34):13835–40.
4. Bales KR, Verina T, Dodel RC, Du Y, Altstiel L, Bender M, Hyslop P, Johnstone EM, Little SP, Cummins DJ, Piccardo P, Ghetti B, Paul SM. Lack of apolipprotein E dramatically reduces amyloid β-peptide deposition. Nature Genet. 1997;17:263–4.
5. Bales KR, Liu F, Wu S, Lin S, Koger D, DeLong C, Hansen JC, Sullivan PM, Paul SM. Human APOE isoform-dependent effects on brain beta-amyloid levels in PDAPP transgenic mice. J Neurosci. 2009;29(21):6771–9.
6. Ballatore C, Lee VM, Trojanowski JQ. Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. Nat Rev Neurosci. 2007;8:663–72.
7. Bates K, Vink R, Martins R, Harvey A. Aging, cortical injury and Alzheimer's disease-like pathology in the guinea pig brain. Neurobiol Aging. 2014;35(6):1345–51.
8. Baum D. Reading a phylogenetic tree: the meaning of monophyletic groups. Nature Educ. 2008;1(1):190.
9. Beck M, Bigl V, Roßner S. Guinea pigs as a nontransgenic model for APP processing in Vitro and in Vivo. Neurochem Res. 2003;28(3–4):637–44.
10. Beck M, Müller D, Bigl V. Amyloid precursor protein in guinea pigs–complete cDNA sequence and alternative splicing. Biochim Biophys Acta. 1997;1351(1–2):17–21.
11. Bekris LM, Yu CE, Bird TD, Tsuang DW. Genetics of Alzheimer disease. J Geriatr Psychiatry Neurol. 2010;23(4):213–27.
12. Bons N, Rieger F, Prudhomme D, Fisher A, Krause KH. Microcebus murinus: a useful primate model for human cerebral aging and Alzheimer's disease? Genes, Brain and Behavior. 2006;5(2):120–30.
13. Borchelt DR, Thinakaran G, Eckman CB, Lee MK, Davenport F, Ratovitsky T, Prada CM, Kim G, Seekins S, Yager D, Slunt HH, Wang R, Seeger M, Levey AI, Gandy SE, Copeland NG, Jenkins NA, Price DL, Younkin SG, Sisodia SS. Familial Alzheimer's disease-linked presenilin 1 variants elevate Abeta1-42/1-40 ratio in vitro and in vivo. Neuron. 1996;17(5):1005–13.
14. Borchelt D, Ratovitski T, Van Lare J, Lee M, Gonzales V, Jenkins N, Copeland N, Price D, Sisodia S. Accelerated Amyloid Deposition in the Brains of Transgenic Mice Coexpressing Mutant Presenilin 1 and Amyloid Precursor Proteins. Neuron. 1997;19:939–45.
15. Braidy N, Munoz P, Palacios AG, Castellano-Gonzalez G, Inestrosa NC, Chung RS, Sachdev P, Guillemin GJ. Recent rodent models for Alzheimer's disease: clinical implications and basic research. J Neural Transm. 2012;119(2):173–95.
16. Buffenstein R. The naked mole-rat: a new long-living model for human aging research. The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences. 2005;60(11):1369–77.
17. Buffenstein R, Yahav S. Cholecalciferol has no effect on calcium and inor- ganic phosphorus balance in a naturally cholecalciferol-deplete subterranean mammal, the naked mole rat (*Heterocephalus glaber*). J Endocrinol. 1991;129:21–6.
18. Calenda A, Jallageas V, Silhol S, Bellis M, Bons N. Identification of a unique apolipoprotein E allele in Microcebus murinus; ApoE brain distribution and co-localization with beta-amyloid and tau proteins. Neurobiol Dis. 1995;2(3):169–76.
19. Cárdenas AM, Ardiles AO, Barraza N, Baéz-Matus X, Caviedes P. Role of tau protein in neuronal damage in Alzheimer's disease and Down syndrome. Arch Med Res. 2012;43(8):645–54.
20. Castellano JM, Kim J, Stewart FR, Jiang H, DeMattos RB, Patterson BW, Fagan AM, Morris JC, Mawuenyega KG, Cruchaga C, Goate AM, Bales KR, Paul SM, Bateman RJ, Holtzman DM. Human apoE isoforms differentially regulate brain amyloid-β peptide clearance. Sci Transl Med. 2011;3(89):89ra57.
21. Chang EH, Savage MJ, Flood DG, Thomas JM, Levy RB, Mahadomrongkul V, Shirao T, Aoki C, Huerta PT. AMPA receptor downscaling at the onset of Alzheimer's disease pathology in double knockin mice. Proc Natl Acad Sci U S A. 2006;103(9):3410–5.
22. Citron M, Westaway D, Xia W, Carlson G, Diehl T, Levesque G, Johnson-Wood K, Lee M, Seubert P, Davis A, Kholodenko D, Motter R, Sherrington R, Perry B, Yao H, Strome R, Lieberburg I, Rommens J, Kim S, Schenk D, Fraser P, St George Hyslop P, Selkoe DJ. Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice. Nat Med. 1997;3(1):67–72.
23. Clarke RF, Hutton M. and the Alzheimer's Collaborative Group. The structure of the presenilin-1 (S182) gene and identification of six novel mutations in early onset AD families. Nature Genet. 1995;11:219–22.
24. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science. 1993;261(5123):921–3.
25. Coulson EJ, Paliga K, Beyreuther K, Masters CL. What the evolution of the amyloid protein precursor supergene family tells us about its function. Neurochem Int. 2000;36:175–84.
26. De Strooper B. Loss-of-function presenilin mutations in Alzheimer disease. Talking Point on the role of presenilin mutations in Alzheimer disease. EMBO Rep. 2007;8(2):141–6.
27. De Strooper B, Saftig P, Craessaerts K, Vanderstichele H, Guhde G, Annaert W, Von Figura K, Van Leuven F. Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. Nature. 1998;391(6665):387–90.
28. De Strooper B, Simons M, Multhaup G, van Leuven F, Beyreuther K, Dotti CG. Production of intracellular amyloid-containing fragments in hippocampal neurons expressing human amyloid precursor protein and protection against amyloidogenesis by subtle amino acid substitutions in the rodent sequence. EMBO J. 1995;14:4932–8
29. Deacon RM, Altimiras FJ, Bazan-Leon EA, Pyarasani RD, Nachtigall FM, Santos LS, Tsolaki AG, Pednekar L, Kishore U, Biekofsky RR, Vasquez RA, Cogram P. Natural AD-Like neuropathology in Octodon degus: impaired burrowing and neuroinflammation. Curr Alzheimer Res. 2015;12(4):314–22.
30. Deane R, Sagare A, Hamm K, Parisi M, Lane S, Finn MB, Holtzman DM, Zlokovic BV. ApoE isoform – specific disruption of amyloid β peptide clearance from mouse brain. J Clin Investig. 2008;118(12):4002–13.
31. Dhenain M, Chenu E, Hisley CK, Aujard F, Volk A. Regional Atrophy in the Brain of Lissencephalic Mouse Lemur Primates: measurement by automatic histogram-based segmentation of MR images. Magn Reson Med. 2003;50(5):984–92.
32. Dodart JC, Mathis C, Bales KR, Paul SM. Does my mouse have Alzheimer's disease? Genes Brain Behav. 2002;1(3):142–55.
33. Dong LM, Parkin S, Trakhanov SD, Rupp B, Simmons T, Arnold KS, Newhouse YM, Innerarity TL, Weisgraber KH. Novel mechanism for defective

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Research Article

The $\alpha_2\delta$-1 subunit remodels $Ca_V1.2$ voltage sensors and allows Ca^{2+} influx at physiological membrane potentials

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Excitation-evoked calcium influx across cellular membranes is strictly controlled by voltage-gated calcium channels (Ca_V), which possess four distinct voltage-sensing domains (VSDs) that direct the opening of a central pore. The energetic interactions between the VSDs and the pore are critical for tuning the channel's voltage dependence. The accessory $\alpha_2\delta$-1 subunit is known to facilitate $Ca_V1.2$ voltage-dependent activation, but the underlying mechanism is unknown. In this study, using voltage clamp fluorometry, we track the activation of the four individual VSDs in a human L-type $Ca_V1.2$ channel consisting of α_{1C} and β_3 subunits. We find that, without $\alpha_2\delta$-1, the channel complex displays a right-shifted voltage dependence such that currents mainly develop at nonphysiological membrane potentials because of very weak VSD–pore interactions. The presence of $\alpha_2\delta$-1 facilitates channel activation by increasing the voltage sensitivity (i.e., the effective charge) of VSDs I–III. Moreover, the $\alpha_2\delta$-1 subunit also makes VSDs I–III more efficient at opening the channel by increasing the coupling energy between VSDs II and III and the pore, thus allowing Ca influx within the range of physiological membrane potentials.

INTRODUCTION

Calcium influx through voltage-activated calcium (Ca_V) channels translates electrical signals into a variety of physiological outcomes such as cell contraction, neurotransmitter or hormonal release, and gene expression (Catterall, 2011; Zamponi et al., 2015). The specificity of the Ca^{2+} signal relies on the activity of the Ca_V channel complex being perfectly tuned to voltage signals. Ca_V channels are multimeric proteins formed by the pore-forming α_1 subunit and at least three auxiliary subunits, β, $\alpha_2\delta$, and calmodulin, in a 1:1:1:1 stoichiometry, resulting in an asymmetric structural architecture (Fig. 1; Findeisen and Minor, 2010; Catterall, 2011; Dolphin, 2013; Ben-Johny and Yue, 2014; Neely and Hidalgo, 2014; Campiglio and Flucher, 2015; Wu et al., 2015). The $\alpha_2\delta$ auxiliary subunit is a large (~170 kD), mostly extracellular protein with a single membrane-anchoring segment (Davies et al., 2010) that binds to the α_1 subunit from the extracellular side (Cassidy et al., 2014). α_2 and δ proteins are the products of the same gene as a preprotein that is posttranslationally proteolysed and then linked by a disulfide-bond to form the mature $\alpha_2\delta$ protein (Calderón-Rivera et al., 2012). Four genes (CACNA2D1–4) encode for distinct $\alpha_2\delta$ isoforms ($\alpha_2\delta$-1–4), which are all expressed in the brain (Dolphin, 2013). In addition to brain tissue, $\alpha_2\delta$-1 is strongly expressed in cardiac, smooth, and skeletal muscles, whereas $\alpha_2\delta$-4 is found in endocrine tissues and the retina. Mutations in the $\alpha_2\delta$-1 gene can lead to Brugada (Burashnikov et al., 2010) and short QT (Templin et al., 2011; Bourdin et al., 2015) syndromes and are associated with epilepsy and mental disability (Vergult et al., 2015). In mice, naturally occurring mutations in the $\alpha_2\delta$-2 gene lead to ataxia and epilepsy (Barclay et al., 2001), whereas the $\alpha_2\delta$-3 protein is important for synaptic morphogenesis (Kurshan et al., 2009) and nociception (Neely et al., 2010). Mutations in $\alpha_2\delta$-4 can result in night blindness (Wycisk et al., 2006). Moreover, $\alpha_2\delta$-1 and -2 have been identified as the molecular targets of gabapentinoid drugs (such as gabapentin and pregabalin), mediating their analgesic action in neuropathic pain (Field et al., 2006; Hendrich et al., 2008; Uchitel et al., 2010). Finally, it has been shown that $\alpha_2\delta$ proteins also play an important role in synapse formation (Eroglu et al., 2009).

Several studies report that the interaction of $\alpha_2\delta$-1 with the pore-forming α_{1C} subunits (L-type $Ca_V1.2$) favors channel activation, as manifested by a hyperpolar-

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Abbreviations used: cryo-EM, cryo-electron microscopy; MES, methanesulfonate; VCF, voltage clamp fluorometry; VSD, voltage-sensing domain.

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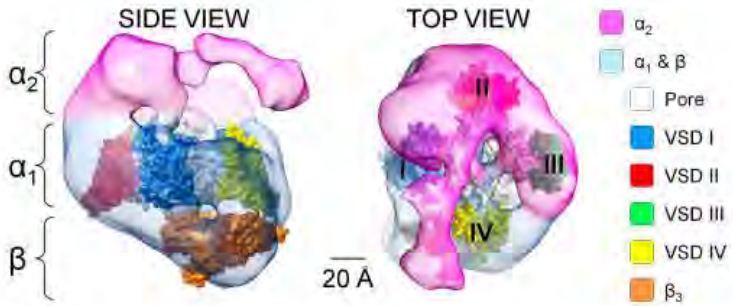

Figure 7. **The $\alpha_2\delta$ subunit covers ~3/4 of the $Ca_V1.2$ pore-forming subunit, excluding VSD IV.** Side and top views of the $Ca_V1.2$ ($\alpha_{1C}/\alpha_2/\beta$) channel volume (pink and light blue) modified from cryo-EM data from Walsh et al. (2009a) are shown. The atomic structures of Na_VAb (PDB accession no. 4EKW; Payandeh et al., 2012), with subunits arranged clockwise, and the atomic structure of AID-associated β_3 subunit (PDB accession no. 1VYT; Chen et al., 2004) were manually positioned in the cryo-EM volume. Because VSD IV is not perturbed by $\alpha_2\delta$-1, we propose that the resolved α_{1C} volume not covered by α_2 is occupied by VSD IV, whereas VSDs I–III are encompassed by the $\alpha_2\delta$-1 subunit, which alters their biophysical properties and, in the case of VSDs II and III, enhances their coupling to the channel pore.

for which there is yet no experimental evidence. Perhaps future studies could explore the possibility.

Conclusions

In summary, we have used VCF to optically track the molecular rearrangements of the individual VSDs of a human $Ca_V1.2$ channel in the presence or absence of $\alpha_2\delta$-1. VCF is now a well-established method to assess voltage-dependent conformational changes, allowing us to track the movement of individual VSDs and to resolve slow conformational changes (as those observed in VSD I) that are extremely difficult to capture by gating current measurements. In this work, we have not systematically recorded gating currents, as they could not reveal the individual contributions of each VSD to $Ca_V1.2$ activation. Perhaps the most important advantage of VCF is that all recordings could be performed in conducting channels, whereby ionic currents and VSD movements were sampled simultaneously, without the use of pore-blockers. We found that the $\alpha_2\delta$-1 auxiliary subunit significantly alters the voltage dependence of VSDs I–III, facilitating their activation, but not that of VSD IV. A 32-state allosteric model, consistent with the $Ca_V1.2$ molecular architecture, predicts the major kinetic and steady-state features of the experimental data, revealing that the association of $\alpha_2\delta$-1 with α_{1C} (in the presence of β_3) specifically increased the coupling energy of VSDs I–III to the pore, as well as effective gating charge in segments I and II. Without the enhanced gating properties brought about by $\alpha_2\delta$-1 association, $Ca_V1.2$ channels could not operate at physiological membrane potentials.

Table 3. **Fitting parameters for the model predictions in Fig. 6**

	Parameter	No $\alpha_2\delta$	With $\alpha_2\delta$-1*
Pore	q_t (e^0)	0.99	0.76
	V_t (mV)	103	140
	x_0	1.0	0.49
	ω_t (s^{-1})	1,356	670
VSD I	q_1 (e^0)	1.4	2.0
	V_1 (mV)	37	8.5
	x_1	0.72	0.89
	ω_1 (s^{-1})	17	110
VSD II	q_2 (e^0)	1.2	2.5
	V_2 (mV)	−6.9	−27
	x_2	0.78	0.99
	ω_2 (s^{-1})	35	44
VSD III	q_3 (e^0)	0.83	1.9
	V_3 (mV)	3.7	−11
	x_3	1.0	0.93
	ω_3 (s^{-1})	145	160
VSD IV	q_4 (e^0)	0.92	1.1
	V_4 (mV)	−54	−52
	x_4	0.85	0.55
	ω_4 (s^{-1})	10	11
Energetic interaction	W_1 (meV)	−8.0	−16
	W_2 (meV)	−16	−50
	W_3 (meV)	−19	−45
	W_4 (meV)	4.1	−0.87

*Parameters in this column are from Pantazis et al., 2014.

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Bioconjugate Chemistry

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Communication

Effect of several HIV-antigens simultaneously loaded with G2-NN16 carbosilane dendrimer in the cell uptake and functionality of human dendritic cells

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REFERENCES

Ahern, C.A., and R. Horn. 2005. Focused electric field across the voltage sensor of potassium channels. *Neuron.* 48:25–29. http://dx.doi.org/10.1016/j.neuron.2005.08.020

Asamoah, O.K., J.P. Wuskell, L.M. Loew, and F. Bezanilla. 2003. A fluorometric approach to local electric field measurements in a voltage-gated ion channel. *Neuron.* 37:85–98. http://dx.doi.org/10.1016/S0896-6273(02)01126-1

Bangalore, R., G. Mehrke, K. Gingrich, F. Hofmann, and R.S. Kass. 1996. Influence of L-type Ca channel alpha 2/delta-subunit on ionic and gating current in transiently transfected HEK 293 cells. *Am. J. Physiol.* 270:H1521–H1528.

Barclay, J., N. Balaguero, M. Mione, S.L. Ackerman, V.A. Letts, J. Brodbeck, C. Canti, A. Meir, K.M. Page, K. Kusumi, et al. 2001. Ducky mouse phenotype of epilepsy and ataxia is associated with mutations in the Cacna2d2 gene and decreased calcium channel current in cerebellar Purkinje cells. *J. Neurosci.* 21:6095–6104.

Barish, M.E. 1983. A transient calcium-dependent chloride current in the immature *Xenopus* oocyte. *J. Physiol.* 342:309–325. http://dx.doi.org/10.1113/jphysiol.1983.sp014852

Ben-Johny, M., and D.T. Yue. 2014. Calmodulin regulation (calmodulation) of voltage-gated calcium channels. *J. Gen. Physiol.* 143:679–692. http://dx.doi.org/10.1085/jgp.201311153

Bezanilla, F. 2008. How membrane proteins sense voltage. *Nat. Rev. Mol. Cell Biol.* 9:323–332. http://dx.doi.org/10.1038/nrm2376

Bourdin, B., B. Shakeri, M.P. Tétreault, R. Sauvé, S. Lesage, and L. Parent. 2015. Functional characterization of CaVα2δ mutations associated with sudden cardiac death. *J. Biol. Chem.* 290:2854–2869. http://dx.doi.org/10.1074/jbc.M114.597930

Burashnikov, E., R. Pfeiffer, H. Barajas-Martinez, E. Delpón, D. Hu, M. Desai, M. Borggrefe, M. Häissaguerre, R. Kanter, G.D. Pollevick, et al. 2010. Mutations in the cardiac L-type calcium channel associated with inherited J-wave syndromes and sudden cardiac death. *Heart Rhythm.* 7:1872–1882. http://dx.doi.org/10.1016/j.hrthm.2010.08.026

Calderón-Rivera, A., A. Andrade, O. Hernández-Hernández, R. González-Ramírez, A. Sandoval, M. Rivera, J.C. Gomora, and R. Felix. 2012. Identification of a disulfide bridge essential for structure and function of the voltage-gated Ca(2+) channel α(2)δ-1 auxiliary subunit. *Cell Calcium.* 51:22–30. http://dx.doi.org/10.1016/j.ceca.2011.10.002

Campiglio, M., and B.E. Flucher. 2015. The role of auxiliary subunits for the functional diversity of voltage-gated calcium channels. *J. Cell. Physiol.* 230:2019–2031. http://dx.doi.org/10.1002/jcp.24998

Cassidy, J.S., L. Ferron, I. Kadurin, W.S. Pratt, and A.C. Dolphin. 2014. Functional exofacially tagged N-type calcium channels elucidate the interaction with auxiliary α2δ-1 subunits. *Proc. Natl. Acad. Sci. USA.* 111:8979–8984. http://dx.doi.org/10.1073/pnas.1403731111

Catterall, W.A. 2010. Ion channel voltage sensors: Structure, function, and pathophysiology. *Neuron.* 67:915–928. http://dx.doi.org/10.1016/j.neuron.2010.08.021

Catterall, W.A. 2011. Voltage-gated calcium channels. *Cold Spring Harb. Perspect. Biol.* 3:a003947. http://dx.doi.org/10.1101/cshperspect.a003947

Cha, A., and F. Bezanilla. 1997. Characterizing voltage-dependent conformational changes in the Shaker K+ channel with fluorescence. *Neuron.* 19:1127–1140. http://dx.doi.org/10.1016/S0896-6273(00)80403-1

Cha, A., P.C. Ruben, A.L. George Jr., E. Fujimoto, and F. Bezanilla. 1999. Voltage sensors in domains III and IV, but not I and II, are immobilized by Na+ channel fast inactivation. *Neuron.* 22:73–87. http://dx.doi.org/10.1016/S0896-6273(00)80680-7

Chanda, B., and F. Bezanilla. 2008. A common pathway for charge transport through voltage-sensing domains. *Neuron.* 57:345–351. http://dx.doi.org/10.1016/j.neuron.2008.01.015

Chanda, B., O.K. Asamoah, R. Blunck, B. Roux, and F. Bezanilla. 2005. Gating charge displacement in voltage-gated ion channels involves limited transmembrane movement. *Nature.* 436:852–856. http://dx.doi.org/10.1038/nature03888

Chen, Y.H., M.H. Li, Y. Zhang, L.L. He, Y. Yamada, A. Fitzmaurice, Y. Shen, H. Zhang, L. Tong, and J. Yang. 2004. Structural basis of the alpha1-beta subunit interaction of voltage-gated Ca2+ channels. *Nature.* 429:675–680. http://dx.doi.org/10.1038/nature02641

Claydon, T.W., and D. Fedida. 2007. Voltage clamp fluorimetry sudies of mammalian voltage-gated K(+) channel gating. *Biochem. Soc. Trans.* 35:1080–1082. http://dx.doi.org/10.1042/BST0351080

Colquhoun, D., and A.G. Hawkes. 1981. On the stochastic properties of single ion channels. *Proc. R. Soc. Lond. B Biol. Sci.* 211:205–235. http://dx.doi.org/10.1098/rspb.1981.0003

Dalhan, D.S., M.I. Dibas, E.J. Petersson, V.C. Auyeung, B. Chanda, F. Bezanilla, D.A. Dougherty, and H.A. Lester. 2004. A fluorophore attached to nicotinic acetylcholine receptor βM2 detects productive binding of agonist to the αδ site. *Proc. Natl. Acad. Sci. USA.* 101:10195–10200. http://dx.doi.org/10.1073/pnas.0301885101

Davies, A., I. Kadurin, A. Alvarez-Laviada, L. Douglas, M. Nieto-Rostro, C.S. Bauer, W.S. Pratt, and A.C. Dolphin. 2010. The α2δ subunits of voltage-gated calcium channels form GPI-anchored proteins, a posttranslational modification essential for function. *Proc. Natl. Acad. Sci. USA.* 107:1654–1659. http://dx.doi.org/10.1073/pnas.0908735107

DeCaen, P.G., V. Yarov-Yarovoy, T. Scheuer, and W.A. Catterall. 2011. Gating charge interactions with the S1 segment during activation of a Na+ channel voltage sensor. *Proc. Natl. Acad. Sci. USA.* 108:18825–18830. http://dx.doi.org/10.1073/pnas.1116449108

Dolphin, A.C. 2012. Calcium channel auxiliary α2δ and β subunits: Trafficking and one step beyond. *Nat. Rev. Neurosci.* 13:542–555. http://dx.doi.org/10.1038/nrn3317

Dolphin, A.C. 2013. The α2δ subunits of voltage-gated calcium channels. *Biochim. Biophys. Acta.* 1828:1541–1549. http://dx.doi.org/10.1016/j.bbamem.2012.11.019

Eroglu, C., N.J. Allen, M.W. Susman, N.A. O'Rourke, C.Y. Park, E. Ozkan, C. Chakraborty, S.B. Mulinyawe, D.S. Annis, A.D. Huberman, et al. 2009. Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. *Cell.* 139:380–392. http://dx.doi.org/10.1016/j.cell.2009.09.025

Fang, K., and H.M. Colecraft. 2011. Mechanism of auxiliary β-subunit-mediated membrane targeting of L-type (Ca(V)1.2) channels. *J. Physiol.* 589:4437–4455. http://dx.doi.org/10.1113/jphysiol.2011.214247

binding of more peptides, especially obstructing the binding of a second gp160 peptide. NEF(1) in this case is not obstructing the binding of a second p24 peptide(1). This evidence can explain why mixtures of peptides including NEF are decreasing the cell uptake of the other peptides, as experimental assays showed in this study, being more dramatic the decreasing of gp160 uptake. Eventually, in a system with only p24 peptides, G2-NN16 can encapsulate three of these peptides, differently that in a solution with a mixture of different peptides, where only two p24 peptides are captured, which explains the cell uptake decreases in such conditions. More p24 peptides were encapsulated and protected by G2-NN16 in presence of other peptides, which can explain that their cell uptake do not decay at the level of gp160. The binding energy of G2-NN16/peptides is not directly correlated with the degree of coverage of the peptide elicited by G2-NN16. SASA parameter as a function of MD simulation time seems a more predictive parameter for the degree of protection. Therefore, our results show that G2-NN16 associated with two or more HIV-derived peptides could not used as a vaccine because it does not offer the potential and correct ability to deliver peptides as non-viral vectors.

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SUPPORTING INFORMATION

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Short Communication

Organotypic Retinal Explant Cultures as *In Vitro* Alternative for Diabetic Retinopathy Studies

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Summary

Diabetic retinopathy (DR) is a major cause of vision loss and one of the most common and debilitating complications of diabetes. Research to prevent DR is hindered by a lack of experimental model systems that faithfully reproduce the disease pathology, in particular for type 2 diabetes, which requires prolonged disease progression in animals to develop some hallmarks of DR. Here, we introduce an alternative *in vitro* model system for DR, based on serum-free, organotypic rodent retinal explant cultures, which allow physiological and pharmacological manipulation of the retina for up to two weeks under tightly controlled conditions. Retinal explant cultures have the advantage of isolating direct neuronal consequences of diabetic conditions from indirect systemic effects mediated via the retinal vasculature or the immune system. Exposed to conditions emulating type 1 or type 2 diabetes, retinal explants displayed elevated cell death rates among inner retinal neurons as well as photoreceptors, with a particularly strong loss of cone photoreceptors. Our results support a direct impact of diabetic conditions on retinal neurons and may help explain color vision defects observed in DR patients. This serum-free *in vitro* DR model avoids the animal suffering of established DR models and reduces the overall number of animals needed for such research. It should prove useful to study the mechanisms of neuronal cell death caused by DR and to screen for potential future DR treatments.

Keywords: retina, diabetes, animal models, photoreceptors, cell death

1 Introduction

Diabetic retinopathy (DR) is one of the most common complications of diabetes and a leading cause of vision impairment worldwide. Although it is generally considered a microvascular disorder, studies in humans and animal models have found evidence for retinal neurodegeneration occurring before the onset of vascular alterations (Barber et al., 2011; Vujosevic and Midena, 2013). Thus, a central question in DR research is whether neuroretinal pathology is a consequence of vascular defects or whether diabetic conditions directly cause retinal neurodegeneration. Currently, DR research is hindered by a lack of disease models that faithfully reproduce the retinal phenotype of diabetes, in particular type 2 diabetes (Lai and Lo, 2013). One of the most commonly used type 1 diabetes models, the injection of streptozotocin in rodents, usually produces a subtle retinal phenotype, with thinning of the ganglion cell layer (Robinson et al., 2012; Martin et al., 2004). Genetic DR models include the Ins2Akita mouse, a type 1 diabetes model carrying a mutation in the insulin-2 gene. Among type 2 diabetes animal models, mice with mutations in the leptin gene or receptor (ob/ob, db/db) may be useful to study DR (Lai and Lo, 2013; Robinson et al., 2012; Ly et al., 2014). However, a general problem with type 2 diabetes models is the long time-frame and high interindividual variability in their pathophysiology. Overall, most type 1 and 2 diabetes

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Robinson, R., Barathi, V.A., Chaurasia, S. S. et al. (2012). Update on animal models of diabetic retinopathy: From molecular approaches to mice and higher mammals. *Dis Model Mech 5*, 444-456. http://dx.doi.org/10.1242/dmm.009597

Sahaboglu, A., Tanimoto, N., Bolz, S. et al. (2014). Knockout of PARG110 confers resistance to cGMP-induced toxicity in mammalian photoreceptors. *Cell Death Dis 5*, e1234. http://dx.doi.org/10.1038/cddis.2014.208

Sanyal, S. and Bal, A. K. (1973). Comparative light and electron microscopic study of retinal histogenesis in normal and rd mutant mice. *Z Anat Entwicklungsgesch 142*, 219-238. http://dx.doi.org/10.1007/BF00519723

Stitt, A. W. (2010). AGEs and diabetic retinopathy. *Invest Ophthalmol Vis Sci 51*, 4867-4874. http://dx.doi.org/10.1167/iovs.10-5881

van Dijk, H. W., Kok, P. H., Garvin, M. et al. (2009). Selective loss of inner retinal layer thickness in type 1 diabetic patients with minimal diabetic retinopathy. *Invest Ophthalmol Vis Sci 50*, 3404-3409. http://dx.doi.org/10.1167/iovs.08-3143

Vujosevic, S. and Midena, E. (2013). Retinal layers changes in human preclinical and early clinical diabetic retinopathy support early retinal neuronal and Muller cells alterations. *J Diabetes Res 2013*, 905058. http://dx.doi.org/10.1155/2013/905058

Wolff, B. E., Bearse, M. A., Jr., Schneck, M. E. et al. (2015). Color vision and neuroretinal function in diabetes. *Doc Ophthalmol 130*, 131-139. http://dx.doi.org/10.1007/s10633-014-9476-4

Zhou, T., Zhou, K. K., Lee, K. et al. (2011). The role of lipid peroxidation products and oxidative stress in activation of the canonical wingless-type MMTV integration site (WNT) pathway in a rat model of diabetic retinopathy. *Diabetologia 54*, 459-468. http://dx.doi.org/10.1007/s00125-010-1943-1

Conflict of interest

The authors declare that there is no duality of interest associated with this manuscript.

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Electrophysiological fingerprints of OFF bipolar cells in rat retina

Alex H. Vielma & Oliver Schmachtenberg

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Retinal bipolar cells (BCs) divide photoreceptor output into different channels for the parallel extraction of temporal and chromatic stimulus properties. In rodents, five types of OFF BCs have been differentiated, based on morphological and functional criteria, but their electrophysiological characterization remains incomplete. This study analyzed OFF BCs with the patch clamp technique in acute slices of rat retina. Their specific voltage-dependent currents and glutamate responses are shown to represent individual fingerprints which define the signal processing and filtering properties of each cell type and allow their unequivocal identification. Two additions to the rat BC repertoire are presented: OFF BC-2′, a variation of BC-2 with wider axonal arbours and prominent Na^+ currents, is described for the first time in rodents, and OFF BC-3b, previously identified in mouse, is electrophysiologically characterized in rat. Moreover, the glutamate responses of rat OFF BCs are shown to be differentially sensitive to AMPA- and kainate-receptor blockers and to modulation by nitric oxide (NO) through a cGMP-dependent mechanism. These results contribute to our understanding of the diversity and function of bipolar cells in mammals.

In the mammalian retina, five classes of neurons provide extensive processing and filtering of raw data input, extracting spatial, temporal and chromatic information from the visual scene. The discovery of parallel processing of visual signals by ON and OFF channels in the retina, responding antagonistically to light stimulus increments or decrements, has been a milestone in vision research[1,2]. This division of visual input is achieved at the first retinal synapse, formed by photoreceptors, horizontal and bipolar cells (BCs). While ON BCs respond to glutamate liberation from photoreceptors with sign-inverting membrane hyperpolarization mediated by mGluR6 receptors coupled to TRPM1 channels[3,4], OFF BCs express ionotropic glutamate receptors at this synapse, generating sign-conserving depolarizing cationic currents in response to glutamate[5–7].

Within OFF BCs, the information is shaped by differential contributions of voltage-dependent Na^+, Ca^{2+}[8,9] and K^+ conductances[10], including hyperpolarization-activated cyclic nucleotide-gated (HCN) channels[11]. Inhibitory signalling from different types of amacrine cells, mediated by GABA and glycine receptors[12], as well as retinal neuromodulators like acetylcholine (ACh) and nitric oxide (NO) contribute to the conditioning of BC responses[13,14]. Finally, the processed information is passed on via glutamate release to ganglion cells in the outer half of the inner plexiform layer (IPL).

A large body of evidence, accumulated during the last two decades, has revealed significant differences among mammals regarding the number and relative percentage of OFF BC types, their morphology and glutamate receptor subunit composition at the photoreceptor synapse[15–18]. In mouse, the five established types of OFF BCs are labelled 1, 2, 3a, 3b and 4, and their axonal arbours stratify in sublayers 1 and 2 of the IPL[19,20], comprising about 40% width of this synaptic stratum[21,22]. Although important progress in the functional differentiation of OFF BCs in different species, including mouse, ground squirrel and rat, has been achieved during recent years[15,16], the organizational scheme of BCs is still mostly based on morphological criteria, particularly axonal arbour shape and localization with respect to the IPL sublayers. Physiological studies of BC function are hindered by the fact that these cells cannot be reliably identified in retinal whole mounts and slice preparations without dye filling or immunohistochemical processing, and transgenic mouse lines expressing fluorescent markers in specific BC types, although becoming increasingly popular, may not always be available or experimentally suitable. Here, we present an electrophysiological approach to unequivocally distinguish OFF BCs in rat retina, based on their voltage-gated currents and responses to glutamate stimuli under voltage and current clamp. This study expands the number of known OFF BC types from 4[6,11,18] to 5 in rat, as in ground squirrel[23] and mouse[15,21], and presents a variation of BC-2 expressing prominent Na^+ currents. Moreover, our results show that glutamate responses in BC-2, 3b and 4 depend on both AMPA and kainate receptor activation and are subject to inhibitory modulation by NO.

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16. Ichinose, T. & Hellmer, C. B. Differential signalling and glutamate receptor compositions in the OFF bipolar cell types in the mouse retina. *J Physiol.* in press, doi: 10.1113/JP271458 (2015).
17. Masland, R. H. The neuronal organization of the retina. *Neuron* **76,** 266–280 (2012).
18. Hartveit, E. Functional organization of cone bipolar cells in the rat retina. *J Neurophysiol.* **77,** 1716–1730 (1997).
19. Wassle, H., Puller, C., Muller, F. & Haverkamp, S. Cone contacts, mosaics, and territories of bipolar cells in the mouse retina. *J Neurosci.* **29,** 106–117 (2009).
20. Ghosh, K. K., Bujan, S., Haverkamp, S., Feigenspan, A. & Wassle, H. Types of bipolar cells in the mouse retina. *J Comp Neurol.* **469,** 70–82 (2004).
21. Helmstaedter, M. *et al.* Connectomic reconstruction of the inner plexiform layer in the mouse retina. *Nature* **500,** 168–174 (2013).
22. Famiglietti, E. V. Jr. & Kolb, H. Structural basis for ON- and OFF-center responses in retinal ganglion cells. *Science* **194,** 193–195 (1976).
23. Lindstrom, S. H., Ryan, D. G., Shi, J. & DeVries, S. H. Kainate receptor subunit diversity underlying response diversity in retinal Off bipolar cells. *J Physiol.* **592,** 1457–1477 (2014).
24. Muller, F. *et al.* HCN channels are expressed differentially in retinal bipolar cells and concentrated at synaptic terminals. *Eur J Neurosci.* **17,** 2084–2096 (2003).
25. Fyk-Kolodziej, B. & Pourcho, R. G. Differential distribution of hyperpolarization-activated and cyclic nucleotide-gated channels in cone bipolar cells of the rat retina. *J Comp Neurol.* **501,** 891–903 (2007).
26. Mataruga, A., Kremmer, E. & Muller, F. Type 3a and type 3b OFF cone bipolar cells provide for the alternative rod pathway in the mouse retina. *J Comp Neurol.* **502,** 1123–1137 (2007).
27. Harris, N. C. & Constanti, A. Mechanism of block by ZD 7288 of the hyperpolarization-activated inward rectifying current in guinea pig substantia nigra neurons *in vitro*. *J Neurophysiol.* **74,** 2366–2378 (1995).
28. Haverkamp, S. *et al.* Type 4 OFF cone bipolar cells of the mouse retina express calsenilin and contact cones as well as rods. *J Comp Neurol.* **507,** 1087–1101 (2008).
29. Puthussery, T. *et al.* Kainate receptors mediate synaptic input to transient and sustained OFF visual pathways in primate retina. *J Neurosci.* **34,** 7611–7621 (2014).
30. Borghuis, B. G., Looger, L. L., Tomita, S. & Demb, J. B. Kainate receptors mediate signaling in both transient and sustained OFF bipolar cell pathways in mouse retina. *J Neurosci.* **34,** 6128–6139 (2014).
31. Pang, J. J., Gao, F. & Wu, S. M. Light responses and morphology of bNOS-immunoreactive neurons in the mouse retina. *J Comp Neurol.* **518,** 2456–2474 (2010).
32. Vielma, A. H., Retamal, M. A. & Schmachtenberg, O. Nitric oxide signaling in the retina: What have we learned in two decades? *Brain Res.* **1430,** 112–125 (2012).
33. Pan, Z. H. Differential expression of high- and two types of low-voltage-activated calcium currents in rod and cone bipolar cells of the rat retina. *J Neurophysiol.* **83,** 513–527 (2000).
34. Hu, C., Bi, A. & Pan, Z. H. Differential expression of three T-type calcium channels in retinal bipolar cells in rats. *Vis Neurosci.* **26,** 177–187 (2009).
35. Baden, T., Berens, P., Bethge, M. & Euler, T. Spikes in mammalian bipolar cells support temporal layering of the inner retina. *Curr Biol.* **23,** 48–52 (2013).
36. Matzen, L. *et al.* AMPA receptor agonists: synthesis, proteolytic properties, and pharmacology of 3-isothiazolol bioisosteres of glutamic acid. *J Med Chem.* **40,** 520–527 (1997).
37. Puller, C., Haverkamp, S. & Grunert, U. OFF midget bipolar cells in the retina of the marmoset, Callithrix jacchus, express AMPA receptors. *J Comp Neurol.* **502,** 442–454 (2007).
38. Bowie, D. & Smart, T. G. Thiocyanate ions selectively antagonize AMPA-evoked responses in Xenopus laevis oocytes microinjected with rat brain mRNA. *Br J Pharmacol.* **109,** 779–787 (1993).
39. Fisher, J. L. & Mott, D. D. Modulation of homomeric and heteromeric kainate receptors by the auxiliary subunit Neto1. *J Physiol.* **591,** 4711–4724 (2013).
40. Vielma, A. *et al.* Nitric oxide amplifies the rat electroretinogram. *Exp Eye Res.* **91,** 700–709 (2010).
41. Ding, J. D. & Weinberg, R. J. Distribution of soluble guanylyl cyclase in rat retina. *J Comp Neurol.* **502,** 734–745 (2007).
42. Suzuki, S., Tachibana, M. & Kaneko, A. Effects of glycine and GABA on isolated bipolar cells of the mouse retina. *J Physiol.* **421,** 645–662 (1990).
43. Grunert, U. Distribution of GABA and glycine receptors on bipolar and ganglion cells in the mammalian retina. *Microsc Res Tech.* **50,** 130–140 (2000).
44. Haverkamp, S., Muller, U., Zeilhofer, H. U., Harvey, R. J. & Wassle, H. Diversity of glycine receptors in the mouse retina: localization of the alpha2 subunit. *J Comp Neurol.* **477,** 399–411 (2004).
45. Eggers, E. D., McCall, M. A. & Lukasiewicz, P. D. Presynaptic inhibition differentially shapes transmission in distinct circuits in the mouse retina. *The J Physiol.* **582,** 569–582 (2007).
46. Wassle, H. *et al.* Glycinergic transmission in the Mammalian retina. *Front Mol Neurosci.* **2,** 6 (2009).
47. Shields, C. R., Tran, M. N., Wong, R. O. & Lukasiewicz, P. D. Distinct ionotropic GABA receptors mediate presynaptic and postsynaptic inhibition in retinal bipolar cells. *J Neurosci.* **20,** 2673–2682 (2000).
48. Euler, T. & Wassle, H. Different contributions of GABAA and GABAC receptors to rod and cone bipolar cells in a rat retinal slice preparation. *Jof Neurophysiol.* **79,** 1384–1395 (1998).
49. Ivanova, E., Muller, U. & Wassle, H. Characterization of the glycinergic input to bipolar cells of the mouse retina. *Europ J Neurosci.* **23,** 350–364 (2006).

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Author Contributions

A.H.V. performed the experiments while O.S. supervised the study. Both authors participated in data analysis, manuscript writing and revision.

Additional Information

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Brief Communications

Metabotropic Glutamate Receptors Induce a Form of LTP Controlled by Translation and Arc Signaling in the Hippocampus

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Activity-dependent bidirectional modifications of excitatory synaptic strength are essential for learning and storage of new memories. Research on bidirectional synaptic plasticity has largely focused on long-term potentiation (LTP) and long-term depression (LTD) mechanisms that rely on the activation of NMDA receptors. In principle, metabotropic glutamate receptors (mGluRs) are also suitable to convert synaptic activity into intracellular signals for synaptic modification. Indeed, dysfunction of a form of LTD that depends on Type I mGluRs (mGluR-LTD), but not NMDARs, has been implicated in learning deficits in aging and mouse models of several neurological conditions, including Fragile X syndrome and Alzheimer's disease. To determine whether mGluR activation can also induce LTP in the absence of NMDAR activation, we examined in hippocampal slices from rats and mice, an NMDAR-independent form of LTP previously characterized as dependent on voltage-gated Ca^{2+} channels. We found that this form of LTP requires activation of Type I mGluRs and, like mGluR-LTD but unlike NMDAR-dependent plasticity, depends crucially on protein synthesis controlled by fragile X mental retardation protein and on Arc signaling. Based on these observations, we propose the coexistence of two distinct activity-dependent systems of bidirectional synaptic plasticity: one that is based on the activity of NMDARs and the other one based on the activation of mGluRs.

Key words: arc; bidirectional; metabotropic glutamate receptors; protein synthesis; synaptic plasticity; translation

Significance Statement

Bidirectional changes of synaptic strength are crucial for the encoding of new memories. Currently, the only activity-dependent mechanisms known to support such bidirectional changes are long-term potentiation (LTP) and long-term depression (LTD) forms that rely on the activation of NMDA receptors. Metabotropic glutamate receptors (mGluRs) are, in principle, also suitable to trigger bidirectional synaptic modifications. However, only the mGluR-dependent form of LTD has been characterized, there we report that an NMDAR-independent form of LTP, initially characterized as dependent on voltage-gated Ca^{2+} channels, also requires the activation of mGluRs. These finding suggest the coexistence of two distinct activity-dependent systems of bidirectional synaptic plasticity: one that is based on the activity of NMDARs and the other one based on the activation of mGluRs.

Introduction

The capacity of synapses to undergo lasting increases or decreases in strength in response to activity patterns is thought to be essential for the process of learning and memory formation. Currently, the most comprehensive (and most studied) models of bidirectional synaptic modification are activity-dependent forms of long-term potentiation (LTP) and long-term depression (LTD) that require the activation NMDA receptors to initiate insertion or removal of AMPA receptors from the synapse (Bliss and Collingridge, 1993). The focus on NMDAR-dependent LTP and LTD (NMDAR-LTP/D) results in part because their molecular mechanisms have been worked out to a great [illegible]

Annex 4
Organization of Scientific Events

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BIOLOGY OF NEUROPSYCHIATRIC DISORDERS

April 27 -29, 2016 Valparaíso, Chile

SPEAKERS

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Pablo Castillo, USA
Pablo Moya, Chile
Andrew Holmes, USA
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9: 00 Welcome and Introduction
9: 30 Teymuras Kurzchalia
10:10 Marek Mlodzik
10:50 Claire Benard
11:30 -11:50 Coffee break
11:50 Brian Smith
12:30 Geraldine Wright
13:10-14:30 Lunch
14:30 Mark Alkema
15:10 Patrick Emery
15:50 Carolina Rezaval
16:30-16:45 Coffee Break
16:45 Ulrike Heberlein
17:30 Scott Waddell
18:10 Closing Remarks
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Annex 7.1
Outreach activities throughout the period


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Andrés Chávez (Neurocientífico del CINV)
Jorge Dahm (Ministro de la Corte Suprema)
Annelise Dörr (Doctora en Psiquiatría)
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Cristián Warnken
ORGANIZA
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27 SEPT
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Ziley Mora (Especialista cosmovisión ancestral mapuche)
Armando Roa (Poeta, abogado y profesor de literatura)
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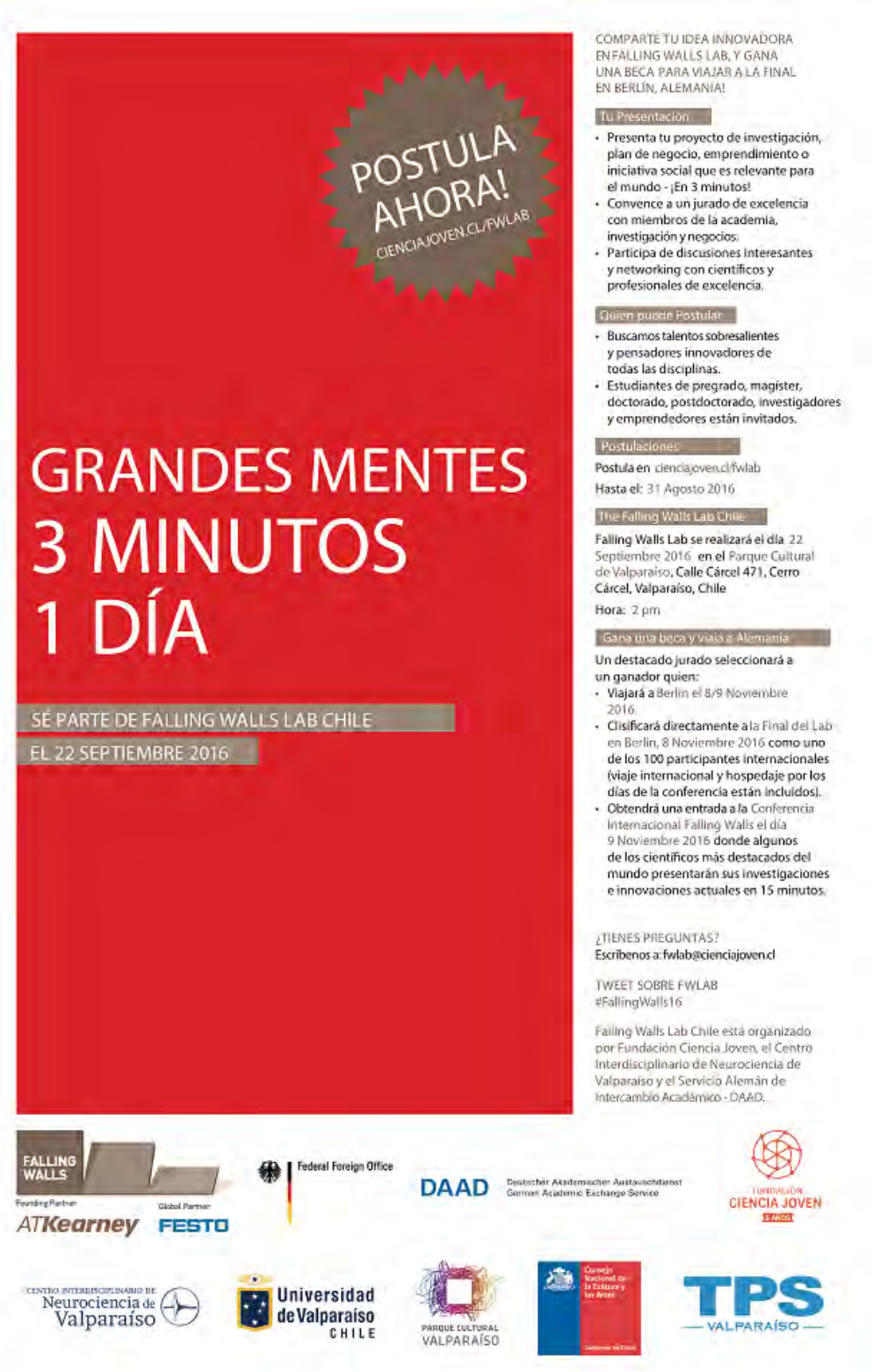
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Annex 7.3
Articles and Interviews

1) **Interview with Dr. Andrés Chávez about the Cannabinoides**
   Newspaper: The Clinic
   Date: March 16, 2016
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EXPERTO EN SUSTANCIAS CANNÁBICAS

Andrés Chávez, neurocientífico: "Los cannabinoides son nuestro cable a tierra"

Recién en 1991 se descubrió el sistema endocannabinoide, a través del cual sustancias muy parecidas al THC regulan nuestro cerebro. En Chile, nadie conoce mejor sus efectos que Andrés Chávez (38), quien después de trece años en Estados Unidos –hizo un postdoctorado en el Albert Einstein College of Medicine– volvió al país en 2015 para sumarse al Centro Interdisciplinario de Neurociencia de Valparaíso (CINV), donde hoy lidera una investigación sobre este "cannabis endógeno" y los posibles beneficios de la planta. Aquí explica qué se sabe hasta ahora y lamenta las "cabezas de pescao" que aún se dicen en Chile acerca de la marihuana, sobre todo los siquiatras de la tele. El 10 de abril estará en el Festival de Ciencia de Antofagasta organizado por Puerto de Ideas.

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María Elena Troncoso, la abuela grafitera que lucha contra el aborto: "Espero que Bachelet pare la matanza"

2) **Interview to Dr. John Ewer about the criticism for the changing the local time.**
Web page: Diario Electrónico el Mostrador
Date: May 19, 2016
Link:http://cinv.uv.cl/cientifico-critica-cambio-de-horario-por-insuficiente-y-advierte-DDAsobre-efectos-negativos-sobre-la-salud/
Scope: National

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NOTICIAS CIENCIA

John Ewer, de Instituto Milenio CINV de Valparaíso

Cultura

Científico critica cambio de horario por "insuficiente" y advierte sobre efectos negativos sobre la salud

por VÍCTOR COSSIO Y MARCO SAJARDO | 19 mayo, 2016

"Sería ideal que Chile adopte como horario único, el de invierno", puntualizó investigador del Centro Interdisciplinario de Neurociencia, de la Universidad de Valparaíso.

"La propuesta del gobierno por el cambio de horario, sigue siendo insuficiente", afirma enfático el Dr. John Ewer, científico del Instituto Milenio, Centro Interdisciplinario de Neurociencia, de la Universidad de Valparaíso (CINV). Las declaraciones se realizaron ante la inminente llegada del horario de invierno, medida que comenzó a regir el pasado sábado.

Si bien a raíz de esto, los chilenos nos despertaremos una hora más tarde y con menos oscuridad, para el investigador, lo óptimo sería mantener un solo horario durante todo el año, retrasando en 120 minutos relojes.

Videos

[VIDEO] Un descuido desafortunado: el hombre al que se le cayó una bolsita de cocaína ante el juez

[VIDEO] Los primeros 100 días de Donald Trump vistos por la crítica de "Los Simpsons"

[VIDEO] "Pude haber sido yo": el hijo de Tarek William Saab, Defensor del Pueblo, condena la represión en Venezuela y el hijo del presidente Maduro le responde

[VIDEO] El pueblo sirio en el que derrotaron a Estado Islámico y que muestra por qué la guerra en Siria no acabará pronto

Más Noticias

Barraza acusa campaña de desinformación por perdonazo del SII: "Está en juego el prestigio que nos hemos ganado con nuestro trabajo riguroso y responsable"

3) **"Brilliant minds", with Dr. Ramón Latorre**
Web page: Diario Electrónico el Mostrador
Date: July 15, 2016
Link://cinv.uv.cl/mentes-brillantes-acusan-que-la-crisis-de-la-ciencia-no-es-un-problema-solo-de-plata-sino-de-miopia-politica-y-cultural/
Scope: National

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NOTICIAS | CIENCIA

Junto a este artículo se emite el segundo capítulo de la serie "Mentes brillantes" dedicado al biólogo y Premio Nacional Ramón Latorre

Cultura

"Mentes brillantes" acusan que la crisis de la ciencia no es un problema solo de plata, sino de miopía política y cultural

por MATÍAS RIVAS R. Y HÉCTOR COSSIO | 14 julio, 2016

Que el año pasado, científicos jóvenes y experimentados investigadores denunciaran, mediante protestas, la profunda crisis que vive el sector, no fue obra de la impulsividad asociada simplemente a la falta de recursos o a su estancamiento. Lo que se intentó remarcar, involucraba un problema mayor: la subestimación de la importancia de la ciencia en el desarrollo económico del país. En este artículo, Premios Nacionales y parlamentarios debaten sobre los polos más críticos del financiamiento. Conicyt, en tanto, niega que la ciencia chilena esté en crisis.

El 2015 quedó como el año en que los científicos -doctores cesantes, postdoctorados a boleta y jefes de centros o núcleos de investigación que ven cómo su financiamiento agoniza- dijeron: ¡Ya basta!

Vestidos con batas blancas y con la consigna "Nuestros gobiernos han elegido la ignorancia", más de 300 científicos protestaron en diferentes puntos del país, llamando incluso la atención de las prestigiosas revistas científicas *Nature* y *Science*, que dedicaron sendos artículos a la crisis de financiamiento en Chile, un escenario en bancarrota que resultaba ser absolutamente insólito para los países miembros de la OCDE, de cual Chile es parte.

¿Dónde están los polos críticos del financiamiento de la ciencia? ¿Es solo un

Islámico y que muestra por qué la guerra en Siria no acabará pronto

Más Noticias

Barraza acusa campaña de desinformación por perdonazo del SII: "Está en juego el prestigio que nos hemos ganado con nuestro trabajo riguroso y responsable"

Ossandón afila el cuchillo de cara a las primarias de Chile Vamos: "Yo puedo salir a la calle, mi contendor no"

Caval: Sebastián Dávalos es sobreseído por acusaciones de delito informático

4) **CORE approved the remaining 2700 million pesos to construct the building Abate Molina.**
Newspaper: El Mercurio
Date: September 23, 2016
Link: http://cinv.uv.cl/core-aprueba-los-2700-millones-de-pesos-que-faltaban-para-construir-el-edificio-molina/
Scope: National

EL MERCURIO DE VALPARAÍSO

Alcaldes que postulan a reelección dejan cargos para iniciar campañas

Región. Aprueban fondos para Instituto de Neurociencia y posta en Pascua

Fiscal Gómez decide pedir formalización de ME-O

Bianchi daría el voto clave para elección de intendentes

Crece pugna por obras inconclusas en G. Carreño

Murga uruguaya estrena obra que mezcla estilos y rompe esquemas

CEDILLO: "NO NOS EQUIVOCAMOS AL CONFORMAR EL PLANTEL"

8 | Actualidad

Core aprueba casi $ 6 mil millones para equipamiento en salud pública y obras

REGIÓN. Centro de Neurociencia de la UV recibirá $ 2.476 millones adicionales

5) In Valparaíso set up a new scientific Alliance
Newspaper: El Mercurio de Valparaíso
Date: September 23, 2016
Link:http://cinv.uv.cl/establecen-nuevas-alianzas-cientificas-en-valparaiso/
Scope: National

Vida social

Establecen nuevas alianzas científicas en Valparaíso

6) **Neuronews : "Scientists alter ideological, political and religious conceptions, using magnetic stimulation of the brain"**
Webpage: Diario Electrónico El Mostrador
Date: August 18, 2016
Link:http://cinv.uv.cl/cientificos-alteran-concepciones-ideologicas-politicas-y-religiosas-usando-estimulacion-magnetica-del-cerebro/
Scope: National

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NOTICIAS | CIENCIA

Convenio con el Centro Interdisciplinario de Neurociencia de la Universidad de Valparaíso

Cultura

Científicos alteran concepciones ideológicas, políticas y religiosas, usando estimulación magnética del cerebro

por ADOLFO AGURTO/CINV | 10 agosto, 2016

Neurocientíficos de EE.UU. concluyeron que la estimulación de una región del cerebro, efectivamente afecta la opinión o valoración de ideologías luego de un estímulo amenazante, como lo es el recordatorio de su propia muerte. Los resultados demostraron que la estimulación magnética aumentó la valoración de las ideologías antinacionalistas en un 28%, pero no así la tendencia en las ideologías pro nacionalistas. Sin embargo, los autores afirman que se necesitan estudios específicos al respecto, como el uso de otros estímulos o si, en ausencia de estos, también se modifica la valoración de ideologías.

Videos

[VIDEO] La BBC explica las claves para entender la crisis en Venezuela: cómo empezó y cómo podría acabar

[VIDEO] Un descuido desafortunado: el hombre al que se le cayó una bolsita de cocaína ante el juez

[VIDEO] Los primeros 100 días de Donald Trump vistos por la crítica de "Los Simpsons"

[VIDEO] "Pude haber sido yo": el hijo de Tarek William Saab, Defensor del Pueblo, condena la represión en Venezuela y el hijo del presidente Maduro le responde

7) Neuroscientist teach children how to build an solar oven with an umbrella
Newspaper: Las Últimas Noticias
Date: December 16, 2016
Link:http://www.lun.com/Pages/SupplementDetail.aspx?dt=2016-12-17&SupplementID=55&BodyID=0
Scope: National

maestra

Kathleen Whitlock además pelea contra el machismo

La gringa que enseña ciencia a los niños de Valparaíso

Llegó a Valparaíso a trabajar como profesora en un doctorado y rápido se dio cuenta de que en Chile ser mujer y científica "es lo más chancho en misa que hay".

Valeria Milesi

[illegible]

"Me he encontrado con chicas que me dicen que ellas no asisten a eventos después de las 18 horas porque se dedican a sus hijos. Eso no está bien, tienen que dejar que los hombres se hagan cargo".