University of Glasgow | College of Medical, Veterinary & Life Sciences

Head of College Scholars List Scheme

Summer Studentship

Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: jill.morrison@glasgow.ac.uk within four weeks of the end of the studentship.

1. **Student**

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2. **Supervisor:**

   Surname: Todd Forename: Andrew

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3. **Research Project Report**

   3.1 Project Title (maximum 20 words):

   Characterization of calretinin-expressing interneurons in the spinal dorsal horn

   3.2 Project Lay Summary (copied from application):

   The spinal cord receives information from nerve fibres that respond to various types of sensory stimulus, including stimuli that are normally perceived as painful or itchy. This is processed through complex circuits involving nerve cells that can either transmit or suppress this information. Recent work has shown that these cells can be assigned to functional populations that have specific roles in sensory processing, for example suppressing certain types of pain. This project will characterise a large population of nerve cells that have recently been identified based on their expression of the calcium-binding protein, calretinin.

   3.3 Start Date: 28/06/18 Finish Date: 31/07/18

   3.4 Original project aims and objectives (100 words max):

The main aim of this research project is to characterise the superficial dorsal horn excitatory interneurons that express calretinin. To do this, we will investigate if there is any co-localization between the protein and two neuropeptides: neurotensin (NTS) and gastrin-releasing protein (GRP). The reason for this is that NTS+ and GRP+ cells have been proposed to be two of the non-overlapping populations of excitatory interneurons described in laminae I and II.

3.5 Methodology: Summarise and include reference to training received in research methods etc. (250 words max):

Co-expression between CR and NTS/GRP was determined via multilabel immunofluorescence. Three Wild-Type and three transgenic GRPeGFP mice were used in this study. 60µm transverse sections were dissected from the spinal cord lumbar region (L1-L3) using the vibrating blade microtome.

Sections were treated with 50% ethanol solution to improve antibody penetration. Table 1 illustrates the antibody combinations, primary and secondary, and their dilutions.

	Primary Antibodies Dilutions (5 overnights incubation)		**Secondary Antibodies Dilutions (1 overnight incubation)**	
Reaction 1	**PKCγ (rb)**	1:500	**rb-PB**	1:200
	CR (gt)	1:1K	**gt-A488**	1:500
	NT (rat)	1:5K	**rat-RR**	1:100
	NeuN (mo)	1:500	**mo-A648**	1:500
Reaction 2	**GFP (ch)**	1:1K	**ch-A488**	1:500
	CR (gt)	1:1K	**gt-RR**	1:100
	Pax2 (rb)	1:1K	**rb-A647**	1:500

Table 1: Antibody dilutions for the indirect detection of CR, GFP and NTS. The samples used for immunoreaction 2 belonged to transgenic animals whose GRP+ cells were manipulated to express green fluorescent protein (GFP). Additional biomarkers protein kinase Cγ (PKCγ), neuronal marker N (NeuN) and transcription factor Pax2. NeuN is present in the nuclei of all neurons and lamina II, PKCγ is present in virtually all NTS cells. Because of this PKCγ+NeuN+ cells can be used to identify those that are NTS+. Tissue sections were rinsed with double salt phosphate buffer solution (PBS) before every incubation as well as after the last one. All antibody cocktails were made in 5% normal donkey serum (NDS) in PBS and 0.3% triton X-100 to further enhance antibody penetration.

Sections were mounted using Vectashield (which delays photobleaching) and scanned with the confocal microscope through the whole depth of the tissue. Confocal image stacks were analysed with Neurolucida for confocal. Due to its “restricted to lamina-II” expression, CR was employed to delimit laminae I-II.

In Reaction 1, because of the high co-expression of PKCγ on NTS in laminae I-II, the former was employed to facilitate the identification of NTS+ cells though all the depth of the tissue, and then categorized as CR+ or CR-.

For Reaction 2, GRPeGFP+ cells were identified through all tissues' depth and labelled as CR+ or CR-. Due to the identification of GRPeGFP+CR+ cells, a second analysis was carried out via the dissector method. The reason: the number of CR-immunoreactive neurons was too great to count through all the tissues' depth and with the dissector we could still be able to see the unbiased proportion of overlap. CR+ cells within a 20μm range were marked and characterised as GRPeGFP+ or GRPeGFP-. Because a small percentage of CR+ interneurons is inhibitory we used Pax2 (marker for inhibitory interneurons) to distinguish them.

3.6 Results: Summarise key findings (300 words max). Please include any relevant tables or images as an appendix to this report:

As the Table 2 below shows, in the case of sections belonging to immunoreaction 1, 91 (81-111) NTS expressing cells were counted. None of these cells were found to be CR+ and as a result, there was a 0% co-localization between the two neuromarkers.

	Total number of peptide neurons examined	**Total number of CR+ examined**	**% of peptide neurons with CR**	**% of CR+ neurons with peptide**	**% of Pax2-/CR+ neurons with peptide**
NT+	91 *(81-111)*	-	0%	-	-
GRPeGFP+	217 *(174-256)*	222 *(172-263)*	55% *(48.9-61%)*	18.9% *(17.1-21.1%)*	21.7% *(18.5-24.6%)*

Table 2: Co-expression of neuropeptides with CR. In the case of GRP, only two tissue sections were analysed per animal. For NT, it was three sections per animal.

The first analysis concerning GRPeGFP revealed a total number of 217 (174-256) GRPeGFP+ neurons through all the tissues depth. A similar number of was counted in the case of CR+ (second analysis), 222 (172-263) cells. Around 55% of GRP interneurons counted were CR+. Conversely, a 19% of CR+ were found to be GRPeGFP+. We saw that 15% of CR+ were also Pax2+. After their exclusion, the percentage of excitatory CR+ that were also GFP+ ascended to 21.7%.

3.7 Discussion (500 words max):

In general terms, the superficial dorsal horn is a waypoint, an area where sensory information from primary afferent fibers is relayed to projection neurones. The actual situation though, is far more complex, mainly due to the existence of interneurons, which constitute around 90-95% of the nerve cells in laminae I and II.

Interneurons make up elaborate local circuits which, have been shown to be involved in many aspects of somatosensation, including the processing of nociceptive input. Considering that pain control is one of the top priorities in any medical procedure, it is no wonder that targeting these circuits could lead to the development of more effective analgesics. This possibility however, is still out of reach principally due to the limited knowledge we currently have about the neural organization of the dorsal horn. It is for this reason that over the

years, the identification of the different interneuron populations in the dorsal horn has become a major concern in the field of neuroscience.

Within the superficial laminae, the characterization of interneurons has been attempted both morphologically and electrophysiologically. Unfortunately, the results obtained are sometimes inconclusive and difficult to interpret. Another approach is examining the pattern of expression of certain neurochemicals via immunohistochemistry. Thanks to this technique, it is now known that calretinin, one of the proteins of interest if this study, is widely expressed in the superficial laminae.

In fact, it has been suggested that ablation of calretinin-expressing cells resulted in increased withdrawal threshold for von Frey hairs. Despite these findings, our group believes that suggesting that CR+ neurons should not be treated as a homogeneous population. This was the starting point of this study.

By looking for markers that are expressed in non-overlapping subsets, we can identify discrete interneuron populations. In the case of excitatory interneurons, up to four independent groups, making up 50% of excitatory interneurons, have been recognized: neurotensin, GRP, neurokinin B and substance P. Comparison of the expression of these markers, in this study we focused on NTS and GRP, with CR revealed no co-expression between the first marker and the calcium-binding protein. On the other hand, 18.9% of CR+ neurons also express GRP, ascertaining that CR is indeed a heterogenous population.

In addition, this percentage ascended when excluding Pax2, the inhibitory interneuron marker. With this we can conclude that within the calretinin neurons, we find some that are inhibitory (Pax2+) and others that are excitatory (Pax2-) which can either possess or not GRP.

Overall it is clear that once these results are combined with those obtained for neurokinin B and substance P, we will be one step closer to understanding the pattern of expression of calretinin as well as to be able to investigate the function of each of the different groups of neurons that express it.

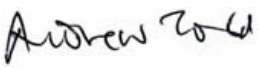
4. Reflection by the student on the experience and value of the studentship (300 words max):

I believe this internship has allowed me to grow as a future neuroscientist. Not only it has boosted my employability prospects, but it has also given me the chance to become familiar with a great range of techniques that are fundamental in the field of research. I have also been able to work as part of a team and get to know what working in a laboratory really is. Overall, I conclude this experience feeling more confident about myself and my skills; better prepared.

Last but not least, I would like to thank the Head of Scholar committee for this wonderful opportunity, as well as my supervisor Dr. Andrew Todd and Dr. Maria Gutierrez-Mecinas for all the support I have received during my time in their laboratory.

5. Dissemination: (note any presentations/publications submitted/planned from the work):

This work forms part of a larger study, which is currently being prepared for submission to the journal Neuroscience (this will include Keila as a co-author).

6. Signatures: Supervisor  Date 11/08/18

Student  Date 07/08/18