September 20, 2005

Yves Brun
Systems Biology/ Microbiology Faculty Search
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1001 E 3rd Street
Bloomington, IN 47405-7005

Dear Dr. Brun,

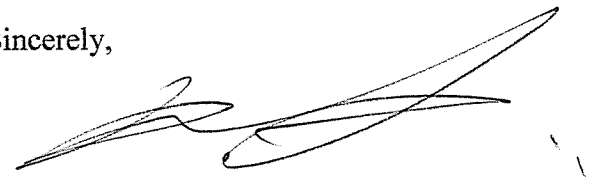
In response to your advertisement in your website and *Science*, I would like to apply for the faculty position at the rank of Assistant Professor in the Department of Biology and Biocomplexity Institute at Indiana University Bloomington. I am currently a Postdoctoral Research Associate in the laboratory of Dr. David Clouthier in the Department of Molecular, Cellular and Craniofacial Biology at the University of Louisville. In this lab, I have used a variety of techniques to investigate the function of endothelin-A receptor (Ednra) signaling during craniofacial and cardiovascular development. This research has focused on understanding the cellular and molecular changes that occur within neural crest cells in the absence of Ednra signaling, which leads to facial and heart birth defects. This work has been partially supported by a Post-Doctoral Research Award to me from the Heart and Stroke Foundation of Canada. I plan on continuing and expanding this research in my own laboratory.

My background and proposed research compliment existing research programs in your department. As a graduate student, I examined the role of peptide elongation factor 1A in controlling apoptosis and caspase protease activation in differentiated cultured muscle cells and regenerating skeletal muscles models. In my current post-doctoral position, I have examined the function of Ednra signaling during development using both conventional and conditional knockout mouse lines. I am currently performing fate analysis of neural crest cells in *Ednra* mutant embryos and am initiating a screen for downstream effectors of Ednra signaling. I can envision my work ultimately leading to a better understanding of the genetic basis of human birth defects involving the head and heart and potentially giving rise to new therapeutic strategies to treat these defects.

I believe that I would be a great asset to the Department of Biology and Biocomplexity Institute, with both my research and technical expertise contributing to a solid departmental foundation. In addition, my background would enable me to teach or contribute to the teaching of courses in cell and molecular biology, genetics and developmental biology. I have attached my curriculum vitae, a statement of proposed research and the names of four references.

Thank you for your consideration of my application. Should you have any questions, please do not hesitate to contact me.

Sincerely,



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Enclosed/
CV
Research proposal
Teaching Statement

STATEMENT OF RESEARCH INTEREST

INTRODUCTION

One of every 33 children is born with birth defects. Cardiovascular malformations are the most common, affecting one child for every 300-400 births and craniofacial defects affect about one child for every 700 births. While corrective surgery can be used in some cases to improve the quality of life of affected children, it is associated with high medical cost and cannot be used to treat all defects. To address these drawbacks, *in utero* treatment regimens are needed to correct these defects prior to birth. However, we must first elucidate the hierarchical signaling mechanisms that direct normal craniofacial and cardiovascular development and understand how perturbation of these signals leads to birth defects. The main goal of my research is to understand the function of endothelin-A receptor signaling in cephalic and cardiac neural crest cells (NCCs) during development.

BACKGROUND

Neural crest cells are a distinct population of cells that delaminate from the neural fold and migrate ventrally in the embryo, giving rise to various anatomical structures. Cephalic NCCs migrate from the posterior midbrain and anterior hindbrain into the maxillary and mandibular portions of the first arch and the second, third and fourth arches to form most of the craniofacial skeleton and cranial ganglions. Cardiac NCCs migrate from the posterior hindbrain into pharyngeal arches 3, 4 and 6, where they participate in asymmetric remodeling of the pharyngeal arch arteries into the great vessels and also form the vascular smooth muscle surrounding the great vessels. A subpopulation of cardiac NCCs also migrates into the cardiac outflow tract, where they contribute to the septation of the outflow tract into the pulmonary and aortic outflows. Improper NCC development leads to a variety of birth defect syndromes, including Carpenter syndrome, DiGeorge syndrome and CHARGE syndrome (1). Since cephalic and cardiac NCCs originate from different environments, it is quite likely that their patterning in regulated by a distinct array of transcripton factors and signaling molecules

One or more signaling pathways crucial for NCC development are regulated by the endothelin-A receptor (Ednra)/endothelin 1 (Edn1) system. During embryonic development, Ednra (a G protein-coupled receptor) is expressed in cephalic and cardiac NCCs while its peptide ligand Edn1 is expressed in the pharyngeal arch ectoderm, core paraxial mesoderm and pharyngeal pouch endoderm. Targeted deletion of the genes encoding *Ednra*, *Edn1* or *Ece1* (endothelin converting enzyme 1, responsible for the production of active Edn1) in the mouse causes severe craniofacial and cardiovascular defects of NCC-derived structures, resulting in early postnatal lethality (2-6). Absence of Ednra signaling in cephalic NCCs results in homeotic transformation of lower jaw structures into upper jaw–like structures. This homeosis is partly caused by the downregulation of normal mandibular arch gene expression and upregulation of maxillary arch gene expression in the mandibular arch (7). Cardiovascular defects include interruption of the aortic arch (type B), transposition of the great vessels, persistent truncus arteriosus (PTA), double outlet right ventricle (DORV) and ventricular septal defects (VSD) (2-6). While Ednra signaling is crucial for both of these NCC populations, intracellular transmission of the Ednra signal appears to occur via different G-protein alpha subunits (8). However, effects of these G-protein alpha subunit changes in NCCs on gene expression and response to Edn1 binding remains are not known.

To further characterize Ednra signaling during embryonic development, we are using a conditional knockout approach to inactivate the *Ednra* gene in NCCs. To accomplish this, mice carrying a conditional mutation in the *Edrna* gene ($Ednrd^{flox}$) are crossed with *Wnt1-Cre* transgenic mice. Our early results show that embryos with two conditional alleles ($Ednrd^{flox/flox}$;*Wnt1-Cre*) have similar craniofacial defects to $Ednra^{-/-}$ embryos. However, they do not exhibit any cardiovascular defect. Cardiovascular defects are only observed in embryos carrying both a conditionally inactivated allele and one mutant allele ($Ednrd^{flox/-}$;*Wnt1-Cre*) (9). Other preliminary data from our lab, in which an Ednra-specific antagonist is used to block Ednra function *in utero*, reveals that Ednra signaling is crucial between E8.5 and E9.5 for proper craniofacial NCC development. However, cardiovascular defects were not observed in these embryos.

While these results indicate that cephalic and cardiac NCCs respond differently to endothelin signaling, the mechanisms regulating such differences are unclear.

HYPOTHESIS AND SPECIFIC AIMS

Based on the background presented above, it appears that Ednra signaling is crucial for cephalic and cardiac NCC development. However, mechanisms regulating *Ednra* expression and activity are unknown. **We hypothesize that *Ednra* expression is differentially regulated in cephalic and cardiac neural crest cells by local factors that induce different transcriptional regulators. This regulation is further tightened by different G-protein alpha subunits. These two levels of regulation determine the sensitivity of cephalic and cardiac NCCs to Edn1 and hence the downstream activators induced by Ednra signaling.** This hypothesis will be tested by three specific aims.

Aim 1: Determine how *Ednra* expression is regulated in neural crest cells. Several conserved sequences are present upstream of the *Ednra* promoter sequence, likely indicating cis-acting elements (enhancers). We will use a transient transgenic mouse approach to identify the transcriptional regulators of Ednra in cardiac and cephalic NCCs. First a *lacZ* reporter gene will be introduced by homologous recombination into a bacterial artificial chromosome (BAC) containing the upstream and coding sequences of *Ednra.* The construct will then be injected into fertilized mouse eggs and expression assessed by β-galactosidase staining of E9.5 mouse embryos. After screening identified elements for putative transcription factor binding sites, we will examine the function of these sites using a mutagenesis / transient transgenic approach. Binding of transcription factors to sequences will be confirmed using a variety of biochemical methods, including luciferase assays and immunoprecipitations. To confirm the relevance of identified binding sites during development, chromatin immunoprecipitation (ChIP) as well as other methods will be used.

Aim 2a: Determine the kinetic changes induced by different G-protein alpha subunits associated with Ednra. Given that cardiac and cephalic NCCs use different G-protein alpha subunits (Gα12/13 *vs.* Gαq/11, respectively) to transmit Ednra signaling (8), it is possible that their differential responses to Ednra signaling are dictated by the different G-protein alpha subunits. To test this possibility, we will investigate the role of different G-protein alpha subunits on Ednra response to Edn1 binding using BRET (bioluminescence resonance energy transfer) technology, which allows real time protein-protein interactions analysis in living cells (10). For this purpose, a luciferase-linked *Ednra* gene and different GFP10-linked G-protein alpha subunit cDNAs will be co-transfected into cultured cells. Transfected cells will be treated with Edn1 and/or a specific antagonist to the receptor. These experiments will determine the sensitivity of Ednra for its ligand when coupled with different G-proteins. Other kinetic parameters for binding can also be measured.

Aim 2b: Determine differences in gene expression resulting from Ednra signaling mediated via different G-protein alpha subunits. Data suggest that the transduction of Ednra signaling is mediated by a series of downstream effectors which varies according to the G-protein alpha subunit used. As the use of different downstream effectors likely results in the induction of different sets of genes in cardiac and cephalic NCCs, changes in gene expression resulting from changes in G-protein alpha subunits will be analyzed using microarray gene analysis. Samples from cultured cells transfected with *Ednra* and various G-protein alpha subunits and subjected to Edn1 activation will be used for these assays. Similarly, cephalic and cardiac NCCs from wild type and *Ednra*$^{-/-}$ embryos will be analyzed. Comparison between these two approaches may indicate potential downstream effectors of Ednra signaling. *In vivo* and *in vitro* results will be confirmed by *in situ* hybridization analysis.

Aim 3: Determine how G-protein alpha subunits change NCC response to Ednra signaling. Targeted inactivation of the genes encoding Gα13 and Gα12 or Gαq and Gα11 recapitulates cardiac or craniofacial defects observed in *Ednra*$^{-/-}$ embryos, respectively (8). It is not clear whether the subunits

associated with Ednra-regulated cardiac development can substitute those involved in Ednra-regulated facial development nor whether such substitution affects NCC gene expression and subsequent development. To investigate these questions, we will create transgenic mice that express different G-alpha protein subunits in NCCs under control of the enhancers identified in Aim 1. Gα13 or Gα12 will be expressed in cephalic NCCs and Gαq and Gα11 in cardiac NCCs. Since transgenic expression can lead to elevated levels of the transgene, the G-alpha subunits should act in a dominant manner. Cardiac and craniofacial development will be closely followed at a variety of ages. This will include in situ hybridization analysis of genes identified in Aim 2 as being downstream of specific G-proteins. Further, NCC migration, proliferation and viability will also be assessed. Should embryonic lethality be observed in founder transgenic mouse embryos, we will take a Tet-On approach to regulate transgene expression. For this purpose two transgenic lines will be created as previously described (11). The first transgenic line will express the reverse tetracycline-responsive transcriptional activator (rtTA) under the control of the enhancers identified in Aim1 to ensure spatial regulation of the Tet-on system while the second will express G-protein alpha subunits under the control of the tetracycline-responsive responsive element (i.e. TRE-*Pmin*CMV-Gα13). By breeding these two transgenic lines and administrating doxycyclin to pregnant female between E8.0 to E10.5 we will ensure temporal expression of the G-protein alpha subunits in specific population of NCCs (9, 12).

SIGNIFICANCE AND LONG-TERM GOAL

The studies proposed above should increase our understanding of the role of Ednra signaling during development and possible etiologies leading to specific human malformations involving NCCs (Aims 1 and 2). By changing G-alpha protein expression in cephalic and cardiac NCCs (Aim 3), we will hopefully elucidate how a differential response to Ednra signaling regulates NCC development at both the cellular and molecular levels. In addition, we will continue to elucidate signaling pathways activated by Ednra signaling and crosstalk with other pathways to understand how NCC development is regulated. Our long term goal is to use our findings to develop novel cell-based corrective therapies to treat craniofacial and cardiovascular birth defects. Findings from these studies might also help the treatment of other disease systems involving endothelin signaling, such as high arterial blood pressure, pulmonary hypertension, cardiac hypertrophy, renal failure, pre-eclampsia and cancer.

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STATEMENT OF TEACHING INTEREST AND PHILOSOPHY

My background would enable me to teach or contribute to the teaching of courses in cell and molecular biology, biochemistry, genetics and developmental biology. While my class lecture experience is somewhat limited, I have spent numerous hours mentoring students in the lab. This experience, along with the mentoring I received during my graduate and post-graduate education, is partly responsible for my decision to become an academic researcher. Academia offers the opportunity to conduct independent research as well as influence the life of students. Teachers have the responsibility to pass on knowledge, help students develop and/or acquire skills and most importantly, challenge them. For these reasons, I strongly believe that teaching should be more than standing in front of a class and delivering a monologue. Lectures should be well prepared and allow for interactions between teacher and students in the form of discussions and/or hands on experience whenever possible. Further, new teaching technologies and applications must be embraced to allow further student leaning and to enhance the lecture experience. To enhance my teaching abilities I would routinely welcome the opportunity to participate in ongoing training sessions for faculty members.

When mentoring students and fellows, it is essential to ensure that their stay in the laboratory provides them with a valuable experience. Successful mentoring should allow students and fellows not only to develop their research skills and improve their scientific knowledge but also develop their uniqueness by achieving individual goals and pursuing new research ideas. It is also important for the mentor to acknowledge that the performance of each trainee must be assessed on an individual basis. While one-on-one supervisor/trainee meetings are essential, group settings such as laboratory meetings are also necessary to develop communication skills and stimulate the exchange of ideas. In my opinion, a good mentor guides his trainees by giving them enough freedom to explore their individuality and develop ideas while ensuring progress.

As a teacher, it is also important to realize that each level of training serves a different purpose. Summer students may be interested in satisfying their curiosity and determining whether research is an appropriate career choice while graduate students aim to acquire the skills and knowledge necessary to obtain their degrees and postdoctoral fellows the expertise needed to become full fledge independent researchers. As a teacher I want to challenge my trainees on an individual basis in the hope that the experience acquired in my laboratory will serve them later in life wherever their career path leads them.