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Application R322-2019-1955

Name Walden Bjørn-Yoshimoto
Position Assistant Professor

Institution University of Copenhagen
Institute Department of Biomedical Sciences
Department

Budget subtotals & total

Budget line	Year 1	Year 2	Year 3	Total
Salary - post doc	554.750,00 kr.	566.000,00 kr.	577.250,00 kr.	<u>1.698.000,00 kr.</u>
Running costs	243.600,00 kr.	222.550,00 kr.	212.850,00 kr.	<u>679.000,00 kr.</u>
Total	798.350,00 kr.	788.550,00 kr.	790.100,00 kr.	2.377.000,00 kr.

Project title

Conotoxins as novel neuropeptide receptor modulators

Summary of application

I propose to identify and characterize novel peptides that modulate neuropeptide systems, and are involved in a variety of neurological disorders, including schizophrenia, pain, Parkinson's disease, depression, eating disorders, and anxiety.

Cone snails are venomous marine molluscs, with a vast number of peptide toxins (conotoxins) in their arsenal. Only few of the conotoxins that act by mimicking vertebrate signaling peptides have been described to date. This is not due to absence, but rather to difficulties in identifying them rationally. There is, however, an enormous potential in these. To date, 10 peptides mimicking signaling peptides have been described. Of those, 6 are either used clinically, in clinical development, or have been in clinical development. This is a uniquely high rate, something I aim to exploit in this project.

To overcome challenges of identifying conotoxins of interest, I have recently developed a novel computational tool that was able to detect 85% of positive controls in a small library. This, together with my, and our collaborators, expertise in the fields of molecular pharmacology, cone snail biology, GPCR function, and peptide structural studies, I aim to

identify and characterize novel conotoxins from a unique library of 30,000 sequences. Based on previous findings, we expect to identify peptides with unique receptor subtype selectivity, as well as increased plasma stability, making them intriguing tool compounds, or even drug leads.

Project start date	2020-04-01
Project end date	2022-03-31

Personal information

First name	Walden
Last name	Bjørn-Yoshimoto
Email	waldenb@gmail.com
Date of birth	1983-06-27
Address	Rødvig Alle 27
Postal code	2650
City	Hvidovre
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Phone number	+45 24494408
Academic degree	PhD
Position	Assistant Professor
Institution	University of Copenhagen

The project

Project title

Conotoxins as novel neuropeptide receptor modulators

Postdoc in Denmark or abroad?

Based in Denmark and planning to stay in Denmark

Scientific achievements/discoveries

During my Master's degree work, I have helped elucidate the trafficking pathway of endogenously internalized dopamine transporter. We found the endocytic pathway in cells transfected with a tagged dopamine transporter, as well as in cultured dopaminergic neurons, simultaneously elucidating the novel biology and establishing a valid model system for further studies. This was described in one publication.

After my Master's, I worked at a small biotech– and pharmaceutical company. Here, I have developed a novel platform to identify compounds that can induce termination-codon readthrough (one patent filed), as well as identified and optimized a bottleneck in a biosynthetic pathway in the production of resveratrol in a yeast strain. This is described in one publication, as well as a patent application. Further contributions were made to pharmaceutical projects including diabetes, obesity and brown adipose tissue conversion, colorectal cancer, as well as to a different project regarding yeast production. However, the specifics are restricted by a non-disclosure agreement.

During my PhD, I have made significant efforts in elucidating and describing a plethora of novel compounds with activity at excitatory amino acid transporters (EAAT). This includes the continued characterization of the first and second class of EAAT1-selective compounds described (which are still the gold-standard in the field of EAAT research). I also identified, and characterized the most EAAT3-selective compound to date, along with a thorough structure-activity relationship. Further, I played an integral role in developing various compounds acting at various EAAT subtypes, as well as compounds with activity at glutamate receptors. In total, these efforts have so far been published in 7 research articles, and two review articles.

After my PhD, I have worked on G protein-coupled receptors (GPCRs), mainly two serotonin receptors. First, I was part of a large international collaboration that described the basis for GPCR polypharmacology, using the serotonin 2C receptor as a model. Subsequently, I have worked on biased signaling of the serotonin 2A receptor in a large multidisciplinary team comprised of in silico chemists, medicinal chemists, structural biologists, and molecular pharmacologists. My practical role was the functional characterization of the different compounds acting at the receptor. The serotonin 2C efforts have been published in one article, with one more in preparation. The serotonin 2A receptor efforts have not yet been published. However, intriguing compounds have been identified, and the first publication is expected to be prepared within 9-12 months.

Finally, my current work is in the field of conotoxins, where I have recently developed a novel bioinformatics approach to search for short peptide sequences mimicking endogenous signaling peptides. This has showed very promising initial results, and this is part of what this current application aims to continue.

Applicant's CV

1. Personal Data

Name: Walden Emil Bjørn-Yoshimoto
 Address: Rødvig Alle 27, 2650 Hvidovre
 Citizenship: Dual US/DK

2. Education

2017 - PhD in Pharmaceutical Sciences
 2010 - MSc in Biochemistry
 2007 - BSc in Biochemistry

3. Current and Most Recent Positions Held

2019 - 2020 Postdoc, Protein Biology Group, Faculty of Science, KU (Position termination date: March 31st, 2020)
 2017 - 2018 Postdoc, Molecular pharmacology group, Faculty of Health and Medical Science, KU
 2013 - 2017 PhD, Molecular pharmacology group, Faculty of Health and Medical Science, KU
 2017 - 2017 Research assistant, Molecular pharmacology group, Faculty of Health and Medical Science, KU (This was a leave of absence from my PhD in order to do the work on the polypharmacology of the 5-HT_{2C} receptor)
 2011 - 2013 Research assistant (2011) and research scientist (2012 - 2013), Evolva A/S

4. Scientific awards and honours

I was awarded a grant to travel to Cairns, Australia to present a poster at a conference (awarded by, and for attending the ISN annual conference, 2015)

5. Scientific focus areas

Molecular and cellular neuroscience. A fair deal of time and effort spent on neurotransmitter transporters, but lately I have moved towards neuronal GPCRs, where I focus on combining my programming skills with my extensive experience with in vitro neuropharmacology.

6. International relations

I have spent time as a researcher in the lab of **Professor Clifford J. Woolf** at **Harvard Medical School**. Most of my time was in close collaboration with the laboratory manager, **Dr. Barrett Lee**, whom I have a great relationship with, and am still in regular contact with (as late as two weeks ago, at the time of writing). Professor Woolf is a global leader

in pain research, and the director of the F.M. Kirby Neurobiology Center and the Neurobiology Program at Boston Children's Hospital. My work here focused on a screen of 50,000 compounds to identify novel inhibitors of a subtype of excitatory amino acid transporters, EAAT3. The work from late 2014 performed here was recently published, showing the most subtype-selective EAAT3 inhibitor to date, accompanied by a thorough structure-activity relationship study of dozens of analogs.

I have spent 7 months in the lab of **Professor Susan G. Amara** at the **National Institutes of Health**. Besides being the scientific director of the National Institute of Mental Health at the NIH, Professor Amara has been a front figure in global research on dopamine-, and excitatory amino acid transporters for decades. In her lab, I worked closely with **Dr. Suzanne M. Underhill** on elucidating the molecular mechanism underlying an amphetamine-induced, dopamine transporter-mediated reorganization of excitatory amino acid transporter 3. Subsequently, I have co-written a review article with Dr. Underhill on the importance of excitatory amino acid transporter 3.

During my time in Denmark I have worked with **Professor Christopf Fahlke**, University of Düsseldorf on characterization of compounds targeting excitatory amino acids transporters. While much of the work is still unpublished, the collaboration includes a published review article on the therapeutic potential of excitatory amino acid transporters.

Recently, I have collaborated with **Professor Bryan L. Roth**, University of North Carolina and **Professor Raymond C. Stevens**, University of Southern California. Professor Roth has a decade long track record of cutting-edge research in the serotonin receptor field, and serves as director for the National Institute of Mental Health Psychoactive Drug Screening Program. Professor Stevens is has helped pioneer high-throughput structural biology, and his work has been essential for pharmacological drug development for decades, as evident by the enormous number of citations from pharmacological studies and patent filings alike. Our collaboration was regarding a study on the structural basis of GPCR polypharmacology, and was published in the journal *Cell* in 2018.

7. Supervision of students

I have a strong interest in mentoring, seeing this as both important and enjoyable. Important because we need new generations of skilled scientists, and enjoyable to see the progress in young people, to be able to give them something useful to carry forward. This can of course be scientific knowledge, understanding, and technical skills, but I see my role as more important than that. Under my tutelage, they should learn how to think scientifically, yes. But they should also learn what drives them. What inspires them. What type of work they want to dedicate their life to. If they can answer that question better after working with me than before, then I think I have added something truly valuable for

them.

I have been official supervisor of two students, one doing a 1-year research project prior to her MSc, the other being a PhD student. For both students I have supervised, I was the co-supervisor, sharing responsibility for the mentoring, as well as the project planning. Meanwhile, I was the *de facto* technical and practical supervisor for both.

What I have found is that it is extremely important to determine what type of person you are supervising. I do not believe there is a “one size fits all”, and I strongly believe that if you try to force this approach, you will be doing the majority of your students a huge disservice. There are of course a few generalizations I can make, but a supervisor needs to be flexible enough to change the implementation of these to suit the personality they are supervising, and aware enough to identify and understand personality types different from their own.

While I start my mentorship in a more guiding role, I would let the student take more and more control over their project as fast as I believe the student can handle, and if possible, move into a role they would perceive me as more of a sparring partner. That is, leave the supervision more hidden to them. If this does not progress natively, I try to nudge the student towards this. While I am of course happy to help in problem solving and determining the direction of the project, I prefer guiding them to their own solutions (of course more so with students that are further in their career than with students that are doing their first project). One place where my experience has shown that I do need to be astutely aware is with the timeline and feasibility. I still prefer if they can (eventually) do this for themselves, but I insist on being in on their plans. If they do not seem feasible, I will let them know. This is not to micromanage, but rather to prevent having an avoidable bad experience with a project that might unnecessarily discouraging the student. The **unavoidable** ones are, in my experience, usually enough.

While the general trend I see in academia is that most supervisor tutors their student towards a faculty position, I don’t think this is the best approach. I try to make the student reflect upon this while doing an academic research project. If they find that they like this type of work and environment, and want to pursue a career within academia, that’s great. If they find that this type of work is not what they want to do, that’s also great. I don’t take that as a failing of mine, if they find that they prefer work outside if academia, rather as a success that they have reflected, and hopefully are able to make a more informed decision than before I supervised them.

As of now, one of my previous students is still in academia, while the other has moved to the biomedical industry. Both state that they have made the right choice, which I take to mean that I have had a measure of success as a mentor.

8. Invited and keynote lectures

None, though my participation and poster presentation in the 2015 annual International

Society for Neurochemistry (ISN) conference was sponsored by the ISN.

9. Research funding

None directly covering research, however, I was awarded travel grants do a scientific visit at the NIH in 2015 (awarded by Augustinus Fonden).

Full publication list for applicant

Asterisk (*) in front of reference indicates first authorship (either single- or shared).
My specific contributions are described within the square brackets [] below each reference

2019

* Wu, P., Bjørn-Yoshimoto, W.E., Staudt, M., Jensen, A.A., and Bunch, L. (2019). Identification and Structure–Activity Relationship Study of Imidazo[1,2-a]pyridine-3-amines as First Selective Inhibitors of Excitatory Amino Acid Transporter Subtype 3 (EAAT3). ACS chemical neuroscience.
[Shared first author, I performed and designed the screening, most of the in vitro pharmacology, I shared responsibilities for selecting analog compounds to test based on rational analysis of previous rounds, as well as co-wrote article.]

Poulie, C.B., Alcaide, A., Krell-Jørgensen, M., Larsen, Y., Astier, E., Bjørn-Yoshimoto, W.E., Yi, F., Syrenne, J.T., Storgaard, M., and Nielsen, B. (2019). Design and Synthesis of 2, 3-trans-Proline Analogues as Ligands for Ionotropic Glutamate Receptors and Excitatory Amino Acid Transporters. ACS chemical neuroscience.
[I did the uptake assays at EAAT1-3, summarized in table 4]

2018

Peng, Y., McCorvy, J. D., Harpsoe, K., Lansu, K., Yuan, S., Popov, P., Qu, L., Pu, M., Che, T., Nikolajsen, L. F., Huang, X. P., Wu, Y., Shen, L., Bjørn-Yoshimoto, W. E., Ding, K., Wacker, D., Han, G. W., Cheng, J., Katritch, V., Jensen, A. A., Hanson, M. A., Zhao, S., Gloriam, D. E., Roth, B. L., Stevens, R. C., and Liu, Z. J. (2018) 5-HT_{2C} Receptor Structures Reveal the Structural Basis of GPCR Polypharmacology. Cell 172, 719-730 e714
[I did the 5-HT_{2C} mutant binding experiments described in "Membrane Preparation and Radioligand Binding" and "Test for RIT Selectivity Mutations", some of data displayed in fig. 6. I shared responsibility for analyzing this data, writing this part (results and M&M).]

Naesby, M., Simón, V.E., Eichenberger, M., Lehka, B.J., Bjørn-yoshimoto, W.E., Jensen, N.B., and Dyekjær, J.D. (2018). Biosynthesis of Phenylpropanoid and Dihydrophenylpropanoid Derivatives, Patent, pending.

[I identified the double bond reductase responsible for derailing the biosynthetic pathway, leading to the unwanted side-products by a thorough literature study, as well as experimentally testing each in yeast knockout strains. This is similar to the 2017 article below.]

2017

Lehka, B. J., Eichenberger, M., Bjørn-Yoshimoto, W. E., Vanegas, K. G., Buijs, N., Jensen, N. B., Dyekjaer, J. D., Jenssen, H., Simon, E., and Naesby, M. (2017) Improving heterologous production of phenylpropanoids in *Saccharomyces cerevisiae* by tackling an unwanted side reaction of Tsc13, an endogenous double-bond reductase. *FEMS yeast research* 17

[I identified the reductase in question. I did the bioinformatics to identity it, as well as the knock-outs to verify that it as this, and not any of all the others I tested, that resulted in poor production yields.]

2016

Hansen, J. C., Bjørn-Yoshimoto, W. E., Bisballe, N., Nielsen, B., Jensen, A. A., and Bunch, L. (2016) beta-Sulfonamido Functionalized Aspartate Analogues as Excitatory Amino Acid Transporter Inhibitors: Distinct Subtype Selectivity Profiles Arising from Subtle Structural Differences. *Journal of medicinal chemistry* 59, 8771-8786 2016

[I did all functional EAAT assays. Further, I was an integral part pf selecting which compounds to be synthesized, based on each round of structure-activity relationships]

Hansen, S. W., Erichsen, M. N., Fu, B., Bjørn-Yoshimoto, W. E., Abrahamsen, B., Hansen, J. C., Jensen, A. A., and Bunch, L. (2016) Identification of a New Class of Selective Excitatory Amino Acid Transporter Subtype 1 (EAAT1) Inhibitors Followed by a Structure-Activity Relationship Study. *Journal of medicinal chemistry* 59, 8757-8770

[I did all functional EAAT assays. Further, I was an integral part pf selecting which compounds to be synthesized, based on each round of structure-activity relationships]

* Bjørn-Yoshimoto, W. E., and Underhill, S. M. (2016) The importance of the excitatory amino acid transporter 3 (EAAT3). *Neurochemistry international* 98, 4-18. (First author)

[I wrote the article, with helpful input and sparing from the corresponding author, Dr. Underhill]

Huynh, T. H., Erichsen, M. N., Tora, A. S., Goudet, C., Sagot, E., Assaf, Z., Thomsen, C., Brodbeck, R., Stensbol, T. B., Bjørn-Yoshimoto, W. E., Nielsen, B., Pin, J. P., Gefflaut, T., and Bunch, L. (2016) New 4-Functionalized Glutamate Analogues Are Selective Agonists at Metabotropic Glutamate Receptor Subtype 2 or Selective Agonists at Metabotropic Glutamate Receptor Group III. *Journal of medicinal chemistry* 59, 914-924

[I did all functional EAAT assays. This was part of the effort to ensure that the mGluR agonists were selective for these receptors.]

Haym, I., Huynh, T. H., Hansen, S. W., Pedersen, M. H., Ruiz, J. A., Erichsen, M. N., Gynther, M., Bjørn-Yoshimoto, W. E., Abrahamsen, B., Bastlund, J. F., Bundgaard, C., Eriksen, A. L., Jensen, A. A., and Bunch, L. (2016) Bioavailability Studies and in vitro Profiling of the Selective Excitatory Amino Acid Transporter Subtype 1 (EAAT1) Inhibitor UCPH-102. *ChemMedChem* 11, 403-419

[I did all functional EAAT assays.]

Hansen, S. W., Erichsen, M. N., Huynh, T. H., Ruiz, J. A., Haym, I., Bjørn-Yoshimoto, W. E., Abrahamsen, B., Hansen, J., Storgaard, M., Eriksen, A. L., Jensen, A. A., and Bunch, L. (2016) New Insight into the Structure-Activity Relationships of the Selective Excitatory Amino Acid Transporter Subtype 1 (EAAT1) Inhibitors UCPH-101 and UCPH-102. *ChemMedChem* 11, 382-402

[I did all functional EAAT assays, as well as analyzed the structure-activity relationships, to direct the rational design of novel compounds.]

2015

Jensen, A. A., Fahlke, C., Bjørn-Yoshimoto, W. E., and Bunch, L. (2015) Excitatory amino acid transporters: recent insights into molecular mechanisms, novel modes of modulation and new therapeutic possibilities. *Current opinion in pharmacology* 20, 116-123

[I co-wrote the review, with my main emphasis being the introduction, and the "novel generation of EAAT modulators".]

2014

Heim, J., Jensen, S., Klein, J., and Bjørn-Yoshimoto, W. E. (2015) Methods and materials for identifying compounds promoting translational read-through of nonsense mutations. Patent, pending.

[Together with my project manager (Sanne Jensen), I designed the approach. I ran all assays for setting up, verifying, and testing compounds, as well as analyzed the data before presenting to my project manager and chief scientific officer (Jutta Heim).]

2010

Eriksen, J., Bjørn-Yoshimoto, W. E., Jorgensen, T. N., Newman, A. H., and Gether, U. (2010) Postendocytic sorting of constitutively internalized dopamine transporter in cell lines and dopaminergic neurons. *J Biol Chem* 285, 27289-27301

[I did all the experiments involving the hemagglutinin-tagged dopamine transporter, the surface ELISA, and the confocal microscopy of Rab4, -7, and -11 colocalization.]

Project description

In the present proposal, I aim to discover and characterize novel cone snail venom peptides (conotoxins) acting on neuropeptide receptors with high biomedical potential.

Background

Neuropeptides have been implicated in most of the important physiological functions in humans, most of which act at G protein-coupled receptors (GPCRs) [1, 2]. Increasingly, numerous, and seemingly unrelated physiological roles are described to a single neuropeptide. For instance, cholecystikinin has been implicated in hunger, memory consolidation, and anxiety [3, 4], while neurotensin has been implicated in pain, metabolism, and inflammation [5, 6]. While this can, in part, be explained by different functions at different locations in the body, another important factor is the presence of numerous receptor subtypes that serve functionally different roles. In recent years, interest for neuropeptides as potential drugs has increased dramatically. Unfortunately, many endogenous neuropeptides are less than ideal drugs. They often suffer from **low stability, short plasma half-life, and inappropriate selectivity profiles** leading to low efficacy and potential side effects [7].

Cone snails are venomous marine molluscs of the genus *Conus*, found throughout the tropical and sub-tropical oceans, where they hunt either vertebrate- or invertebrate prey (fig 1). More than 700 species have been identified, each producing 100 – 500 different toxin peptides, many of which are physiologically active in mammals [8]. These conotoxins are generally fairly short, highly structured, and often contain multiple disulfide bonds. Conotoxins with activity at ion channels have historically been the easiest to identify, since they often have the most pronounced effect when screening venom fractions by injecting in animals, and observing changes in behavior. However, cone snail venoms also contain signaling peptide mimicking conotoxins (SigMims), such as the recently described cone snail venom insulin, which activates the human insulin receptor [9, 10] and has provided the basis for the design of a novel fast-acting drug lead for diabetes [11] (unpublished data). Another example is conulakin-G, a mimic of the neuropeptide neurotensin, that shows very potent *in vivo* pain relief in mammals [12, 13]. Preliminary evidence from our group suggests that cone snails also use mimetics of the peptide hormone somatostatin for prey capture. Preliminary data suggests that these are **highly stable and selective** for subtypes of the somatostatin receptor found in the peripheral nervous system, including a subtype that has recently been implicated in pain signaling (fig 2) [14].

Despite their significant therapeutic potential, several challenges have hampered the discovery of SigMims. They generally share very little sequence identity with the endogenous signaling peptides, thus traditional bioinformatic search algorithms such as BLAST or hidden Markov models have proved largely ineffective. In fact, most of the SigMims that have been identified to date were discovered by happenstance, or by manually curating identified toxin sequences. With the availability of large toxin sequence datasets, this has become unfeasible.

To overcome this major limitation, I have recently developed a novel bioinformatic method for *in silico* discovery of short toxins with minimal sequence similarity to the endogenous peptide they mimic. The proof-of-concept (POC) method has proven remarkably efficient at identifying our positive controls (fig 3). I aim to fully develop this tool, and apply it to a unique library of 30,000 conotoxin sequences in order to discover new SigMims. I will focus our efforts on 5-10 human neuropeptide systems that show high promise from the *in silico* discovery, emphasizing systems that are prominently associated with high-impact neurological disorders and pathological states including pain, schizophrenia, Parkinson's disease, depression, anxiety, and addiction (fig 4).

This project will consist of three main objectives, that will be conducted over a three-year period, as well as accompanying studies performed by collaborators, or contract research companies (described in objective 3). My overall aim is to develop a computational pipeline that will lead to the identification and functional characterization of new cone snail toxins with high biomedical potential.

Objectives and approach

Objective 1 – *In silico* screening of our unique 30,000 conotoxin sequence library.

These were collected by my group leader (Dr. Helena Safavi) over the last several years, and provide a unique source for the discovery of new bioactive peptides (fig 1). Based on the number of known examples, we estimate that the number of SigMims will be ~0.2 – 0.4%, meaning the potential presence of 60-120 of these in our library. Our novel search algorithm has proven useful in initial tests, identifying ~85% of our positive controls (Fig 3). We therefore expect to find a large percentage of the SigMims present in our database. I will use our novel method in concert with the homology search tool BLAST [15]; relying on our method when searching for very short neuropeptides (<12 amino acids), and using both BLAST and our method for longer neuropeptides. While we have a working prototype, I will require 1-2 months to script parts that are currently performed manually in our proof-of-concept protocol (e.g. ortholog selection and alignments, better determination of cysteine connectivity profiles, etc.). This will enable us to share this tool with the research community in a user-friendly package.

We will screen for conotoxins resembling a total of 421 known or predicted human signaling peptides [16] (unpublished data). From these, I will only proceed with mimics of select neuropeptides, emphasizing neuropeptide systems with a large body of evidence supporting their role in neurological disorders, or their potential for becoming a drug lead

for disease treatment (fig 4). However, by doing the *in silico* screen against a range of targets if we, against expectations, do not find enough relevant hits against one of our top ten preferred targets, we would simply use the next neuropeptide system on the list. Given the previous findings listed above, it is highly unlikely that I will not be able to discover several peptide system mimics to work with.

Objective 2 – *in vitro* verification of *in silico* hits. The vast majority of neuropeptide receptors are GPCRs, something I have abundant experience working with. In objective 2 and 3, I will employ various GPCR activity assays, first to verify the *in silico* hits, then to characterize the verified hits in HEK293T cells. Specifically, I will use an optimized Epac biosensor and the CisBio cAMP HTRF kit for $G_{\alpha_s}/G_{\alpha_{i/o}}$ [17, 18], a classic fluorescent calcium dye approach and the CisBio IPOne kit to assess $G_{\alpha_{q/11}}$ activity [18, 19], whereas $G_{\alpha_{12/13}}$ activity will be assessed using the TGF α -shedding assay [20]. The β -arrestin recruitment will be assessed using the PRESTO-Tango assay, or the enhanced bystander BRET β -arrestin recruitment assay [21, 22]. We have access to the PRESTO-Tango constructs and the Epac sensors, while other receptors or constructs will be purchased from cDNA.org or synthesized by Twist Bioscience. The peptides will be procured by traditional solid-phase peptide synthesis (purchased from Peptide International Inc. or synthesized by our collaborator Prof. Olivera, University of Utah, USA), or by utilizing a novel bacterial expression system developed by Dr. Safavi and our collaborator, Prof. Lars Ellgaard, University of Copenhagen, for the efficient production of conotoxins [23].

Objective 3 – Characterize conotoxins. New conotoxins of interest will be compared to the endogenous ligands of the peptide system, both in terms of potency and selectivity at different receptor subtypes, using receptor assays described under objective 2. The 2-3 most promising compounds will further be subject to structure characterization by nuclear magnetic resonance (NMR) spectroscopy, in collaboration with Associate Prof. Kaare Teilum. This will allow the investigation of their structural stability and receptor binding mode. Finally, select peptides will be assessed for plasma stability to determine if they exhibit better metabolic stability over the endogenous neuropeptide (this service will be procured from BioDuro).

Objective 2 and 3 will be iterative, alternating between screening and characterization, completing the process for 1-2 neurotransmitter systems in each consecutive round. This will allow (1) finishing a work package, where the best SigMims can be transferred to collaborators and contract researchers that will perform the characterizations described above, while I start the next round, and (2) a better flow of dissemination, allowing publications to be prepared with semi-regular intervals, describing each system.

Objective 4 – Link novel members of a conotoxin superfamily to our hits. Conotoxins can be grouped into gene superfamilies based on highly conserved signal peptides, despite the mature peptide sequences often being extremely diverse [24, 25]. These gene superfamilies share common target receptors, and this method of grouping diverse peptides is a powerful and unique tool in the conotoxin field. Additional sequences found in this manner will be added to our pool of proposed ligands targeting a given

neuropeptide system, and be subject to studies described in objective 2 and -3. This allows for expanding our peptide identification process beyond what any simple search algorithm alone could, and greatly increases the chance of identifying conotoxins with diverse modes of action, receptor subtype selectivity profiles, or receptor signaling bias.

While the scope of this project is ambitious, several factors significantly increase the likelihood of success. Firstly, we have procured the assistance of experts in the respective fields. This includes Prof. Hans Bräuner-Osborne, who has been at the very front of molecular GPCR research for decades, Prof. Ellgaard, who has developed the capacity to produce conotoxins without the need for chemical synthesis, Associate Prof. Kaare Teilum, an expert in advanced NMR, as well as Prof. Baldomero Olivera, who is one of the global leaders in conotoxin research. Dr. Safavi, who has trained under Prof. Olivera, has become a very prominent figure in the conotoxin community in her own right. With my experience in molecular and cellular neuropharmacology, and the collaborators we have gathered, we have ensured the expertise to drive this project. Secondly, we have access to a large untapped pool of unique sequences. As described above, novel SigMims are very likely to exist in this library. Thirdly, we have developed a novel search strategy, that allows searching for the types of relations relevant for this study. Fourthly, there is a built-in redundancy in our approach. Despite the high expected hit rate, we are not dependent on this being the case. Even with significantly lower a hit rate than previous findings would suggest, we would still have more than enough hits to drive this project.

Furthermore, I would like to emphasize the **potential impact** on the scientific community that is expected to arise from these efforts. The novel bioinformatics tool developed will be shared with the research community, which will be a tremendous asset to numerous fields of research. We expect one open access publication covering each of the 3-4 neuropeptide systems with the most promising peptides. The novel compounds obtained will certainly serve as tool compounds, and likely also as drug leads. At present, only 10 venom peptides that mimic endogenous signaling peptides in humans have been described to date [26-28]. **Of those 10, 6 are either currently used clinically, currently going through clinical trials, or have been in clinical trials.** That is a staggering percentage, not seen in any other class of compounds.

Finally, it is worth noting that my emerging experience in this field, together with the established expertise of Dr. Safavi, presents a unique opportunity to bring Denmark to the forefront of toxin research. This will also be an invaluable opportunity for myself, as a young researcher, to gain additional experience, allowing ownership of my own project, something that will greatly help propel my future aim of leading my own research group.

Gender dimension

The science itself has no significant gender dimension. Our setup uses HEK293T cells, which are derived from a male fetus, though this is unlikely to be a gender issue for the types of experiments we will utilize them for. Should this project continue, I foresee that select compounds will be tested in animal disease models, and here I will ensure that both female and male animals are used, and any differences thoroughly established.

Outside of the project-specific considerations, I value gender balance, and am acutely aware of gender bias in the workplace, as well as in much of the scientific work carried out. Should this project be funded, I will end up being the sole male in the group of three females (one PI, two PhD students). Adding me will bring a measure of gender balance to the group, and the group dynamics.

Literature references

1. van den Pol, Anthony N., Neuropeptide Transmission in Brain Circuits. *Neuron*, 2012. 76(1): p. 98-115.
2. Borbely, E., B. Scheich, and Z. Helyes, Neuropeptides in learning and memory. *Neuropeptides*, 2013. 47(6): p. 439-50.
3. Nader, K., O. Hardt, and P.V. Miguez, Consolidation and Reconsolidation. *Encyclopedia of Psychopharmacology*, 2015: p. 436-440.
4. Rehfeld, J.F., Cholecystokinin—from local gut hormone to ubiquitous messenger. *Frontiers in endocrinology*, 2017. 8: p. 47.
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Description of the research environment(s)

The bulk of the research will be carried out in the lab of Dr. Safavi. As per January 1st, 2020, this group will move to the Department of Biomedical Sciences at the Faculty of Health and Medical Sciences. This is located on the 9th floor of the Maersk Tower, and is a very open and collaborative environment. Five group leaders share the space, and they promote interactions and knowledge sharing by various approaches. One largely successful approach that has been implemented is to avoid permanent lab benches, and instead have "workstations" for various types of work. The results is a much broader surface of interaction with other groups than in a traditional environment.

Further, there is a highly collaborative spirit within the section, and a tendency for group leaders to take a keen interest in each others work, to the degree that topic-specific collaborations routinely appear. This, of course, is in large part driven by the interactions of the "ground-level" group members, and their interactions.

It seems like a great approach to promote collaborations, and one that seems to actually work, as opposed to many other (quite well-meaning) approaches I have previously experienced, that aim to promote knowledge sharing and collaborations between groups located in the physical vicinity of each other.

Place(s) where the project will be carried out, including duration (months)

1. The lab of Dr. Safavi-Hemami, BMI, Faculty of Health and Medical Sciences, University of Copenhagen.

This is where the majority of the work will be done.

2. The lab of Professor Hans Bräuner-Osborne, Molecular pharmacology group, Faculty of Health and Medical Sciences, University of Copenhagen.

Some of the GPCR functional assays might be carried out here. This lab routinely uses the assays we want to utilize, so this gives us direct access to a large body of knowledge and expertise.

3. The lab of Professor Lars Ellgaard, Protein biology group, Faculty of Science, University of Copenhagen.

This is where most of the peptides will be expressed and purified. The majority of this will not be carried out by myself, however, I will be involved, and I will only spend very limited time here.

Danish institution that will administer the grant

University of Copenhagen, Faculty of Health an Medical sciences, Department of Biomedical Sciences.

Comments to the budget

DKK 50,000 will be provided by the group (used to purchase additional IPOne and cAMP kits from CisBio). Peptides will largely be obtained from collaborators (as Dr. Safavi has done previously). The cost of peptides listed is for a small percentage of peptides that will be purchased from Peptide International Inc., mainly for when we need small amounts faster than our collaborators can provide them.

All reagent and kit costs are estimated based on the feasible workload to undertake. The running costs are estimated based on experience from our collaborators, who have performed similar types of projects, in terms of assay types and scope. The first year, some time is spent on scripting, while the last year, additional time is expected to be spent writing articles and disseminating findings. As such, these costs are expected to be higher the second year, compared to year 1 and 3.

I aim to disseminate our findings at international conferences (e.g. the Gordon Research Conference on Venoms in Vermont, 2021, as well as GPCR-, molecular pharmacology-, or other toxin-specific conferences). However, I have not included this in the budget since I will apply for funding elsewhere, and should that not be granted, funding from the lab will cover this.

Date of PhD degree

15. august 2017

Maternity or paternity leave after PhD degree

0

Other types of leave

0

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Budget

Year Type	Name	Amount of money	Details
1			
	Applied from the Lundbeck Foundation		
	Salary - post doc	554.750,00 kr.	9 months in 2020, 3 months in 2021, see attached salary note from institute administrator
	Running costs	32.000,00 kr.	Tissue culture medium and reagents. Estimated 8 months in the first year.
	Running costs	50.000,00 kr.	Miscellaneous lab consumables and minor purchases (buffers, chemicals, plastics, DNA prep kits, etc.)
	Running costs	64.400,00 kr.	Constructs ordered. This will be an initial posting that will be used throughout the project.
	Running costs	56.500,00 kr.	Peptide synthesis. This will mainly be provided via collaborators, but I want to reserve funding to rush the 5 most promising at each step
	Running costs	40.700,00 kr.	CisBio IPOne and cAMP kits (DKK. 5809 and 4366 each, respectively, each providing 1,000 datapoints). The group will provide 2 of each kit the first year.
		<u>798.350,00 kr.</u>	
2			
	Applied from the Lundbeck Foundation		
	Salary - post doc	566.000,00 kr.	9 months in 2021, 3 months in 2022, see attached salary note from institute administrator
	Running costs	40.000,00 kr.	Tissue culture medium and reagents. Estimated 10 months in the second year.
	Running costs	65.000,00 kr.	Miscellaneous lab consumables and minor purchases (buffers, chemicals, plastics, DNA prep kits, etc.)
	Running costs	56.500,00 kr.	Peptide synthesis. This will mainly be provided via collaborators, but I want to reserve funding to rush the 5 most promising at each step
	Running costs	61.050,00 kr.	CisBio IPOne and cAMP kits (DKK 5809 and 4366 each, respectively, each providing 1,000 datapoints)
		<u>788.550,00 kr.</u>	
3			
	Applied from the Lundbeck Foundation		
	Salary - post doc	577.250,00 kr.	9 months in 2022, 3 months in 2023, see attached salary note from institute administrator
	Running costs	36.000,00 kr.	Tissue culture medium and reagents. Estimated 7 months in the third year.

Year Type	Name	Amount of money	Details
3	Applied from the Lundbeck Foundation		
	Running costs	55.000,00 kr.	Plasma stability studies
	Running costs	45.000,00 kr.	Miscellaneous lab consumables and minor purchases (buffers, chemicals, plastics, DNA prep kits, etc.)
	Running costs	56.500,00 kr.	Peptide synthesis. This will mainly be provided via collaborators, but I want to reserve funding to rush the 5 most promising at each step
	Running costs	20.350,00 kr.	CisBio IPOne and cAMP kits (DKK 5809 and 4366 each, respectively, each providing 1,000 datapoints). The group will provide 4 of each kit the third year.
		<u>790.100,00 kr.</u>	
		<u>2.377.000,00 kr.</u>	

Co-applicant(s) / Mentor

Name	Safavi-Hemami Helena
Email	vjm257@ku.dk
CV	

Publications

34 peer reviewed publications, H-index: 15
Web of Science ResearcherID: [E-7624-2015](#)

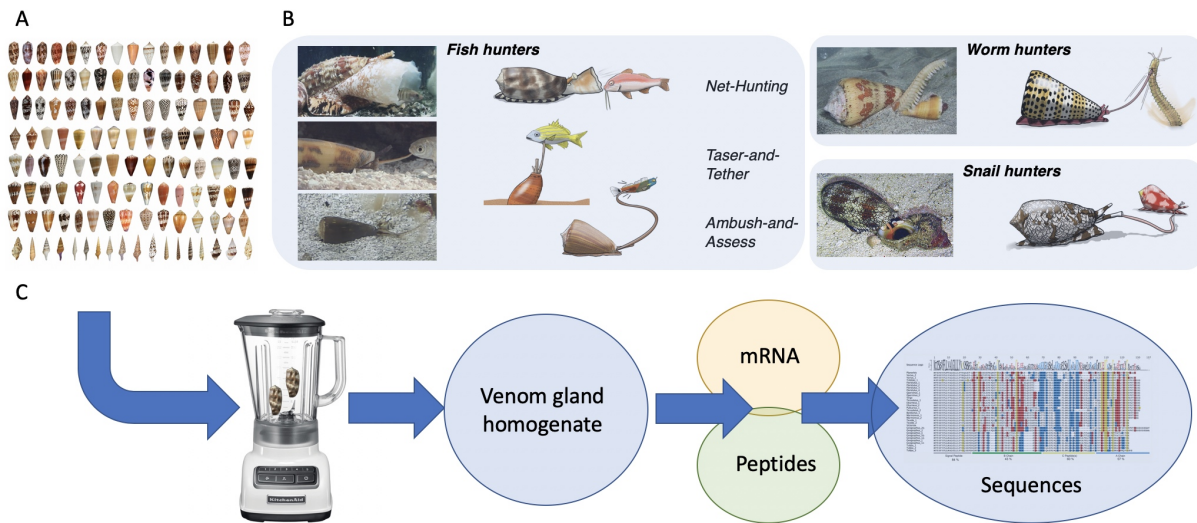


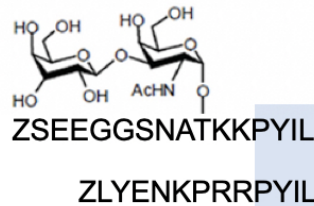
Figure 1 - Cone snails and their peptide toxins.:

A: Shells of the cone snail species that have been sequenced for our peptide library. B: Cone snails have different prey, and different hunting strategies. We focus our efforts on “fish-hunters”, whose toxins have evolved to act on vertebrate receptors. C: Collection of cone snail toxin sequences. Venom glands are homogenized to obtain mRNA and peptides for sequencing.

A

Contulakin-G Toxin

Human Neurotensin



Subtype selective (NTSR2>NTSR1)

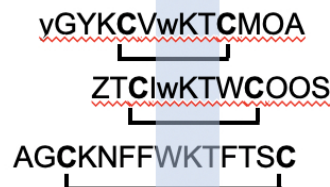
Neurotensin receptor (NTR)
agonists

B

Cont-Ro1 Toxin

Cont-Ro2 Toxin

Human Somatostatin



Subtype selective (SSTR 1, 2, 4)

Likely more metabolically stable

Somatostatin receptor (SSTR)
agonists

Figure 2 - Examples of conotoxins that act as neuropeptide mimics. :

A: Contulakin-G is a neurotensin receptor ligand with subtype selectivity at mammalian receptors, and a potent in vivo analgesic in rodents. B: Somatostatin-like peptides recently discovered from *Conus rolani* activate the mammalian somatostatin receptors, despite sharing very little sequence identity with somatostatin (unpublished sequences). Two of these display subtype selectivity over the endogenous signaling peptide.

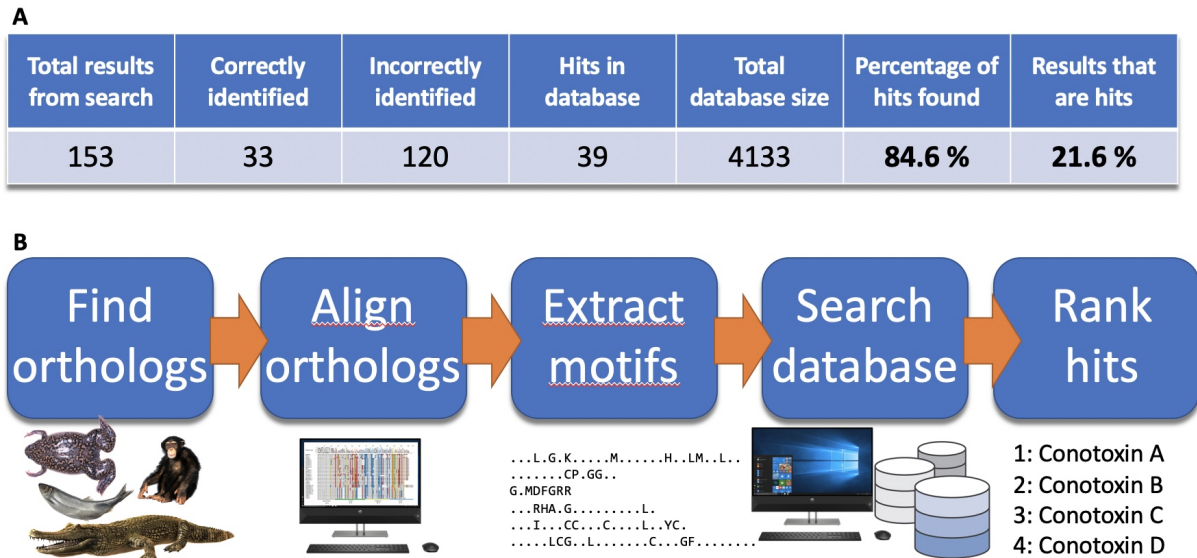


Figure 3 - The “proof of concept” (POC) script I have recently developed:

A: Preliminary data from my POC script. Search was done based on five related human neuropeptides (arginine-vasopressin, copeptin, neurophysin-1, neurophysin-2, and oxytocin), where the corresponding conotoxins are known as “conopressins”. A database of 4133 semi-random conotoxins from fish hunter cone snails was used, wherein all the 39 conopressins have been annotated prior to searching (though the search was done blinded, I did not annotate or see the conopressin sequences when defining the search parameters). The script finds ~85% of all conopressins in the database, and ~22% of all results returned are correct.

B: schematized workflow of the approach. This tool is useful for any researcher looking for modulators of their receptor of interest from any sequencing database, not just for conotoxin research.

Neuropeptide system	Examples of physiological roles	Examples of pathophysiology or proposed therapeutic potential	References
Neurotensin	Pain, cardiovascular control, inflammation, control of hormone release, metabolism	Pain, schizophrenia, addiction	[31-33]
Neuropeptide Y	Cardiovascular regulator, stress coping,	Depression, stress and anxiety, neuroprotective in neurodegenerative disorders such as Alzheimer's-, Parkinson's-, and Huntington's disease.	[34-36]
Substance P	Neuroinflammation, blood-brain barrier permeability, nociception	Parkinson's disease, inflammatory bowel disease, fibrotic diseases	[37-40]
Cholecystokinin	Feeding behavior, gut-brain signaling, anxiety and fear, memory consolidation	Panic disorder, obesity	[34, 41-43]
Agouti-related protein	Apetite, metabolism, inflammation	Addiction, eating disorders	[29, 30]
Melanocyte-stimulating hormones	Apetite, sexual behavior, metabolism, inflammation, alcohol dependence	Addiction, eating disorders, melasma	[29, 44, 45]
Orexin	Wakefulness, addiction, motivation, food intake, metabolism, mood, arousal	Depression, addiction, insomnia, narcolepsy, obesity	[46-49]
Neuromedin-U	Smooth muscle contraction, cardiovascular regulator, nociception, inflammation, bone formation	Neuropathic pain, stress, osteoporosis, inflammatory disorders, obesity	[50, 51]
Nociceptin	Nociception, mood, learning, memory	Addiction, neuropathic pain, anxiety, depression	[52-54]
Cortistatin	Wakefulness, memory, neuroendocrine regulation, brain-gut communication	Insomnia, neuroprotective in ischemia	[55-57]

Figure 4 - Select high-priority neuropeptides:

Examples of high-priority human neuropeptides, with select physiological roles, pathophysiological states, or proposed therapeutic potential listed. This illustrates the diverse roles each neuropeptide can convey, depending on the specific subtype of receptor targeted and/or the location in the organism. We will focus our efforts on (1) neuropeptides with high biomedical relevance to neurological disorders, and (2) neuropeptides that are involved in physiological functions likely to be a valid target for a weaponized peptide toxin, i.e. systems that relate to locomotion, pain or sensory input, motivation, acute cardiovascular effects, fear, metabolism, wakefulness, etc.

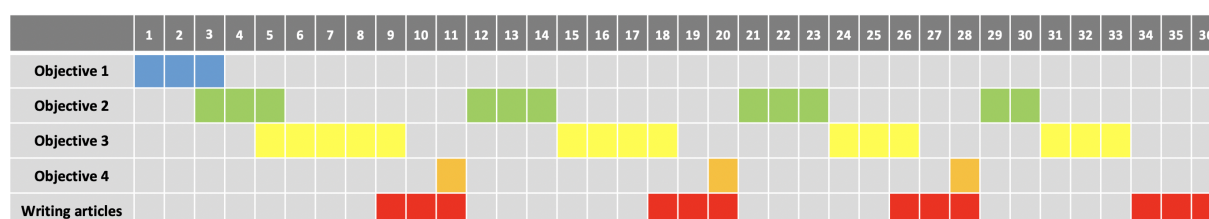


Figure 5 - Proposed time line:

Numbers at the top show months. Colors in a month/quarter means that efforts will be put into a given objective, though not necessarily at full-time. Objective 1: Software development and in silico screening. Objective 2: Peptide production and in vitro screening. Objective 3: Characterization and comparison of conotoxins. Objective 4: Finding novel members of conotoxin superfamily. These will be treated as the hits from objective 1, and entered into subsequent rounds of objective 2 and -3. I propose to prepare articles after the first full round of characterization (starting with the most promising hits), then again after each completed round, though the timing is of course subject to change, based on our findings. The first article will describe the in silico methodology, as well as the findings pertaining to the a given neuropeptide system, while the others will focus on one or more neuropeptide systems, depending on the findings. There will be some overlap of the different objectives in the timeline, especially once the characterization in objective 3 begins. This will allow us to focus on 1-2 neuropeptide systems at a time, and will make the dissemination less condensed, allowing articles concerning specific neuropeptide systems to be prepared continuously, instead of condensing the dissemination at the end of the project.

UNIVERSITY OF COPENHAGEN
FACULTY OF SCIENCE

To the Lundbeck Foundation fellowship selection committee



Helena Safavi, PhD

Assistant Professor, University of Copenhagen

Email: safavihelena@bio.ku.dk, Phone: +45 61 43 60 96



02 NOVEMBER 2019

Subject: Letter of recommendation for Dr. Bjørn-Yoshimoto's fellowship application

Dear members of the fellowship selection committee,

It is with great pleasure that I write this letter of recommendation for Dr. Bjørn-Yoshimoto's fellowship application. Dr. Walden Bjørn-Yoshimoto is an exceptionally talented young scientist and an excellent candidate for the Lundbeck Foundation postdoctoral fellowship program.

I had the great pleasure to first meet Walden earlier this year when he contacted me regarding an interest in joining my lab as a postdoctoral research fellow. Walden was highly recommended by his former mentors and by colleagues in the Department of Drug Design and Pharmacology where he obtained his training.

Walden is an early career scientist but has already accumulated an impressive list of scientific achievements. This includes authorship on 12 peer-reviewed journal articles, including papers in the *Journal of Medicinal Chemistry* and *Cell*, two filed patents, and collaborative visits to prestigious laboratories in the USA, including Prof. Clifford's Woolf lab at Harvard Medical School and Dr. Susan Amara's lab at the National Institutes of Health (NIH) in Maryland.

Walden's previous research focused on elucidating the pharmacology and function of human membrane proteins important for signalling. During his PhD studies Walden studied the molecular pharmacology of excitatory amino acid transporters (EAATs) that mediate the synaptic uptake of glutamate in the central nervous system. Walden's efforts to screen a large library of compounds during his research stay in Harvard led to the identification of several new EAAT-selective inhibitors. Furthermore, he investigated the effects of the psychostimulant amphetamine on a specific subtype of the EAAT. This work led to 5 original research publications and 2 review articles on EAATs that continue to provide an important resource for the scientific community (66 and 30 citations, respectively).

During his first postdoc in Dr. Anders Jensen's group at the Department of Drug Design and Pharmacology Walden developed and implemented pharmacological assays to functionally characterize the 5-HT₂ serotonin receptors that are expressed in the brain and peripheral nervous system where they are involved in neurotransmission. This research was published in the prestigious journal *Cell* last year and several additional publications are in preparation.

Research in our group centers around developing computational and experimental tools to discover novel receptor ligands from animal venoms that can be developed as pharmacological

tools and drug leads. Examples include the discovery of a new and diverse family of insulins in the venoms of predatory marine cone snails. These have been developed as novel, fast-acting drug leads for diabetes. Other work includes the discovery and application of toxins that alleviate pain by specifically targeting different receptors in the peripheral and central nervous system.

Last year, I relocated from the University of Utah, USA to the University of Copenhagen where we continue to identify and characterize novel pharmacological agents from animal venoms. I was absolutely delighted when Walden applied for a postdoctoral position in my lab. Having looked at many applications it was clear that Walden was an exceptional candidate who had by far the strongest track record in molecular pharmacology and in the functional elucidation of ligand binding to membrane receptors and transporters. Additionally, Walden is a self-taught programmer who can easily handle large data and is capable of developing computational tools to improve the discovery of interesting ligands from our large sequencing datasets. This combination of high proficiency in molecular pharmacology and computational programming is extremely rare and, combined with Walden's ability to independently develop and conceptualize innovative research ideas, has provided the basis of the project that Walden is now proposing. While the objectives are ambitious, the research environment in our lab is ideally suited to successfully execute this project, and Walden has the motivation and skillset to drive and accomplish this project.

Having worked with Walden for nearly one year now, it has become clear that he has the personality, drive and intellectual competence to become an independent group leader in the near future. Not only did he develop the prototype tool of the computational pipeline proposed in this project but also independently established several orthogonal receptor assays to pharmacologically evaluate new toxins and researched and implemented safety measures required to work with toxins in our lab. Furthermore, he is very well connected and liked by the pharmacology research community in Copenhagen, including several world-renowned researchers in the field of GPCR structure-function, and is in the process of establishing connections and collaborations with international researchers in the field of venom discovery and application.

Receiving the Lundbeck Foundation postdoctoral fellowship would significantly accelerate his well-deserved path to independence.

I have no doubt that Walden will be successful with this project and beyond, and that his research will not only be well-received but be transformative for the research community. It is with great enthusiasm that I support Walden's fellowship application.

Please do not hesitate to contact me if you require additional information.

Yours sincerely,



(Dr. Helena Safavi, Assistant Professor, University of Copenhagen)

Figure 6 - Letter of support from group leader Helena Safavi:

UNIVERSITY OF COPENHAGEN
BIOMEDICINSK INSTITUT

Lundbeck Foundation – Salary budget



Salary budget for the Postdoctoral Fellow Walden Bjørn-Yoshimoto in
the application for the project

22 OCTOBER 2019

“Conotoxins as novel neuropeptide receptor modulators”

BIOMEDICINSK INSTITUT
ADMINISTRATIONEN

The Salary budget proposed in the application is in agreement with our
estimates of the average yearly Postdoc/Assistant Professor salaries at the
Department of Biomedical Sciences at the Faculty of Health and Medical
Science.

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2200 KØBENHAVN N

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DIR +45 23398726
MOB +45 23398729

2020: 552,000

2021: 563,000

2022: 575,000

2023: 584,000

Ason@sund.ku.dk
www.bmi.ku.dk

REF: ASON

Yours sincerely,


Anders Sondrup
Institute administrator
BIOMEDICINSK INSTITUT
Københavns Universitet
Fakultet for Medicin
Head of Administration
Copenhagen N

Figure 7 - Salary confirmation from institute administrator Anders Sondrup: