Pacemaking in Mouse Locus Coeruleus Neurons: Electrophysiological Properties, Role of Mitochondria and Development

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Statement of originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

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Acknowledgement of Authorship

I hereby certify that the work embodied in this thesis contains a published paper/s/scholarly work of which I am a joint author. I have included as part of my thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publications.

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Paper	Contribution	Signature
de Oliveira, R. B.; Graham, Brett; Howlett, Marcus C.H.; Gravina, Fernanda S.; Oliveira, Max W.S.; Imtiaz, Mohammad S.; Callister, Robert J.; Lim, Rebecca; Brichta, Alan M.; van Helden, Dirk F.. Ketamine anesthesia helps preserve neuronal viability. Journal of Neuroscience Methods, v. 189, p. 230-232, 2010. DOI: 10.1016/j.jneumeth.2010.03.029	80% of the total	Dirk F. van Helden
de Oliveira, Ramatis B.; Howlett, Marcus C.H.; Gravina, Fernanda S.; Imtiaz, Mohammad S.; Callister, Robert J.; Brichta, Alan M.; van Helden, Dirk F.. Pacemaker currents in mouse Locus Coeruleus neurons. Neuroscience, 2010. DOI: 10.1016/j.neuroscience.2010.06.028	80% of the total	Dirk F. van Helden
de Oliveira, Ramatis B.; Gravina, Fernanda S.; Brichta, Alan M.; Callister, Robert J.; van Helden, Dirk F.. Effects of mitochondrial disruption on ionic currents and pacemaking in neurons of the locus coeruleus. *Manuscript under review in "Journal of Physiology".*	90% of the total	Dirk F. van Helden

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Table of Contents

List of Abbreviations

ACSF - artificial cerebrospinal fluid

AHP – after hyperpolarization

AP - Action potential

CCCP - carbonyl cyanide m-chlorophenylhydrazone

CICR - Ca^{2+}-induced Ca^{2+} release

ChTx – Charybdotoxin

CNS – Central nervous system

DDT - Dichlorodiphenyltrichloroethane

DMSO - Dimethyl sulfoxide

EGTA - Ethylene glycol tetra acetic acid

FCCP - p-trifluoromethoxyphenylhydrazone

I_A - fast transient K^+ current (I_A)

I_{Cl} – chloride current

I_H - hyperpolarization-activated current

$I_{K\text{-}Ca}$ - Ca^{2+}-activated K^+ current

K_{Ca} - Ca^{2+}-activated K^+ channel

K_{IR} - K^+ inward rectifier current

LC – Locus coeruleus

MC – Membrane capacitance

MTC – Motor cortex

MMP – Mitochondrial membrane potential

NMDG - N-methyl-D-glucamine chloride

MNCE - Mitochondrial Na^+/Ca^{2+} exchanger

PBS - Physiological buffer solution

PD – Parkinson's diseases

R_{IN} – input resistance

SN – Substantia nigra

TEA – Tetraethylammonium chloride

TH – Tyrosine hydroxylase

TTX – Tetrodotoxin

Vm – membrane potential

4-AP - 4-Aminopyridine

$[Ca^{2+}]_c$ - cytosolic Ca^{2+} concentration

Ψm - Mitochondrial membrane potent

Abstract

This thesis presents studies on the electrophysiological properties of Locus coeruleus (LC), a group of noradrenergic neurons present in the brain stem pons. The specific interest in this neuronal population came from the fact that this neuronal population accounts for the largest noradrenergic nucleus present in the brain, providing noradrenaline input to virtually all cerebral regions and involved in controlling or modulating many types of behaviours. Another important feature is the recent "rediscovery" that LC neurons are involved in Parkinson's disease (PD), where loss of activity and/or decrease in the neuronal population are thought to be a fundamental step to the progression and maintenance of this pathology.

LC neurons release many different types of neurotransmitters, noradrenaline, however, being the most studied. Regardless, of the neurotransmitter involved, release is controlled by intrinsic spontaneous firing activity, which is the result of a combination of pacemaker currents in balance with each other. These currents determine membrane depolarization and action potential initiation, and are responsible for adjusting the neuronal firing rate according to system needs. Even though the electrophysiological properties of LC neurons have been studied for decades, the mechanisms controlling and generating the pacemaker process need further investigation. As this process is a key feature underlying the influence exerted by LC neurons, a better understanding of this process is fundamental to determining how the brain functions and how impairment of LC activity may lead to neurological disorders. For example, it has been demonstrated in PD that along with the massive loss of Substantia Nigra (SN) neurons, the noradrenergic signalling provided by LC neurons to

the SN also suffers a massive impairment. Recently, it was suggested that noradrenaline exerts a neuronal protective effect against oxidative stress and inflammation. This is of great significance, as oxidative stress and inflammation are thought to be significantly involved in the development and maintenance of PD. These finding led to the proposal that impairment in the noradrenergic signalling provided by LC neurons to the SN could be a trigger in the generation and progression of PD.

All these facts inspired me to undertake detailed investigations into LC neuronal function. My aim was to first establish a solid understanding of the pacemaker process and then integrate this knowledge into a broader context. By choosing the mouse model this would allow future detailed studies using targeted mutants. I began by investigating the cellular basis of spontaneous firing of LC neurons with special emphasis on understanding the underlying pacemaker mechanisms, particularly those involved in action potential generation. In addition, I studied the effect of age and the possible involvement of mitochondria as a regulator of this process.

The first research chapter (see chapter 3) presents a methodological approach that was found to significantly improve the viability of LC neurons. This facilitated our electrophysiological studies and should be of general use for all those investigating brain neurons *in vitro*. LC neurons are medium-sized cells with large dendritic arbours. As a result, achieving proper voltage control in voltage clamp experiments in slice preparations is a challenge. It was found that the ketamine-based anaesthesia improved neuronal viability by substantially increasing the membrane input resistance and rate of success in voltage clamp experiments. This was not only found to be the case for LC neurons but was also confirmed in another neuronal population, namely hypoglossal motor neurons.

The second research chapter (chapter 4) characterizes the combination of pacemaker currents responsible for establishing depolarization during the interspike interval voltage range in mouse LC neurons. By using a series of voltage clamp approaches, I demonstrated that the composition of currents included a TTX-sensitive voltage-dependent Na^+ conductance, a high TEA-sensitive K^+ conductance, but no Ca^{2+} conductance. Interestingly, this complement of currents is different to rat LC neurons, the most studied LC preparation, which exhibit an important persistent pacemaker Ca^{2+} current close to the threshold for action potential initiation. A lack of a persistent Ca^{2+} current in mice could represent a significant temporal change in all Ca^{2+}-dependent pathways giving a different perspective for cellular experiments carried out in the two species. This reminds us that direct comparisons between different animal species should be done with care.

The third research chapter (chapter 5) investigates developmental changes in LC pacemaker currents. The two basic conductances (i.e. Na^+ and K^+), responsible for controlling the membrane depolarization underwent changes during development. However, once again there was no evidence for Ca^{2+} conductance in the pacemaker-interval voltage range in LC neurons, either infant or adult mice. Changes in the basic pacemaker currents were accompanied by other changes such as differences in TTX-insensitive spiking activity and the levels of baseline voltage noise. This chapter presents the first detailed report on the way development induces electrophysiological changes in mouse LC neurons.

The fourth paper (chapter 6) of this thesis presents an investigation into the possible participation of mitochondria as an active modulator of LC pacemaking. Aspects of mitochondria were investigated for two main reasons: 1) they act as significant Ca^{2+}

stores and are involved in controlling the intracellular Ca^{2+} dynamic in many different cells, hence they may impact on the pacemaking process of LC neurons; and 2) many neuropathologies such as PD have mitochondrial dysfunction as primary cause, thus the understanding of the role played by mitochondria in the pacemaking process could pave the way to discover new clinical approaches that may be beneficial at very early stages of the disease. Specifically, it was found that disturbance of mitochondrial metabolism activated Ca^{2+} channels that, in turn, activated K^+ channels, which resulted in changes in the spontaneous firing activity of LC neurons. Two significant observations were made. The first was that Ca^{2+} channels activated by mitochondria were nifedipine sensitive, suggesting these were L-type Ca^{2+} channels. However, these voltage-dependent channels were activated at unusual hyperpolarized membrane potentials indicating an alternative pathway for their activation. The second observation was that activation of K^+ channels involved Ca^{2+} entry but was independent of intracellular Ca^{2+} release. These findings are of significant interest since they demonstrate a pathway for K^+ channel activation that is different than previously described by other investigators. Moreover, the fact that mitochondria may be involved in the pacemaker process, by not only buffering internal Ca^{2+} but also modulating ionic channels, indicates an exciting new perspective that could be applied to studies involving PD.

In summary, this thesis has characterized conductances responsible for controlling neuronal pacemaking in infant and adult mouse LC. Investigations of inhibiting mitochondrial function led to the proposal that mitochondria may serve as fine tune regulators of LC pacemaking. These results constitute a detailed description of fundamental electrophysiological properties of mouse LC neurons, which provide a foundation for future LC studies using mutant mice and thus providing new insights into mechanisms that control LC pacemaking. Going still further, the results presented here

can be applied to the study of the mechanisms underlying the progression of neurological diseases, such as PD, where there is direct involvement of LC neurons and their mitochondria.

Publications

The following publications have arisen from data presented in the present thesis:

Journal Articles

- **de Oliveira, R. B.;** Graham, Brett; Howlett, Marcus C.H.; Gravina, Fernanda S.; Oliveira, Max W.S.; Imtiaz, Mohammad S.; Callister, Robert J.; Lim, Rebecca; Brichta, Alan M.; van Helden, Dirk F.. Ketamine anesthesia helps preserve neuronal viability. Journal of Neuroscience Methods, v. 189, p. 230-232, 2010. DOI: 10.1016/j.jneumeth.2010.03.029

- **de Oliveira, Ramatis B.;** Howlett, Marcus C.H.; Gravina, Fernanda S.; Imtiaz, Mohammad S.; Callister, Robert J.; Brichta, Alan M.; van Helden, Dirk F.. Pacemaker currents in mouse Locus Coeruleus neurons. Neuroscience, 2010. DOI: 10.1016/j.neuroscience.2010.06.028

- **de Oliveira, Ramatis B.;** Gravina, Fernanda S.; Brichta, Alan M.; Callister, Robert J.; van Helden, Dirk F.. Effects of mitochondrial disruption on ionic currents and pacemaking in neurons of the locus coeruleus. *Manuscript under review in "Journal of Physiology".*

- **de Oliveira, Ramatis B.;** Gravina, Fernanda S.; Brichta, Alan M.; Callister, Robert J.; van Helden, Dirk F. Developmental changes in pacemaker currents in mouse locus coeruleus neurons. *Manuscript under construction to be sent for publication.*

Conference Abstracts

- Gravina, Fernanda S.; **de Oliveira, R. B.;** Graham, Brett; Howlett, Marcus C.H.; Oliveira, Max W.S.; Imtiaz, Mohammad S.; Callister, Robert J.; Lim, Rebecca; Brichta, Alan M.; van Helden, Dirk F.. Ketamine anaesthesia helps preserve neuronal viability. In: 28th International Australasian Winter Conference on Brain Research 2010, Wanaka, New Zealand, 2010.

- **de Oliveira, Ramatis B.;** Howlett, Marcus C.H.; Gravina, Fernanda S.; Imtiaz, Mohammad S.; Callister, Robert J.; Brichta, Alan M.; van Helden, Dirk F.. Pacemaker Calcium Currents: Difference in Locus Coeruleus Neurons of Mice and Rats. In: 28th International Australasian Winter Conference on Brain Research 2010, Wanaka, New Zealand, 2010.

- **de Oliveira, R. B.;** Howlett, M.; Gravina, F. S.; Callister, R.J.; Brichta, A.M.; van Helden, D.F. Properties of Locus coeruleus neurons in infant and adult mice. In: ANS/AuPS 2010 the 30th Annual Meeting of the Australian Neuroscience Society, in conjunction with the 50th Anniversary Meeting of the Australian Physiological Society, 2010, Sydney, 2010.

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Physiological Sciences (IUPS2009) Function of Life: Elements and Integration, 2009, Kyoto. The Journal of Physiological Sciences, v.59, 2009.

- **de Oliveira, R. B.;** Howlett, M.; Gravina, F. S.; Imtiaz, M.S.; Callister, R.J.; Brichta, A.M.; van Helden, D.F.. Influence of mitochondria in the interspike interval pacemaking currents of mice Locus Coeruleus neurons. In: 2008 Annual Meeting of the Australian Physiological Society, Melbourne, 2008.

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