PI: **ZECEVIC, NADA R**	Title: Development of the Cerebral Cortex in Mammals	
Received: 06/29/2010	FOA: PA10-067	Council: 01/2011
Competition ID: ADOBE-FORMS-B	FOA Title: RESEARCH PROJECT GRANT (PARENT R01)	
2 R01 NS041489-10A1	Dual:	Accession Number: 3311557
IPF: 1506603	Organization: UNIVERSITY OF CONNECTICUT SCH OF MED/DNT	
Former Number:	Department: Neuroscience	
IRG/SRG: NCF	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 10: 293,220 Year 11: 284,005 Year 12: 284,589 Year 13: 284,386 Year 14: 284,174	Animals: Y Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: N Early Stage Investigator: N
Senior/Key Personnel:	*Organization:*	*Role Category:*
Nada Zecevic Ph.D.	The University of Connecticut Health Center	PD/PI

Additions for Review

Updated Pages SupplementalR0110641442

OMB Number: 4040-0001
Expiration Date: 06/30/2011

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	State Application Identifier

1. * TYPE OF SUBMISSION
☐ Pre-application ☒ Application ☐ Changed/Corrected Application

2. DATE SUBMITTED | **Applicant Identifier**

4. a. Federal Identifier: NS041489

b. Agency Routing Identifier:

5. APPLICANT INFORMATION

*** Organizational DUNS:** 0222542260000

* Legal Name: The University of Connecticut Health Center
Department: | Division:
* Street1: 263 Farmington Avenue
Street2:
* City: Farmington | County / Parish:
* State: CT: Connecticut | Province:
* Country: USA: UNITED STATES | * ZIP / Postal Code: 06030-2806

Person to be contacted on matters involving this application
Prefix: Ms. | * First Name: Wendy | Middle Name:
* Last Name: Walsh | Suffix:
* Phone Number: 860-679-5472 | Fax Number: 860-679-2670
Email: wewalsh@up.uchc.edu

6. * EMPLOYER IDENTIFICATION *(EIN) or (TIN):* 1521725543A1

7. * TYPE OF APPLICANT: H: Public/State Controlled Institution of Higher Education
Other (Specify):
Small Business Organization Type ☐ Women Owned ☐ Socially and Economically Disadvantaged

8. * TYPE OF APPLICATION:
☐ New ☒ Resubmission
☐ Renewal ☐ Continuation ☐ Revision

If Revision, mark appropriate box(es).
☐ A. Increase Award ☐ B. Decrease Award ☐ C. Increase Duration ☐ D. Decrease Duration
☐ E. Other (specify):

* Is this application being submitted to other agencies? Yes ☐ No ☒ What other Agencies?

9. * NAME OF FEDERAL AGENCY: National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:
TITLE:

11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:
Development of the Cerebral Cortex in Mammals

12. PROPOSED PROJECT:
* Start Date: 04/01/2011 | * Ending Date: 03/31/2016

*** 13. CONGRESSIONAL DISTRICT OF APPLICANT:** CT-005

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION
Prefix: Dr. | * First Name: Nada | Middle Name:
* Last Name: Zecevic | Suffix: Ph.D.
Position/Title: Associate Professor
* Organization Name: The University of Connecticut Health Center
Department: Neuroscience | Division: School of Medicine
* Street1: 263 Farmington Avenue
Street2:
* City: Farmington | County / Parish:
* State: CT: Connecticut | Province:
* Country: USA: UNITED STATES | * ZIP / Postal Code: 06030-3401
* Phone Number: 860-679-1768 | Fax Number: 860-679-8766
* Email: nzecevic@neuron.uchc.edu

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested	2,186,045.00
b. Total Non-Federal Funds	0.00
c. Total Federal & Non-Federal Funds	2,186,045.00
d. Estimated Program Income	0.00

16. * IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?

a. YES ☐ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ☒ PROGRAM IS NOT COVERED BY E.O. 12372; OR

☐ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious. or fraudulent statements or claims may subject me to criminal, civil, or administrative penalities. (U.S. Code, Title 18, Section 1001)

☒ *** I agree**

**** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.***

18. SFLLL or other Explanatory Documentation

Add Attachment | Delete Attachment | View Attachment

19. Authorized Representative

Prefix: Ms. * First Name: Dana Middle Name:

* Last Name: Carroll Suffix:

* Position/Title: Director, ORSP

* Organization: The University of Connecticut Health Center

Department: Division:

* Street1: 263 Farmington Avenue

Street2:

* City: Farmington County / Parish:

* State: CT: Connecticut Province:

* Country: USA: UNITED STATES * ZIP / Postal Code: 06030-2806

* Phone Number: 860-679-4040 Fax Number: 860-679-2670

* Email: grantaward@adp.uchc.edu

*** Signature of Authorized Representative**: Wendy Walsh

*** Date Signed**: 06/29/2010

20. Pre-application Add Attachment | Delete Attachment | View Attachment

424 R&R and PHS-398 Specific Table Of Contents

OMB Number: 4040-0010
Expiration Date: 08/31/2011

Project/Performance Site Location(s)

Project/Performance Site Primary Location ☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: University of Connecticut Health Center

DUNS Number: 0222542260000

* Street1: 263 Farmington Ave.ue

Street2:

* City: Farmington County:

* State: CT: Connecticut

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: 06030-2806 * Project/ Performance Site Congressional District: CT-005

Project/Performance Site Location 1 ☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name:

DUNS Number:

* Street1:

Street2:

* City: County:

* State:

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: * Project/ Performance Site Congressional District:

Additional Location(s) Add Attachment Delete Attachment View Attachment

RESEARCH & RELATED Other Project Information

1. * Are Human Subjects Involved? ☐ Yes ☒ No

1.a If YES to Human Subjects

Is the Project Exempt from Federal regulations? ☐ Yes ☐ No

If yes, check appropriate exemption number. ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6

If no, is the IRB review Pending? ☐ Yes ☐ No

IRB Approval Date:

Human Subject Assurance Number:

2. * Are Vertebrate Animals Used? ☒ Yes ☐ No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ☒ Yes ☐ No

IACUC Approval Date:

Animal Welfare Assurance Number A3471-01

3. * Is proprietary/privileged information included in the application? ☐ Yes ☒ No

4.a. * Does this project have an actual or potential impact on the environment? ☐ Yes ☒ No

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ☐ Yes ☐ No

4.d. If yes, please explain:

5. * Is the research performance site designated, or eligible to be designated, as a historic place? ☐ Yes ☒ No

5.a. If yes, please explain:

6. * Does this project involve activities outside of the United States or partnerships with international collaborators? ☐ Yes ☒ No

6.a. If yes, identify countries:

6.b. Optional Explanation:

7. * Project Summary/Abstract 1234-Zecevic_Abstract_Resubmission_Fi | Add Attachment | Delete Attachment | View Attachment

8. * Project Narrative 1235-Zecevic_Project narrative final | Add Attachment | Delete Attachment | View Attachment

9. Bibliography & References Cited 1236-Zecevic_Literature_Resubmission_ | Add Attachment | Delete Attachment | View Attachment

10. Facilities & Other Resources 1237-Zecevic_Environment Equipment_Re | Add Attachment | Delete Attachment | View Attachment

11. Equipment | Add Attachment | Delete Attachment | View Attachment

12. Other Attachments Add Attachments | Delete Attachments | View Attachments ☐

Abstract

The long-term objective of this proposal is to improve our understanding of development of the human cerebral cortex, and cortical interneurons in particular. Cortical interneurons are necessary for building and fine-tuning of the cortical microcircuitry as they provide inhibitory input to principal (pyramidal) cells. In rodents, the majority of cortical interneurons is derived from the ventral pallidum (ganglionic eminences, GE). They subsequently migrate tangentially into the dorsal cerebral cortex. In the human brain, however, several reports have shown that cortical interneurons may originate both in the GE and in the neocortical ventricular/subventricular zones. The much longer developmental period, size, complexity of the human cerebral cortex and various subtypes of interneurons implicate inter-species differences. Thus, information derived from animal models, although very useful, can not be directly applied to humans. However, to be able to cure various psychiatric and neurological disorders, we need to better understand human brain.

Three Specific Aims will be addressed: 1. Cortical interneuron's progenitors in the human fetal brain. 2. Ventral transcription factors in the human fetal brain, and 3. Do radial glia and/or intermediate progenitors contribute to human cortical interneuron population? Methods of classical histology, immunohistochemistry, *in situ* hybridization, and *in vitro* genetic manipulation methods will be used. We will relate some of the findings in the human fetal brain to the developing mouse brain so that issues relevant to species-dependent differences can be addressed.

A key premise is that a diversity of cortical progenitor cells is necessary to achieve the complexity of the human cerebral cortex. The experimental approaches proposed here are not typically used to study the human fetal brain, but we expect they will yield important information about basic mechanisms regulating the initial cortical organization in humans. The knowledge about human cortical interneurons is fundamental for understanding normal developmental processes, as well as congenital and psychiatric brain disorders, such as schizophrenia or autism, and may contribute to their prevention and treatment. Having available to us a well-characterized collection of human fetal brains, an opportunity to obtain fresh tissue, and expertise in this field, we are in a favorable position to perform proposed experiments.

Project narrative:

I propose to study the distribution and origin of human cortical interneurons, cell type involved in basic circuitry of the cerebral cortex. The experiments outlined in this application will not only improve our knowledge of normal human cortical development, but will be helpful for understanding the pathogenic mechanisms of congenital and psychiatric brain disorders, such autism and schizophrenia.

Environment

Our proximity to Albert Einstein Tissue Repository makes it possible to obtain fresh brain tissue. After a 1.45 min. drive back, during which time tissue is placed on ice in cell culture medium and continuously oxygenated from a portable tank, tissue is further dissected in our lab.
I can discuss scientific issues with a number of faculty, both in Department of Neuroscience and Genetics and Molecular Medicine. In addition, I will share expertise and have support from Dr Nenad Sestan (Yale University, New haven, CT) and Dr Stewart Anderson (Weil Cornell Medical College, NY)-please see attached letters.

Equipment:

The P.I.'s laboratory has access to all of the equipment in the Department of several Core facilities (Molecular Core, Viral Core, Center for Cell Analysis and Modeling-CCAM; Flow Cytometry Center).

In Dr.Zecevic laboratory there is an Olympus BX52 bright-field and fluorescence microscope with a digital camera, a Olympus dissecting microscope, Olympus CKX41 inverted fluorescent microscope, a Neurolucida stereology system with a computer imaging system, Micromcryostat, Leica VT1000 vibratome, a PCR machine, 2 tissue culture hoods; fume hood; two CO2 incubators, electrophoresis equipment; two -80°C freezers and one -20C freezer; a cold room; 1 shaker incubator for bacteria; an electroporation apparatus, liquid N2 tank; water bath; Bellco Glass Co. shaker, Genie small rocker for the incubator, numerous power supplies, analytical balance and a pH meter,

Conveniently located common core space houses ice machines, x-ray developer machine, ultracentrifuges,spectrophotometers, autoclaves, 2 Zeiss LSM 510 confocal microscopes, gel dryers, Kopf pipette puller, a spectrofluorimeter and sonicators.

OMB Number: 4040-0001
Expiration Date: 06/30/2011

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: Dr. * First Name: Nada Middle Name:

* Last Name: Zecevic Suffix: Ph.D.

Position/Title: Associate Professor Department: Neuroscience

Organization Name: The University of Connecticut Health Center Division: School of Medicine

* Street1: 263 Farmington Avenue

Street2:

* City: Farmington County/ Parish:

* State: CT: Connecticut Province:

* Country: USA: UNITED STATES * Zip / Postal Code: 06030-3401

* Phone Number: 860-679-1768 Fax Number: 860-679-8766

* E-Mail: nzecevic@neuron.uchc.edu

Credential, e.g., agency login: NZECEVIC

*** Project Role:** PD/PI **Other Project Role Category:**

Degree Type: M.D., M.Sc, Ph.D.

Degree Year: 1970, 1974, 1978

***Attach Biographical Sketch** 1243-Zecevic_updated biosketc [Add Attachment] [Delete Attachment] [View Attachment]

Attach Current & Pending Support [Add Attachment] [Delete Attachment] [View Attachment]

PROFILE - Senior/Key Person 1

Prefix: * First Name: Middle Name:

* Last Name: Suffix:

Position/Title: Department:

Organization Name: Division:

* Street1:

Street2:

* City: County/ Parish:

* State: Province:

* Country: USA: UNITED STATES * Zip / Postal Code:

* Phone Number: Fax Number:

* E-Mail:

Credential, e.g., agency login:

*** Project Role:** **Other Project Role Category:**

Degree Type:

Degree Year:

***Attach Biographical Sketch** [Add Attachment] [Delete Attachment] [View Attachment]

Attach Current & Pending Support [Add Attachment] [Delete Attachment] [View Attachment]

Program Director/Principal Investigator (Last, First, Middle): Zecevic, Nada R.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
NADA R. ZECEVIC	Associate Professor - Department of Neuroscience
eRA COMMONS USER NAME (credential, e.g., agency login) NZECEVIC	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
University of Belgrade, Medical School, Belgrade, Yugoslavia (YU)	MD	1970	Medicine
University of Belgrade Center for Multidisciplinary Studies, Belgrade, YU	M.Sc.	1974	Neuroscience
University of Belgrade, Medical School, Belgrade, YU	Ph.D.	1978	Developmental Neurobiology

Personal statement: I have been working in the field of primate cerebral cortex development from the beginning of my career. Previous experience with studies on human fetal brain puts my laboratory in a favorable position to identify scientific questions important for advancing knowledge about normal human brain development. In addition, I can identify specific regions and cell types in a still undifferentiated fetal brain that are of interest for this project. In *vitro* method on the live human brain tissue is established in my lab and allows us to directly study cortical progenitor differentiation and follow cell fate of their progeny. Results collected from the human fetal brain will be very helpful to translational research regarding diseases.

B. RESEARCH AND PROFESSIONAL EXPERIENCE:

Positions and Employment:

1971-1972 Internship, City Hospital of Belgrade, Belgrade, Yugoslavia.
1971-1974 Research Assistant, Dept.Neurobiology, Institute for Biological Research, University of Belgrade, Yugoslavia.
1973-1974 Research Fellow, Dept.Neuroanatomy and Neuropathology, Harvard Medical School, Boston, Mass.
1976-1977 Research Fellow, Dept.Anatomy, Medical School, The Johns Hopkins University, Baltimore, MD.
1978-1987 Investigator, Institute for Biological Research, University of Belgrade.
1978-1991 Lecturer, Medical School and Ctr.Multidisciplinary Studies, University of Belgrade.
1982-1986 Resident, Dept.Neurology, Clinic of Neurology, Medical School University of Belgrade, Yugoslavia.
1983-1985 Visiting Scientist, Sec.Neuroanatomy, Yale University, New Haven, CT
1987- 1993 Senior Investigator, Institute for Biological Research, Belgrade, Yugoslavia.
1991- 1998 Associate professor, Ctr.Multidisciplinary Studies, University of Belgrade,Yugoslavia.
1996-1997 Assistant Professor, Department of Medicine,University of Connecticut Medical School, Farmington, CT.

1997-2000 Assistant Professor, Department of Neurology, University of Connecticut Medical School, Farmington, CT.

2000-2006 Assistant Professor, Department of Neuroscience, Univ.Connecticut Medical School, Farmington, CT.

2006 - Present Associate Professor Tenure, Department of Neuroscience, University of Connecticut Medical School, Farmington, CT.

Other experiences and Professional Memberships:

International Brain Research Organization (IBRO), European Neuroscience Association (ENA), Society for Neuroscience (SNF, US), American Neurochemistry Soc. (ANS)
Reviewer ad hoc for NIH, MS Society of US, Canada, MS Society of Australia.
For Journals: J.Neuroscience, Cer Cortex, Glia, PloS, J Neurosci Res etc.

HONORS:

1967, 1969, 1970 Deans honor at Medical School of Belgrade.
1971-1974 Research Fellow, NSF of the Republic Serbia.
1982 Fellowship for Visiting Scientist, University of Zurich, Pharmacology Dept.
1990, 1992 Fellowship for Visiting Scientist, INSERM U106, Paris, France

2004, 2006 Fellowship the Brain Gain Program (BGP) funded by the WUS, Republic of Austria to teach in an undergraduate college course in Belgrade, Serbia with a number of lectures on Brain and Microglia development.
2006- NIH and MS grant awards

2009- Stem Cell Initiative grant award.

References:

Most relevant for the current application:

1. **Zecevic N.**(1993): Cellular composition of the telencephalic wall in human embryos. Early Human Dev. 32:131-149. PMID: 8486116.

2. **Zecevic,N.** and C.Verney (1995): Development of catecholamine neurons in human embryos and fetuses with special emphasis on the innervation of the cerebral cortex. J.Comp.Neurol.,351:509-535. PMID: 7721981.

3. **Zecevic,N.** and A.Milosevic (1997): The initial development of the GABA-immunoreactivity in the human cerebral cortex. J.Comp.Neurol., 380: 495-506. PMID: 9087528.

4. **Zecevic,N.** Milosevic,A.,S.Rakic and M.Marin-Padilla (1999) Early development and composition of the human Primordial Plexiform Layer.An immunohistochemical study . J.Comp.Neurol.412:241-254. PMID: 10441754.

5. **Zecevic,N**. and Rakic,P. Development of Layer I neurons in the Primate Cerebral Cortex. (2001) J.Neurosc. 21(15):5607-5619. PMID: 11466432.

6. Rakic,S. and **Zecevic N** (2003) Emerging complexity of cortical layer I in humans. Cer.Cortex, 13:1072-1083. PMID: 12967924.

7. **Zecevic, N** (2004) Specific characteristics of radial glia in the human fetal telencephalon. GLIA48:27-35. PMID: 15326612.

8. **Zecevic N,** Chen Y, Filipovic R. (2005) Contributions of Cortical Subventricular Zone to the Development of the Human Cerebral Cortex, J.Comp.Neurol. 491:109-122. PMCID: PMC2628573

9. Howard B, Chen Y, **Zecevic N** (2006) Cortical Progenitor cells in the developing human telencephalon. GLIA. 53:57-66. PMID: 16158418.

10. Mo Z, Moore A, Filipovic R, Ogawa Y, Kazuhiro I, Antic S and **Zecevic N** (2007) Human cortical neurons originate from radial glia and neuron-restricted progenitors. J.Neurosc. 27(15):4132-4145. PMID: 17428991.

.

11. Mo Z and **Zecevic N** (2008) Is Pax6 critical for neurogenesis in the human fetal brain? Cer Cortex 18:1455-1465. doi:10.1093/cercor/bhm181 PMCID: PMC2670483; PMID: 17947347

12. Mo Z and **Zecevic N** (2009) Human fetal Radial glia cells generate oligodendrocytes *in vitro.* GLIA 57:490–498. published on line doi: 10.1002/glia.20775**;** PMCID: PMC2644732

13. Moore A, Filipovic R, Mo Z., Rasband M, **Zecevic N**. Antic S. (2009) Electrical excitability of early neurons in the human cerebral cortex during the second trimester of gestation. Cer Cortex 19(8):1795-805. Epub 2008 Nov 17; PMCID: PMC2705693 PMID: 19015375

Most recent:

14. Luisa Pinto[1], Daniela Drechsel[1,2], Marie-Theres Schmid[1], Jovica Ninkovic[1], Martin Irmler[3], Monika S. Brill[1,4], Laura Restani[5], Laura Gianfranceschi[5], Chiara Cerri[5], Susanne N. Weber[6], Victor Tarabykin[7], Kristin Baer[8], Francois Guillemot[2], Johannes Beckers[3,9], **Nada Zecevic**[10], Colette Dehay[11], Matteo Caleo[5], Hubert Schorle[6] and Magdalena Götz (2009) AP2gama regulates basal progenitor fate in a region- and layer-specific manner in the developing cortex. Nature Neurosci. 12 (10): 1229-1237. PMID: 19749747.

15. **Zecevic N**, Hu F, Jakovcevski I. Cortical interneurons in the developing human neocortex. J.Neurobiology (in press).

D. Research Support:

1. NIH 2006-2010 “Development of the Cerebral Cortex in Mammals” (PI)- no cost extension.

2. 2006-2009 National Multiple Sclerosis Society (PI) (no cost extension)
“Development of Oligodendrocyte in the Human Fetal Brain: Relevance for MS” (RG 3083-C4/1)

3. 2009- 2012 Connecticut Innovation Stem Cell Grant
“Human embryonic stem cells (hESC) as a source of radial glia, neurons and oligodendrocytes”

4. Pending R21: “Shh signaling and Interneuron progenitors in human SVZ”

OMB Number: 4040-0001
Expiration Date: 06/30/2011

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:** 0222542260000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry *** Start Date:** 04/01/2011 *** End Date:** 03/31/2012 **Budget Period** 1

A. Senior/Key Person

	Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.	Dr.	Nada		Zecevic	Ph.D.	PD/PI	109,733.00	4.20			38,407.00	14,594.00	53,001.00
2.													
3.													
4.													
5.													
6.													
7.													
8.													

9. **Total Funds requested for all Senior Key Persons in the attached file**

Total Senior/Key Person 53,001.00

Additional Senior Key Persons: Add Attachment Delete Attachment View Attachment

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
2	Post Doctoral Associates	12.00			79,423.00	30,181.00	109,604.00
1	Graduate Students	12.00			28,000.00	5,600.00	33,600.00
	Undergraduate Students						
	Secretarial/Clerical						
1	Research Assistant 1	6.00			29,057.00	11,042.00	40,099.00
4	**Total Number Other Personnel**					**Total Other Personnel**	183,303.00

Total Salary, Wages and Fringe Benefits (A+B) 236,304.00

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:** 0222542260000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry *** Start Date:** 04/01/2011 *** End Date:** 03/31/2012 **Budget Period 1**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

	Equipment item	* Funds Requested ($)
1.	Nanoinjector II Drummond Sci.Com.,Stereomicroscope Zeiss Stemi	9,000.00
2.		
3.		
4.		
5.		
6.		
7.		
8.		
9.		
10.		
11.	**Total funds requested for all equipment listed in the attached file**	
	Total Equipment	9,000.00

Additional Equipment: Add Attachment Delete Attachment View Attachment

D. Travel

		Funds Requested ($)
1.	Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	5,000.00
2.	Foreign Travel Costs	
	Total Travel Cost	5,000.00

E. Participant/Trainee Support Costs

		Funds Requested ($)
1.	Tuition/Fees/Health Insurance	
2.	Stipends	
3.	Travel	
4.	Subsistence	
5.	Other Tuition and fees	4,140.00
	Number of Participants/Trainees **Total Participant/Trainee Support Costs**	4,140.00

RESEARCH & RELATED Budget {C-E} (Funds Requested)

Tracking Number:GRANT10641442 Funding Opportunity Number:PA-10-067 Received Date:2010-06-29T09:03:58-04:00

RESEARCH & RELATED BUDGET - SECTION F-K, BUDGET PERIOD 1

Next Period

*** ORGANIZATIONAL DUNS:** 0222542260000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry *** Start Date:** 04/01/2011 *** End Date:** 03/31/2012 **Budget Period 1**

F. Other Direct Costs		Funds Requested ($)
1.	Materials and Supplies	19,576.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	other purchased services	15,000.00
9.	animal care	4,200.00
10.		
	Total Other Direct Costs	38,776.00

G. Direct Costs	Funds Requested ($)
Total Direct Costs (A thru F)	293,220.00

H. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC	54.00	280,080.00	151,243.00
2.				
3.				
4.				
	Total Indirect Costs			151,243.00

Cognizant Federal Agency Department of Health and Human Services (DHHS) Mr. Jeffrey War

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	444,463.00

J. Fee	Funds Requested ($)

K. * Budget Justification 1239-Zecevic_Budget Justification_Fina Add Attachment Delete Attachment View Attachment

(Only attach one file.)

OMB Number: 4040-0001
Expiration Date: 06/30/2011

Previous Period

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 2

*** ORGANIZATIONAL DUNS:** 0222542260000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry *** Start Date:** 04/01/2012 *** End Date:** 03/31/2013 **Budget Period** 2

A. Senior/Key Person

	Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.	Dr.	Nada		Zecevic	Ph.D.	PD/PI	113,025.00	4.20			39,559.00	15,032.00	54,591.00
2.													
3.													
4.													
5.													
6.													
7.													
8.													
9.	**Total Funds requested for all Senior Key Persons in the attached file**												

Total Senior/Key Person 54,591.00

Additional Senior Key Persons: Add Attachment Delete Attachment View Attachment

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
2	Post Doctoral Associates	12.00			81,806.00	31,086.00	112,892.00
1	Graduate Students	12.00			28,840.00	5,768.00	34,608.00
	Undergraduate Students						
	Secretarial/Clerical						
1	Research Assistant 1	6.00			29,929.00	11,373.00	41,302.00
4	**Total Number Other Personnel**					**Total Other Personnel**	188,802.00

Total Salary, Wages and Fringe Benefits (A+B) 243,393.00

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 2

*** ORGANIZATIONAL DUNS:** 0222542260000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry *** Start Date:** 04/01/2012 *** End Date:** 03/31/2013 **Budget Period 2**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

	Equipment item	* Funds Requested ($)
1.		
2.		
3.		
4.		
5.		
6.		
7.		
8.		
9.		
10.		
11.	**Total funds requested for all equipment listed in the attached file**	
	Total Equipment	

Additional Equipment: Add Attachment Delete Attachment View Attachment

D. Travel **Funds Requested ($)**

1.	Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	4,000.00
2.	Foreign Travel Costs	
	Total Travel Cost	4,000.00

E. Participant/Trainee Support Costs **Funds Requested ($)**

1.	Tuition/Fees/Health Insurance	
2.	Stipends	
3.	Travel	
4.	Subsistence	
5.	Other Tuition and fees	4,264.00
1	**Number of Participants/Trainees** **Total Participant/Trainee Support Costs**	4,264.00

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTION F-K, BUDGET PERIOD 2

Next Period

* ORGANIZATIONAL DUNS: 0222542260000

* Budget Type: ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry * Start Date: 04/01/2012 * End Date: 03/31/2013 Budget Period 2

F. Other Direct Costs		Funds Requested ($)
1.	Materials and Supplies	15,048.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	Other purchase services	13,000.00
9.	animal care	4,300.00
10.		
	Total Other Direct Costs	32,348.00

G. Direct Costs	Funds Requested ($)
Total Direct Costs (A thru F)	284,005.00

H. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC	54.00	279,741.00	151,060.00
2.				
3.				
4.				
	Total Indirect Costs			151,060.00

Cognizant Federal Agency Department of Health and Human Services (DHHS) Mr. Jeffrey War

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	435,065.00

J. Fee	Funds Requested ($)

K. * Budget Justification 1239-Zecevic_Budget Justification_Fina Add Attachment Delete Attachment View Attachment

(Only attach one file.)

OMB Number: 4040-0001
Expiration Date: 06/30/2011

Previous Period

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 3

*** ORGANIZATIONAL DUNS:** 0222542260000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry *** Start Date:** 04/01/2013 *** End Date:** 03/31/2014 **Budget Period** 3

A. Senior/Key Person

	Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.	Dr.	Nada		Zecevic	Ph.D.	PD/PI	116,416.00	4.20			40,746.00	15,483.00	56,229.00
2.													
3.													
4.													
5.													
6.													
7.													
8.													
9.	**Total Funds requested for all Senior Key Persons in the attached file**												

Total Senior/Key Person 56,229.00

Additional Senior Key Persons: Add Attachment Delete Attachment View Attachment

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
2	Post Doctoral Associates	12.00			84,260.00	32,019.00	116,279.00
1	Graduate Students	12.00			29,705.00	5,941.00	35,646.00
	Undergraduate Students						
	Secretarial/Clerical						
1	Research Assistant 1	6.00			30,827.00	11,714.00	42,541.00
4	**Total Number Other Personnel**					**Total Other Personnel**	194,466.00

Total Salary, Wages and Fringe Benefits (A+B) 250,695.00

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 3

*** ORGANIZATIONAL DUNS:** 0222542260000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry *** Start Date:** 04/01/2013 *** End Date:** 03/31/2014 **Budget Period 3**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

	Equipment item	* Funds Requested ($)
1.		
2.		
3.		
4.		
5.		
6.		
7.		
8.		
9.		
10.		
11.	**Total funds requested for all equipment listed in the attached file**	
	Total Equipment	

Additional Equipment: Add Attachment Delete Attachment View Attachment

D. Travel **Funds Requested ($)**

1.	Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	4,000.00
2.	Foreign Travel Costs	
	Total Travel Cost	4,000.00

E. Participant/Trainee Support Costs **Funds Requested ($)**

1.	Tuition/Fees/Health Insurance	
2.	Stipends	
3.	Travel	
4.	Subsistence	
5.	Other Tuition and fees	4,392.00
1	**Number of Participants/Trainees** **Total Participant/Trainee Support Costs**	4,392.00

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTION F-K, BUDGET PERIOD 3

Next Period

*** ORGANIZATIONAL DUNS:** 0222542260000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry *** Start Date:** 04/01/2013 *** End Date:** 03/31/2014 **Budget Period 3**

F. Other Direct Costs		Funds Requested ($)
1.	Materials and Supplies	12,002.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	Other purchase services	9,000.00
9.	animal care	4,500.00
10.		
	Total Other Direct Costs	25,502.00

G. Direct Costs		Funds Requested ($)
	Total Direct Costs (A thru F)	284,589.00

H. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC	54.00	280,197.00	151,306.00
2.				
3.				
4.				
	Total Indirect Costs			151,306.00

Cognizant Federal Agency Department of Health and Human Services (DHHS) Mr. Jeffrey War

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	435,895.00

J. Fee	Funds Requested ($)

K. * Budget Justification 1239-Zecevic_Budget Justification_Fina Add Attachment Delete Attachment View Attachment

(Only attach one file.)

OMB Number: 4040-0001
Expiration Date: 06/30/2011

Previous Period

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 4

*** ORGANIZATIONAL DUNS:** 0222542260000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry *** Start Date:** 04/01/2014 *** End Date:** 03/31/2015 **Budget Period** 4

A. Senior/Key Person

	Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.	Dr.	Nada		Zecevic	Ph.D.	PD/PI	119,908.00	4.20			41,968.00	15,948.00	57,916.00
2.													
3.													
4.													
5.													
6.													
7.													
8.													

9. **Total Funds requested for all Senior Key Persons in the attached file**

Total Senior/Key Person 57,916.00

Additional Senior Key Persons: Add Attachment Delete Attachment View Attachment

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
2	Post Doctoral Associates	12.00			86,788.00	32,979.00	119,767.00
1	Graduate Students	12.00			30,596.00	6,119.00	36,715.00
	Undergraduate Students						
	Secretarial/Clerical						
1	Research Assistant 1	6.00			31,752.00	12,066.00	43,818.00
4	**Total Number Other Personnel**					**Total Other Personnel**	200,300.00

Total Salary, Wages and Fringe Benefits (A+B) 258,216.00

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 4

*** ORGANIZATIONAL DUNS:** 0222542260000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry *** Start Date:** 04/01/2014 *** End Date:** 03/31/2015 **Budget Period 4**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

	Equipment item	* Funds Requested ($)
1.		
2.		
3.		
4.		
5.		
6.		
7.		
8.		
9.		
10.		
11.	**Total funds requested for all equipment listed in the attached file**	
	Total Equipment	

Additional Equipment: Add Attachment Delete Attachment View Attachment

D. Travel **Funds Requested ($)**

1.	Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	3,000.00
2.	Foreign Travel Costs	
	Total Travel Cost	3,000.00

E. Participant/Trainee Support Costs **Funds Requested ($)**

1.	Tuition/Fees/Health Insurance	
2.	Stipends	
3.	Travel	
4.	Subsistence	
5.	Other Tuition and fees	4,525.00
1	**Number of Participants/Trainees** **Total Participant/Trainee Support Costs**	4,525.00

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTION F-K, BUDGET PERIOD 4

Next Period

*** ORGANIZATIONAL DUNS:** 0222542260000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry *** Start Date:** 04/01/2014 *** End Date:** 03/31/2015 **Budget Period 4**

F. Other Direct Costs		Funds Requested ($)
1.	Materials and Supplies	8,445.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	Other purchase services	6,000.00
9.	animal care	4,200.00
10.		
	Total Other Direct Costs	18,645.00

G. Direct Costs	Funds Requested ($)
Total Direct Costs (A thru F)	284,386.00

H. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC	54.00	279,861.00	151,125.00
2.				
3.				
4.				
	Total Indirect Costs			151,125.00

Cognizant Federal Agency Department of Health and Human Services (DHHS) Mr. Jeffrey War

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	435,511.00

J. Fee	Funds Requested ($)

K. * Budget Justification 1239-Zecevic_Budget Justification_Fina Add Attachment Delete Attachment View Attachment

(Only attach one file.)

OMB Number: 4040-0001
Expiration Date: 06/30/2011

Previous Period

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 5

*** ORGANIZATIONAL DUNS:** 0222542260000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry *** Start Date:** 04/01/2015 *** End Date:** 03/31/2016 **Budget Period** 5

A. Senior/Key Person

	Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.	Dr.	Nada		Zecevic	Ph.D.	PD/PI	123,505.00	4.20			43,227.00	16,426.00	59,653.00
2.													
3.													
4.													
5.													
6.													
7.													
8.													
9.	**Total Funds requested for all Senior Key Persons in the attached file**												

Total Senior/Key Person 59,653.00

Additional Senior Key Persons: Add Attachment Delete Attachment View Attachment

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
2	Post Doctoral Associates	12.00			89,391.00	33,969.00	123,360.00
1	Graduate Students	12.00			31,514.00	6,303.00	37,817.00
	Undergraduate Students						
	Secretarial/Clerical						
1	Research Assistant 1	6.00			32,704.00	12,428.00	45,132.00
4	**Total Number Other Personnel**					**Total Other Personnel**	206,309.00

Total Salary, Wages and Fringe Benefits (A+B) 265,962.00

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 5

*** ORGANIZATIONAL DUNS:** 0222542260000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry *** Start Date:** 04/01/2015 *** End Date:** 03/31/2016 **Budget Period 5**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

	Equipment item	* Funds Requested ($)
1.		
2.		
3.		
4.		
5.		
6.		
7.		
8.		
9.		
10.		
11.	**Total funds requested for all equipment listed in the attached file**	
	Total Equipment	

Additional Equipment: Add Attachment Delete Attachment View Attachment

D. Travel **Funds Requested ($)**

1.	Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	1,000.00
2.	Foreign Travel Costs	
	Total Travel Cost	1,000.00

E. Participant/Trainee Support Costs **Funds Requested ($)**

1.	Tuition/Fees/Health Insurance	
2.	Stipends	
3.	Travel	
4.	Subsistence	
5.	Other Tuition and fees	4,661.00
	Number of Participants/Trainees **Total Participant/Trainee Support Costs**	4,661.00

RESEARCH & RELATED Budget {C-E} (Funds Requested)

Tracking Number:GRANT10641442 Funding Opportunity Number:PA-10-067 Received Date:2010-06-29T09:03:58-04:00

RESEARCH & RELATED BUDGET - SECTION F-K, BUDGET PERIOD 5

*** ORGANIZATIONAL DUNS:** 0222542260000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: The University of Connecticut H

Delete Entry *** Start Date:** 04/01/2015 *** End Date:** 03/31/2016 **Budget Period 5**

F. Other Direct Costs		Funds Requested ($)
1.	Materials and Supplies	5,000.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	Other purchase services	4,051.00
9.	Animal care	3,500.00
10.		
	Total Other Direct Costs	12,551.00

G. Direct Costs	Funds Requested ($)
Total Direct Costs (A thru F)	284,174.00

H. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC	54.00	279,513.00	150,937.00
2.				
3.				
4.				
	Total Indirect Costs			150,937.00

Cognizant Federal Agency Department of Health and Human Services (DHHS) Mr. Jeffrey War

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	435,111.00

J. Fee	Funds Requested ($)

K. * Budget Justification 1239-Zecevic_Budget Justification_Fina Add Attachment Delete Attachment View Attachment

(Only attach one file.)

BUDGET JUSTIFICATION

Personnel

Nada Zecevic, MD,PhD. (PI) As PI, I will be fully responsible for the experiments and studies proposed in this grant. In the first year of the grant, I will spend 4.2 person months of my time and effort in setting up future experiments, identify appropriate cases to be included in this study, optimizing immunostaining protocols for each antibody and selecting the best antibodies. In the second year, I will oversee experiments using double labeling immunohistochemistry, transfection and follow up of these cells. In the last three years I will be involved in interpretation of results, as well as writing papers. I expect that by this time, the post doctoral fellows and the research assistant will be trained and capable to execute majority of experiments by themselves, with my supervision.

Xiajong Yu, PhD. Yu is trained in molecular biology with experience in cell culture, Western Blots, immunocytochemistry, and thus she will be extremely useful for this project and will spend 12.0 person months of time and effort. In particular she will be engaged in immuno-labeling of the human tissue, and in vitro studies of cell and slice cultures. She also will be in charge of slice preparation and electroporation experiments. These procedures will be very time consuming and technically demanding but probably very important for the outcome of this study. In addition, she will analyze results, write papers, and supervise others in the laboratory.

TBN Postdoctoral fellow. I am looking for a postdoctoral fellow who will spend 12.0 person months of his time during each of five years of this project. He/she will perform most of single and double labeling immunohistochemical experiments, transfection of progenitor cells and their follow up in cell cultures. Post doc will be responsible to develop and execute combination of immunolabelling techniques, as well as the best method for quantifying immunolabeling of our tissue. He/she will be trained to do electroporation in slice cultures in order to label cortical progenitor cells, and follow their differentiation. In addition, he/she will supervise the Research Assistant in experiments that involve double immuno-Iabeling and confocal microscopy. He/she will be engaged in interpretation and writing of results in the last three years of the grant.

Nicole Mayer, Research Assistant II. In order to successfully execute proposed experiments, we will need a help of a Research Assistant with 6 person months of the time and effort. Nicole will be in charge of ordering supplies, ordering and keeping track of antibodies, maintaining order in the lab, autoclave dishes, help with immunostainig, prepare solutions, do Western Blots and help with collecting the tissue.

Graduate Student (100% effort or 12 months) will perform experiments related to making human specific constructs and transfecting them to human cortical progenitors. Graduate student will do immunohistochemical studies, Western Blot studies and in situ hybridization. Fringe benefit rates are 34%. Personnel costs are increased by 3% in years 2-4.

Due to the high amount of technical assistance required (with associated salary and significant fringe rates here at UCHC), I am requesting a non modular budget.

Stewart Anderson, MD, Associate Professor Dept. of Psychiatry Weill Cornel Medical Collage, NY, is a collaborator who will help with training a postdoc for applying electroporation on brain slices and with other aspects of genetic labeling of *in vitro* cells. He will have 0 time on this project. Please see the attached letter.

RESEARCH & RELATED BUDGET - Cumulative Budget

		Totals ($)
Section A, Senior/Key Person		281,390.00
Section B, Other Personnel		973,180.00
Total Number Other Personnel	20	
Total Salary, Wages and Fringe Benefits (A+B)		1,254,570.00
Section C, Equipment		9,000.00
Section D, Travel		17,000.00
1. Domestic	17,000.00	
2. Foreign		
Section E, Participant/Trainee Support Costs		21,982.00
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other	21,982.00	
6. Number of Participants/Trainees	3	
Section F, Other Direct Costs		127,822.00
1. Materials and Supplies	60,071.00	
2. Publication Costs		
3. Consultant Services		
4. ADP/Computer Services		
5. Subawards/Consortium/Contractual Costs		
6. Equipment or Facility Rental/User Fees		
7. Alterations and Renovations		
8. Other 1	47,051.00	
9. Other 2	20,700.00	
10. Other 3		
Section G, Direct Costs (A thru F)		1,430,374.00
Section H, Indirect Costs		755,671.00
Section I, Total Direct and Indirect Costs (G + H)		2,186,045.00
Section J, Fee		

Tracking Number:GRANT10641442 Funding Opportunity Number:PA-10-067 Received Date:2010-06-29T09:03:58-04:00

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

1. Project Director / Principal Investigator (PD/PI)

Prefix:	Dr.	* First Name:	Nada
Middle Name:			
* Last Name:	Zecevic		
Suffix:	Ph.D.		

2. Human Subjects

Clinical Trial? ☒ No ☐ Yes

* Agency-Defined Phase III Clinical Trial? ☐ No ☐ Yes

3. Applicant Organization Contact

Person to be contacted on matters involving this application

Prefix:	Ms.	* First Name:	Wendy
Middle Name:			
* Last Name:	Walsh		
Suffix:			

* Phone Number:	860-679-5472	Fax Number:	860-679-2670
Email:	wewalsh@up.uchc.edu		

* Title: University of Connecticut Health Center

* Street1:	263 Farmington Avenue
Street2:	
* City:	Farmington
County/Parish:	
* State:	CT: Connecticut
Province:	
* Country:	USA: UNITED STATES
* Zip / Postal Code:	06030-2806

PHS 398 Cover Page Supplement

4. Human Embryonic Stem Cells

* Does the proposed project involve human embryonic stem cells? ☒ No ☐ Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://stemcells.nih.gov/research/registry/. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Cell Line(s): ☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Tracking Number:GRANT10641442 Funding Opportunity Number:PA-10-067 Received Date:2010-06-29T09:03:58-04:00

OMB Number: 0925-0001

PHS 398 Research Plan

1. Application Type:

From SF 424 (R&R) Cover Page. The response provided on that page, regarding the type of application being submitted, is repeated for your reference, as you attach the appropriate sections of the Research Plan.

*Type of Application:

☐ New ☒ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

2. Research Plan Attachments:

Please attach applicable sections of the research plan, below.

1. Introduction to Application (for RESUBMISSION or REVISION only)	1240-Zecevic_Introduction_R	Add Attachment	Delete Attachment	View Attachment
2. Specific Aims	1241-Zecevic_Specific Aims_R	Add Attachment	Delete Attachment	View Attachment
3. *Research Strategy	1242-Zecevic_Significance In	Add Attachment	Delete Attachment	View Attachment
4. Inclusion Enrollment Report		Add Attachment	Delete Attachment	View Attachment
5. Progress Report Publication List		Add Attachment	Delete Attachment	View Attachment

Human Subjects Sections

6. Protection of Human Subjects	1244-Zecevic_Human subjects	Add Attachment	Delete Attachment	View Attachment
7. Inclusion of Women and Minorities		Add Attachment	Delete Attachment	View Attachment
8. Targeted/Planned Enrollment Table		Add Attachment	Delete Attachment	View Attachment
9. Inclusion of Children		Add Attachment	Delete Attachment	View Attachment

Other Research Plan Sections

10. Vertebrate Animals	1245-Zecevic_Vertebrate anim	Add Attachment	Delete Attachment	View Attachment
11. Select Agent Research		Add Attachment	Delete Attachment	View Attachment
12. Multiple PD/PI Leadership Plan		Add Attachment	Delete Attachment	View Attachment
13. Consortium/Contractual Arrangements		Add Attachment	Delete Attachment	View Attachment
14. Letters of Support	1246-Zecevic_Ltrs of support	Add Attachment	Delete Attachment	View Attachment
15. Resource Sharing Plan(s)		Add Attachment	Delete Attachment	View Attachment

16. Appendix Add Attachments | Remove Attachments | View Attachments

Introduction

I would like to thank the reviewers for their thoughtful and constructive comments. I am pleased that all three reviewers found studies of the human brain significant and worth pursuing. In the revised application, I made numerous changes to the entire text according to reviewer's comments, and the need to shorten the application. Some new preliminary results (figures 3,9,11) are included, whereas some experiments are eliminated (reduced number of constructs and the effect of SHH). I believe these changes made this application more focused.

1. Concerns were raised about the use of mouse promoters in human brain tissue studies by all three reviewers. These are valid concerns in the absence of preliminary data. At the same time, the application was viewed as being overly ambitious (Rev. #3). To adequately answer these critiques, I reduced the number of constructs to two promoter-reporters: GFP-Lhx6 and GFP-Nkx2.1, for which we either have preliminary results (Lhx6) or have made Cre plasmid with the commercially available human Nkx2.1 promoter (Switch Gear Genomics). Similarly, for loss-and-gain of functions I will focus on Nkx2.1 and Pax6 transcription factors. We have preliminary data for Pax6, and we cloned human shNkx2.1. We will proceed with additional promoter-reporters if they become available for human tissue. Our main goal is to study Nkx2.1 lineage to better characterize cortical $Nkx2.1^{+}$ and $calretinin^{+}$ progenitors at mid-term, as they may be human-specific and thus may have clinical relevance.
2. Reviewers suggested better controls for loss-of-function studies. We now include the following text: "We will do several immunolabeling and WB analysis to verify protein expression and examine possible off-target effects, PCR to verify levels of mRNAs, in addition of transfecting scrambled RNA. BLAST will be used to confirm that oligonucleotide strands used to make shRNA are specific for the particular gene to knock-down, and that the scrambled controls match no known genes."
3. Preliminary results on the effect of over-expression of Pax6 on cell proliferation and neuron production were missing (Rev#2), These are now shown in new Fig.8.
4. The viability of slice cultures and the likelihood of observing changes in short culturing time was questioned (Rev# 1, 2). Reports on primates, including our published studies, used similarly short culturing time to generate important results on human brain development. However, we now have cultured slices for 16 days, employing a different method (Simoni and Yu, 2006). Moreover, transfection experiments will also be done in dispersed cell cultures which are maintained for 2-3 weeks (Mo and Zecevic, 2008, 2009). Our studies with TUNEL show that cell death in slices is not excessive.
5. Slices are made and electroporated in our lab, while tissue is collected 2 hours away (Rev#3).
6. Progress on the previous grant is now presented in more details (Rev#2 and 3). Although the majority of proposed experiments were successfully finished and published, we did experiments with Pax6 transcription factor instead of Notch and Numb distribution (original SA1) since Pax6 seemed more relevant for understanding neurogenetic fate of radial glia. Partial results on Pax6 are published (Mo and Zecevic, 2008), and this line of study will be continued in new SA3. Experiments on the effect of FGF2 on cortical progenitors (SA2) are in the final stage (Yu et al., in prep).
7. Rev#2 commented on descriptive nature of experiments and lack of explanation of mechanisms. Indeed, many baseline data on human brain development are currently still missing, so that even descriptive details are needed. For example, very little is known about new progenitor subtypes, $Nkx2.1^{+}$ and $calretinin^{+}$ dorsal progenitors in the cortical SVZ. In addition we are proposing state-of-the art experiments on <u>live human tissue</u>: loss-and-gain of function experiments as well as transfection/infection with promoter-reporter genes or shRNA. These types of experiments are very rarely attempted with human brain tissue and so far have been done only by a handful of laboratories. Yet, these experiments are the only way to obtain knowledge about novel evolutionary traits that might characterize human brains.

A. SPECIFIC AIMS

Understanding the cellular and molecular mechanisms of normal human brain development is an essential first step towards the prevention and therapeutic intervention of neurodevelopmental disorders.

Our main focus is to study human cortical interneurons, one of the two major subtypes of neurons in the cerebral cortex. Cortical interneurons are necessary for building and fine-tuning of the cortical microcircuitry as they provide inhibitory input to principal (pyramidal) cells. In rodents, most or all of the cortical interneurons are derived from the pallidum (ganglionic eminences, GE) and migrate tangentially into the dorsal cerebral cortex. In the human brain the origins and detailed characteristics of cortical interneurons remain unresolved. In fact, cortical interneurons in humans may have dual origins, from the GE as well as from the neocortical ventricular/subventricular zones (VZ/SVZ). The much longer developmental period, size, complexity of the human cerebral cortex, and molecular characteristics of interneurons all implicate species-specific differences. Thus, in addition to studies of animal models that are very useful, we need to better understand the human brain in order to be able to cure various psychiatric and neurological disorders. Although the main goal of these studies is to characterize interneuron generation in the human fetal brain, we will relate some of these findings to the developing mouse brain so that issues relevant to species-dependent differences can be addressed.

Specific Aim 1. Cortical interneuron's progenitors in the human fetal brain. We will characterize human cortical interneurons and their progenitors in terms of their cellular morphology, antigen phenotype and spatiotemporal distribution from early to late developmental stages (5-24 gestation weeks, g.w.). Next, we will test the hypothesis that, apart from the GE, an additional source of cortical interneurons is the neocortical SVZ, which is especially active in later phases of neurogenesis. We will assess the proliferation of potential cortical interneuron progenitors (Dlx, Mash1, GAD65, and calretinin positive cells) in the GE and cortical SVZ.

Specific Aim 2. Ventral transcription factors in human fetal brain. A corollary to the hypothesis about the dual origin of cortical interneurons is that ventral transcription factors (TF), Dlx1/2, Nkx2.1, Mash1 and Lhx6 are differently regulated and distributed in the human brain. *In vitro* experiments with tissue from the GE and neocortical VZ/SVZ will be used for retroviral lineage analysis, loss-and gain-of-function experiments and single and double immunolabeling with interneuron and proliferation markers.

Specific Aim 3. Do radial glia and/or intermediate progenitors contribute to human cortical interneuron population? We postulate that multiple neuronal progenitor cells in the human fetal neocortical VZ/SVZ, including radial glia (RG) and intermediate progenitors (IPCs), generate subpopulations of cortical interneurons. Methods to be employed include classical histology, immunohistochemistry, *in situ* hybridization, and *in vitro* transfection/infection.

A key premise driving this application is that a diversity of cortical progenitor cells is necessary to achieve the complexity of the human cerebral cortex. The experimental approaches proposed here are not typically used for studying the human fetal brain. We expect that our results will yield important information, not presently available, about basic mechanisms regulating the initial cortical organization in the human brain. The information about cortical interneurons is fundamental, not only for understanding normal developmental processes, but also for understanding pathogenesis of congenital and psychiatric disorders, and may contribute to their prevention and treatment.

Abbrevations to be used throughout the application: BLBP- brain lipid binding protein; CalR- calretinin; CB- calbindin; GE- ganglionic eminence (ventral pallidum in rodents), GFP- green fluorescent protein; RG- radial glia; g.w.-gestation weeks; IPCs-the intermediate progenitor cells; NPY-neuropeptide Y; PV- parvalbumin; VIP- vasointestinal polypeptide; Sst-somatostatin; TF-transcription factor; VZ/SVZ- ventricular/subventricular zone.

Research Strategy

a. SIGNIFICANCE

The cerebral cortex is considered the principal structure that makes humans different from any other species. Yet, far less progress has been made to define the cellular and molecular properties of cortical development in humans than in rodents. Our knowledge on human brain development is lagging behind animal models since new experimental approaches, such as *in vivo* electroporation or creation of mutant and transgenic mice, are not possible in humans. We however need to advance our understanding of various aspects of human brain development if we want to prevent or cure human diseases. I propose to focus on cortical interneurons, their origin, distribution and molecular characteristics in the human fetal brain. Cortical interneurons play a crucial role in the functioning of cortical microcircuitry as they provide inhibitory input to projection (pyramidal) neurons. They also have a role in normal cortical development through their influence on cell proliferation and migration (Haydar et al., 2000; Owens and Kriegstein, 2002). Importantly, cortical interneurons are implicated in neuropsychiatric disorders that range from cortical ectopias with epilepsy (DeFelipe, 1999; Gleeson and Walsh, 2000) to schizophrenia, autism or bipolar disorder (Levitt et al., 2004; Baraban and Tallent, 2004; Lewis et al., 2005). Combined results from several laboratories suggest that the cortical interneuron progenitor population is more complex in primates relative to rodents (Zecevic and Rakic, 2001, Rakic and Zecevic, 2003; Letinic et al., 2002; Petanjek et al., 2009; Fertuzinhos et al., 2009). The increased complexity of progenitors is probably an evolutionary adaptation necessary for the development of higher brain functions in primates, and particularly in humans.

In rodents, most cortical interneurons originate in the ventral pallidum (ganglionic eminence-GE) and migrate tangentially to the dorsally located cerebral cortex (Anderson et al., 1997; Parnavelas et al., 2000). In contrast to rodents, a long developmental period and a larger human brain with new cortical areas and expanded upper cortical layers II-IV (Hill and Walsh, 2005; Molnár et al., 2006) is likely to need additional sources of interneurons in late corticogenesis. The secondary proliferative zone is the neocortical subventricular zone (SVZ) which evolutionarily expands in primates, including humans (Smart et al., 2002; Lukaszewicz et al., 2006; Zecevic et al., 2005, Bayatti et al., 2008) and is a source of late born cortical neurons (Rakic 2009).

We postulate here that late born calretinin ($CalR^+$) interneurons destined for upper cortical layers, are generated from distinct progenitors in the outer neocortical SVZ in humans. These progenitors by their location, migration pattern and antigen profile are different from the interneurons generated in the embryonic forebrain, which are of GE origin and migrate tangentially to deep cortical layers. Importantly, bipolar $CalR^+$ neurons are much more abundant in upper cortical layers in human (50%) than in rodents (15%) (DeFelipe et al., 2006). Upper cortical layers are implied in development of human specific higher brain functions, such as learning, memory, abstract thinking, and language (Hill and Walsh, 2005). Their significance is further underscored by reports that neurons in these layers are selectively damaged in psychiatric pathologies (e.g., Lewis et al., 2005). Thus, a better understanding and appreciation of human interneuronal diversity and how it is created could help in devising future targeted therapies for psychiatric diseases, as well as for use of stem-cell technology in replacement therapies of neurological disorders.

Transcription factors are useful markers of various progenitors during development (Marin and Rubenstein, 2001; Wonders and Anderson, 2006; Molyneaux et al., 2007), but their regulation may differ in primates, as discussed in several reports (Letinic et al., 2002; Rakic and Zecevic, 2003; Zecevic et al., 2005; Bystron et al., 2006). This point is well illustrated with Nkx2.1 expression which in mice is down-regulated in cells that migrate to the neocortex (Nobrega-Pereira et al., 2008). However, in human brain we have demonstrated early presence of GABA/$Nkx2.1^+$ cells in the cortical preplate (Rakic and Zecevic, 2003), and at mid-term (20 gestational weeks) (Fig.3, 4), suggestive of their local origin. In SA 2 we will examine the expression of ventral transcription factors, and study the role of Nkx2.1 in interneuron specification and differentiation in humans. These results will be compared to rodents to see how much is conserved during evolution.

Additional interneuron progenitors might be generated by radial glia (RG) and intermediate progenitors (IPC), which were reported to generate cortical projection neurons both in rodents (e.g., Noctor et al., 2001, 2004; Haubensak et al., 2004) and in human brain (Mo et al., 2007, Hansen et al., 2010). There is very little data on the IPCs progeny in human brains whereas they are extensively studied in mouse models (rev. Hevner et al., 2006; Kowalczyk et al., 2009). We will use genetic labeling to follow progeny of RG/IPCs and determine

whether they can also generate cortical interneurons, as our preliminary data suggest. Dorsal transcription factor Pax6 (paired box 6), a determinant of neuronal fate of the RG in mice (Gotz et al., 1998, Osumi et al., 2008) and humans (Mo and Zecevic, 2008), will be further studied in SA3 in relation to cortical interneurons.

b. Innovation. I propose to study a novel concept that in humans multiple progenitors located in the cortical VZ/SVZ, including radial glia or/and intermediate progenitors, generate cortical interneurons. Studies on fresh human brain tissue are not often conducted, since good quality tissue necessary for this approach is hard to obtain. To partially overcome problems related to studies on human tissue, I proposed to apply *in vitro* methods which are used in a handful of labs including mine to generate results beyond descriptive ones (Letinic et al., 2002, Mo et al., 2007; Mo and Zecevic, 2008, 2009, Hansen et al., 2010; our Preliminary data).
Our preliminary results demonstrate that it is possible to genetically label cells in human brain slices by electroporation and follow them for linage analysis. Transfection/infection studies will be also done *in vitro* for loss- and gain-of-function experiments. It is not clear whether reporter-promoter genes or particular shRNA, routinely used in mice, could work in human tissue. Based on our preliminary results with electroporation (Fig.7) and over expression of Pax6 (Fig.11), as well as our published results on genetic labeling of radial glia cells (Mo et al., 2007) and loss of Pax6 function (Mo and Zecevic, 2008), we are confident that these experiments can be done on human tissue. Since these studies are very important for further understanding of human brain development, and congenital malformation of the CNS, the available mouse constructs have to be tested and, if necessary, human-specific ones have to be made. So far we have made the human Lhx6 probe, shRNA Nkx2.1, human promoter-reporter Nkx2.1-GFP and Lhx6-GFP (Fig.7). However, classical immunohistochemistry and *in situ* hybridization techniques will also be applied, and although these are not novel approaches, a collection of well preserved cryosections of human fetal forebrains in different stages of development, from embryonic (5g.w.) to fetal (20-24 g.w.) is relatively unique. Longitudinal analysis of this tissue will allow us to obtain a dynamic picture, not presently available, of human cortical interneurons differentiation during development.

c. Approach. To study the development of human cortical interneurons we will apply methods of classical histology, immunohistochemistry and molecular biology techniques (*in situ* hybridization, PCR and Western Blot). *In vitro* system will be used for retroviral lineage analysis and loss-and gain-of function experiments combined with single and double immunolabeling with interneurons and proliferation markers.
Material:
A) Human fetal tissue for immunohistochemical analysis is obtained from various Brain Banks. Our fetal brain collection consists of 85 cases ranging in age from 5 to 24 gestational weeks. Fetal brains were cut either in coronal (frontal) or in sagittal plane. This material was previously used in our publications (e.g. Zecevic, 1998; 2004; Zecevic et al., 2005).
B) Fresh human fetal brain hemispheres (n=28) have to date been obtained from The Tissue Repository of the Albert Einstein University, The Bronx, NY, and we will continue to obtain fetal tissue from the same source on an average rate of 1-2 per month (please see the attached letter by Dr. B. Poulos). Handling of tissue is carried out in accordance with all regulations set forth by the institutional ethics committees. Only tissue where no evidence of disease or developmental abnormalities is discovered after ultrasonic and neuropathological examination is analyzed. Tissue is put in oxygenated ice-cold Hank's balanced salt solution (HBSS, Sigma, St. Louis, MO) with 0.75% glucose, transported and dissected within 2 h after extraction.

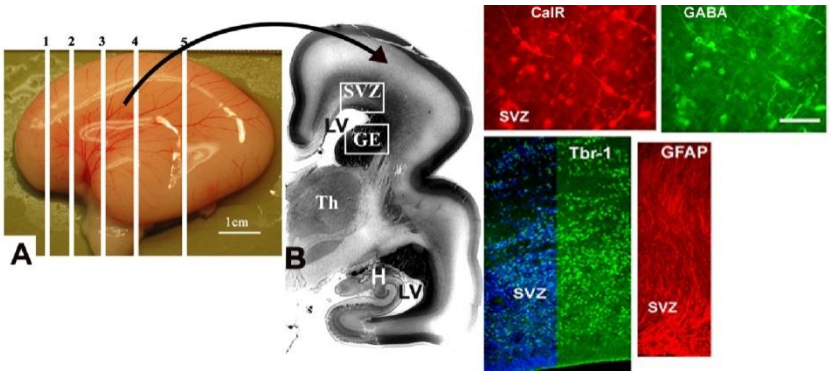


Fig. 1 A. Left brain hemisphere at 20gw. White bar mark position of frontally cut blocks. B. Typical section stained with Nissl. Slices or tissue for cell culture is dissected from the boxed area regions. Other blocks are fixed and frozen for immunolabeling, Note that in human brain temporal lobe is ventral thus we use terms GE and neocortical VZ/SVZ instead of ventral and dorsal telencephalon used in rodents .

Gender, age and left/right hemisphere is noted. An example of a typical fetal brain at mid-term is shown in Fig1. From the same brain, tissue is

dissected for cell and slice cultures, and frozen for immuno-histochemistry and Western Blots. Ultrasound and anatomical landmarks (Mueller and O'Rahilly, 1990) were used to determine the gestational age of embryos and fetuses.

Note: Preliminary results are included with the appropriate Specific Aim sections.

Specific Aim 1: Cortical interneuron's progenitors in the human fetal brain.

Hypothesis: Cortical interneurons have dual origin in the human brain: an initial origin from the GE and a later origin from the expanded neocortical VZ/SVZ.

Experimental Design: Immunohistochemistry and *in situ* hybridization will be done to determine distribution of various subtypes of interneurons and proliferation of their progenitors in human fetal forebrain. These studies will continue in SA 2.

Immunohistochemical method is described in detail in our publications (e.g., Zecevic 2004,; 2005; Mo and Zecevic, 2008). After fixation (4% paraformaldehyde and 0.1% glutaraldehyde), cryoprotection in 30% sucrose, tissue blocks were frozen in isopentane at -70^{0}C, and cut into 14μm sections. Primary antybodies to be used are: calretinin (rabbit, 1:2000, Swant, Belliziona, Switzerland), calbindin and Map2, mouse, from Sigma (St. Louis, MO), GABA (mouse, 1:1000, and rabbit, 1:2000, Sigma), NPY (rabbit, 1:5000, Hudson, WI), VIP (rabbit, 1:500, ImmunoStar), somatostatin, NeuN, GAD65 and GAD67 mouse, from Chemicon, parvalbumin (mouse, 1:1000, Sigma), Ki67 (rabbit, 1:100, Anaspec, San Jose, CA), PH3 (mouse, 1:500, Imgenex), BrdU (mouse 1:50, Becton Dickinson, CA), Nkx2.1 (rabbit, 1:200, Epitomics, Burlingame, CA), pan Dlx (gift Dr. Y.Morozov, Yale Univ., New Haven, CT., rabbit, 1: 2000), Tbr2 and Tbr1 (rabbit, 1:2000, gift from Dr. Hevner, Univ. WA.) Pax6 (mouse, 1:500, Dev.Hybridoma Banks, Iowa City, IA), Lhx6 (rabbit, 1:1000, Abcam), Mash1 (mouse, 1:500, BD Pharmingen), doublecortin (rabbit, 1:200, Santa Cruz), caspase 3 (rabbit, 1:500, Sigma). For double or triple labeling experiments, a cocktail of primary antibodies generated in different animal species and corresponding secondary antibodies (from Molecular Probes, Eugena, Oregon, U.S) will be used. Brief counterstaining with 0.1% bis-bensamide (Sigma) is performed to label all cell nuclei.

To **control** for antibody specificity we use the Western Blot from the same age fetal brains, replacement of primary antibody by the normal serum from the same animal species, and predigesting of primary antibodies. All of these should result in lack of immunostaining.

In situ hybridization will be performed using the digoxigenin method, as described in our previous publication (Jakovcevski and Zecevic 2005).

Morphometric study of immunofluorescent cells. The number of immuno-positive cells will be calculated per surface area in the fetal SVZ, using Neurolucida and Stereoinvestigator (version 9.0, MicroBrightField,Inc., VT) on randomly selected 15 μm thick frozen sections or cell cultures. When possible, the optical disector principle will be used to estimate cell densities, as described previously (Jakovcevski et al., 2009). At least 3 fetal brains and 3 sections per age/region will be analyzed. Numerical densities are estimated by counting nuclei of labeled cells within systematically randomly spaced optical disectors. The parameters for this analysis will be: guard space depth 2 μm, base and height of the disector 3,600μm^2 and 10 μm, respectively, distance between the optical disectors 60 μm, objective 40× Plan-Neofluar 40×/0.75. We will also use two-dimensional counts of the immunolabeled cell/ nuclear profiles will be performed in each delineated field and profile number will be normalized to area. Relative proportion of immunolabeled cells, will be calculated from all cells (as assessed by nuclear staining), because during development absolute cell numbers vary greatly due to comparatively large differences in cell densities among various areas and at different time-points.

Analysis of variance (ANOVA) for non-correlated and partially correlated data and post-ANOVA tests will be applied. For small number of cases the non-parametric Mann-Whitney test will be used to compare immunolabeled cells at various ages. Values of $p<0.05$ will be considered significant.

Distribution of interneurons and their progenitors in two forebrain regions

We will identify interneuron's progenitors and mature interneurons (GABAergic cells, CalR, GAD65, NPY, Sst, PV, CB) in the GE and neocortical SVZ of three gestational age groups: embryonic (5-7 g.w., n=5), early fetal (7-15 g.w., n=6), and mid-term (17-24 g.w., n=10). Progenitor cells will be identified by co-labeling with proliferation markers (Ki67, PH3), or pulse labeling with BrdU *in vitro*. *In situ* hybridization will be added if necessary to determine distribution of specific RNAs.

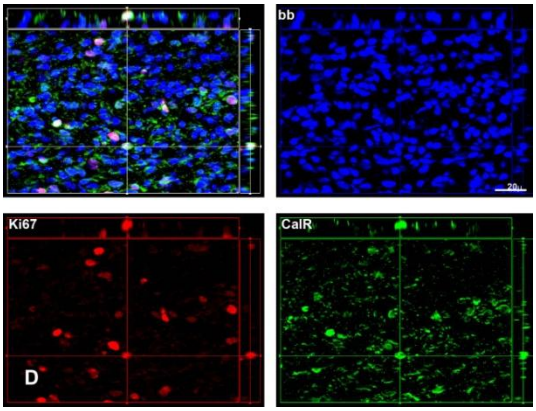


Fig.2. Immunolabeling of cryosections of 20gw fetal brain. Optical sectioning shows double-labeled CalR$^+$/Ki67$^+$ proliferating cells in the SVZ. bb-blue, nuclear stain.

We will start the analysis with CalR$^+$ cells, a subtype of interneurons which is more prevalent in primates relative to rodents. Notably, proliferating CalR$^+$ cells are demonstrated in neocortical SVZ at mid-term (Fig.2). These progenitors were not described before, in either fetal primates or rodents. Characterization of these progenitors will be done with additional double and triple labeling, using ventral transcription factors (Nkx2.1, Dlx, Mash1, Lhx6) and other interneuron markers. The proportion of GABAergic and CalR$^+$ cells in the upper cortical layers will be determined at mid-term. We will chart the distribution of CalR/GABA+ cells in the neocortical SVZ and the GE from embryonic (5g.w.) to fetal (15, 20 and 24 g.w.) stages. At least five cases per age group will be examined. Quantification of immunolabeled cells will be done on fixed sections and in acute cell cultures from the mid-term VZ/SVZ. Acute cell culture (Fig. 3) will provide baseline data about these molecules for studies planed in SA2 and 3.

Anticipated results/interpretations

We expect that careful characterization of the spatiotemporal distribution of cortical interneurons and their progenitors in the human fetal forebrain will expand ours and other laboratories reports (Zecevic and Milosevic, 1997, Zecevic et al., 1999, Letinic et al., 2002; Rakic and Zecevic, 2003; Bayatti et al., 2008; Fertuzinhos et al., 2009). The developmental gradients for cells of interest will be determined throughout the rostro-caudal axis of the fetal brain. We will spend the most time exploring CalR$^+$ cell populations in the SVZ at mid-term, which is the time when upper cortical layers are formed. We expect that additional CalR$^+$ cells for these layers are generated in the outer SVZ, which would shorten the migration route for late born interneurons. This idea is consistent with our finding that GE proliferative zone at mid-term is reduced while neocortical outer SVZ is expanded (Zecevic et al., 2010). Moreover, *in vitro* results suggest that at mid-term cortical VZ/SVZ contains more CalR$^+$ progenitors than the GE (Fig.3). We anticipate further that various interneuron subtypes are generated at specific developmental stages. In contrast to rodents, both CalR and CB are among the first GABAergic cells to appear in the human telencephalic wall, whereas only sparse parvalbumin (PV) and somatostatin (Sst) neurons were demonstrated at midgestation (Zecevic et al., 2010).

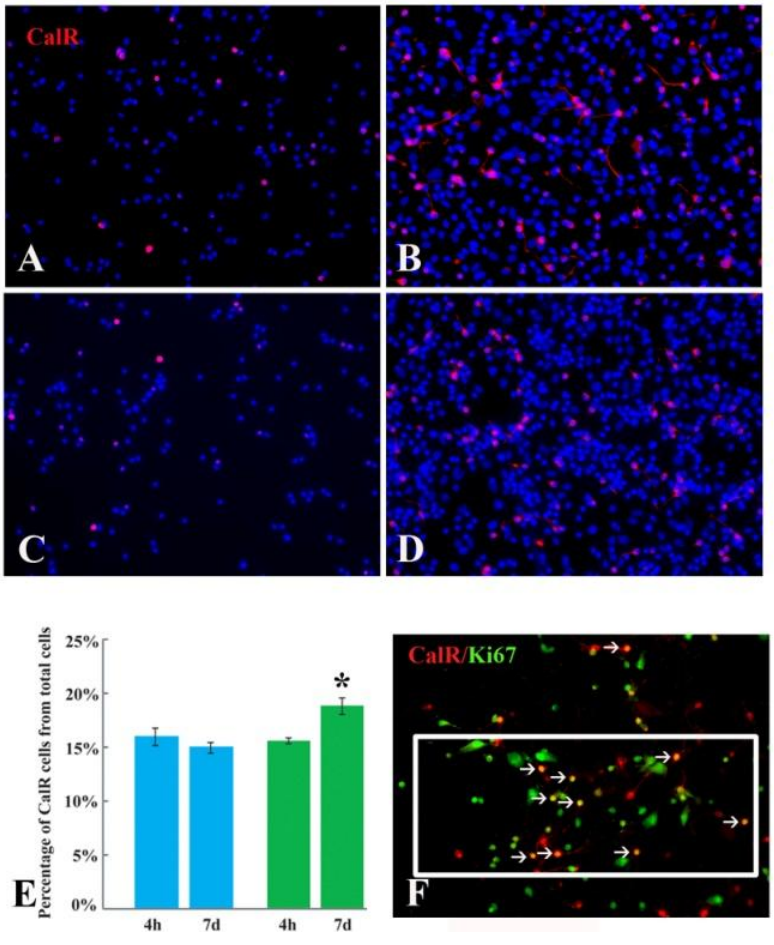


Fig. 3. A,B) Cells from SVZ, and C,D) from GE are kept for 4 hrs (A,C) or 7DIV (B,D) and immunolabeled for CalR (red). E) Quantification shows significant increase of CalR$^+$ cells only in SVZ cultures after 7div (green bar). F) In SVZ CalR cells are proliferating as seen with their co-labeling with proliferation marker Ki67 (green, arrows).

Double and triple labeling with ventral TF, Nkx2.1, Dlx, Lhx6, and Mash1 will better characterize cortical CalR$^+$ cells. In rodents the Nkx2.1 lineage from MGE differentiates into PV and Sst neurons, and not in CalR$^+$

interneurons (Wonders and Anderson, 2005), but this might not be true for human. It is possible, even probable, that the cortical CalR$^+$ population is heterogeneous as it is derived from different sites at different developmental times. For example, the CalR$^+$ population is spared in the cortex of patients with a severe holoprosencephaly with missing GE, suggesting that they originate in the cortical SVZ (Fertuzinhos et al., 2009). It is still not clear whether the CalR$^+$ progenitors will remain as mature CalR$^+$ neurons in the cortical plate, or alternatively start expressing some other interneuron markers, such as PV or Sst.

We expect to generate baseline data on the proportion of GABA/CalR interneurons in upper cortical layers at mid-term, and to compare their numbers with other subtypes of interneurons (CB, NPY, VIP) in the same cortical layers using specific antibodies. Similar immunoreactions will be done on coronally cut embryonic mouse brains (E16, P-3) and results will be compared between species. These experiments will be done applying a well established technique and antibodies we have used with great success, thus we do not expect any problems with this SA.

Specific Aim 2: Ventral transcription factors (TF) in human fetal brain

Hypothesis: Ventral transcription factors Dlx, Nkx2.1, Lhx6 and Mash1 in human brain are expressed in neocortical interneuron's progenitors. Special emphasis will be on cortical Nkx2.1$^+$ cells identified in our preliminary study to proliferate in the cortical SVZ at mid-term.

Experimental Design: Single and double immunoreactions on frozen sections will be done over the first half of intrauterine development (5-22gw). Western Blot analysis and *in situ* hybridization will be used to compare the quantity of protein and distribution of mRNA for ventral TF in the GE and the dorsal VZ/SVZ at mid-term. The role of Nkx2.1 in potential cortical interneuron progenitors will be explored using Nkx 2.1 promoter-reporters and loss-and-gain of function experiments.

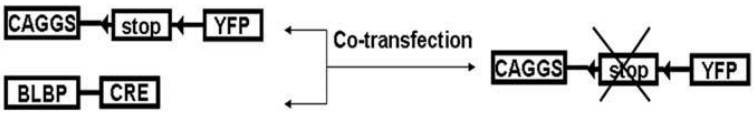
Dissociated cell cultures. The protocol has been previously described in our papers (Zecevic et al. 2005, Mo et al., 2007, Mo and Zecevic, 2008). Cultures are prepared from the VZ/SVZ of the fetal forebrain, dissected from the frontally cut hemispheres as a tissue band approximately 2000 μm high from the VZ surface, dissociated and resuspended in DMEM/F12 (Invitrogen) and either subjected to immunopanning (see below) or seeded onto 35 mm plastic culture dishes or coverslips for the acute immunocytochemistry studies. We are able to maintain cells in cultures for more than a month without significant cell death, as tested with caspase 3 immunolabeling and TUNAL test.

Organotypic slice cultures. Tissue blocks (1x1x0.5cm) containing cortical VZ/SVZ or GE are dissected from frontally cut fetal hemispheres, embedded in 3% low-melted agarose (Invitrogen), and cut into 200-400 μm-thick slices in cold sterile HBSS containing 0.75% D-glucose and saturated with a 95% O_2-5% CO_2 mixture, using a Vibroslice (World Precision Instruments). Slices are transferred onto 30 mm membrane inserts (0.4mm porosity, Millipore Corp), and placed in 6-well plates containing serum-free defined growth medium (5mg/ml of insulin, 100mg/ml of transferrin, 20nM progesterone, 100mM putrescine and 30nM selenium). Using the interface technique (Stoppini et al., 1991, Simoni and Yu, 2006), slices are kept in a humidified incubator at 37ºC with 5% CO2 for up to 2 weeks. Slices will be fixed in 4% paraformaldehyde for 6 h., cryoprotected with 30% sucrose, frozen at -70ºC, and re-sectioned into 15μm thick sections for immunohistochemistry. During the first 5div there was an increasing number of proliferating cells in the slices while dying cells remained below 5% of all cells (Filipovic and Zecevic, 2009).

Transfection/infection with retrovirus-GFP and GFP- Pax6. Cells from the GE and neocortical VZ/SVZ proliferative zones will be plated on poly-L-lysinated 12mm coverslips in DMEM/F12/B27 supplemented with either 1ng or 10ng/mL FGF2, at the density of $0.24x10^6$cells/well and will be left overnight to recover. Transfection with Lipofectamin 2000 will be done using the manufacturer's protocol (Invitrogen, Carlsbad, California) or AMAXA system (Amaxa Nuclefector II, Gaithersburg, MD). As a positive control for transfection efficacy the GFP construct under the cytomegalovirus (CMV) promoter, constitutively active in all cells, will be used (Mo and Zecevic, 2008).

Retrovirus production. Replication-incompetent GFP-expressing retrovirus was produced from a stably transfected packaging cell line (293gp NIT-GFP, gift from Dr. Fred Gage, Salk Institute, La Jolla, CA). 293gp cells were transfected at ~75% confluence with pVSV-G using lipofectamine (Invitrogen). Supernatant was harvested 48h after transfection, filtered through 0.45 μm low-protein binding filters (Millipore, Billerica, MA), and stored at −80°C. Volumes of NIT-GFP virus sufficient to infect 50 cells are added to 0.5 ml of cells (50,000 cells) and then incubated for 30 minutes at 37°C. To specifically track the proliferation and differentiation of cells of interest we will use promotor-Cre/Floxed and CAGGS-loxP-LacZ-loxP-YFP co-

transfection, the way we used BLBP-Cre (brain lipid binding protein) and Floxed yellow fluorescent protein (YFP) plasmids to label radial glia progenitors (Mo et al., 2007- diagram). Other necessary plasmids-Cre will be made in a similar way. Controls will be done to determine whether the Cre antibody labels only BLBP$^+$ cells, and another to determine whether YFP fluorescence is observed only in Cre$^+$ cells (Mo and Zecevic, 2008).



The schematic diagram of the pCAGGS-LoxP-LacZ-LoxP-YFP and BLBP-Cre [LoxP sites are indicated by triangles].

Electroporation 200-μm-thick slices from human cortical SVZ or GE are injected (0.5-1 μl of plasmids per injection site at 5mg/ml concentration) with RFP (red fluorescent protein) pCAGGS vector where chicken β-actin promoter drives the expression, and Lhx6-GFP plasmid using nanoinjector (Drummond, Nanoinject II)-Fig.7. BTX electroporation system (Harvard Apparatus, Square Porator T830) with charging voltage 100-150V, delivering 5 pulses of 5 ms duration each, is used. GFP cells will be visualized as green using the confocal microscope and time lapse imaging. After electroporation, slices will be cultured for another 5-7 days or longer if necessary in the neurobasal medium and processed as described above.

Loss-and-gain of function experiments. Interference RNA (shRNA) We successfully used this method in experiments with Pax6 shRNA in fetal brain tissue (Mo and Zecevic, 2008). In short, a 60-bp oligonucleotide containing Bgl II and Hind III restriction sites was inserted into the multiple cloning sites of pSuper-EGFP (Oligoengine, Seattle, WA). As a control, a scrambled version of RNA was designed, that does not recognize any coding regions in the human genome. In the cells transfected with Pax6 shRNA, the expression of protein Pax6 was eliminated whereas BLBP expression was not changed. The oligonucleotide strands were purchased from IDT Genosys (Coralville, IA) and were annealed before cloning into pSuper-EGFP. The transfection efficiency with lipofectamine was about 10%. We now cloned human primer Nkx2.1 RNAi according to published results (Weir et al., 2007). We will do several immunolabeling and Western Blot analyses to verify protein expression and examine possible off-target effects, PCR to verify levels of mRNAs, and transfection with scrambled RNA. BLAST will be used to check specificity of oligonucleotide strands used to make shRNA for the particular gene to knock-down, and that the scrambled controls match no known genes. **Overexpression** of **Nkx2.1**. Human fetal brain cDNA will be used with the Nkx2.1 primer designed to contain the coding sequence (1206bp) downloaded from NCBI database, and ligated to pMXIG plasmid-GFP reporter gene. For **Pax6 overexpression**: Pax6-expressing retrovirus was produced from a stably transfected packaging cell line (293gpg) transfected at ~90% confluence with pMIX-Pax6 using lipofectamine (Invitrogen). Supernatant was harvested 48 h after transfection, filtered through 0.45 μm low-protein binding filter. Volumes of retrovirus sufficient to infect 500 cells were added to cell culture and incubated for 24 hours at 37°C. Then the infection medium was replaced with expansion medium, in which the cells were cultured for another 48 hours, and immunolabeled with the appropriate marker.

BrdU labeling. To assess proliferation in *in vitro* systems, 20 μM BrdU will be added for 4-12 h. in acutely dissociated cell or slice cultures as described previously (e.g., Mo and Zecevic, 2008). **Confocal microscopy and live imaging.** Sections will be viewed with a confocal laser-scanning microscope (Carl Zeiss, LSM 510) using excitation and emission filters: 488/515 for fluorescine and GFP, and 568/590-610 for Rhodmine. Z-serial images will be collected at 0.5-1μm steps and 3D reconstructions will be done using Imaris 3D software (Bitplane AG). Zeiss 510 LSM Confocal microscope fitted with a Zeiss XL LSM incubator will be used for live imaging; cells are maintained at 37^{0}C and supplied with humidified air (5% C02). BLBP-GFP transfected radial glia cells are illuminated with a 488 nm argon laser using a 40x objective. Images were acquired every minute with the pinhole open.

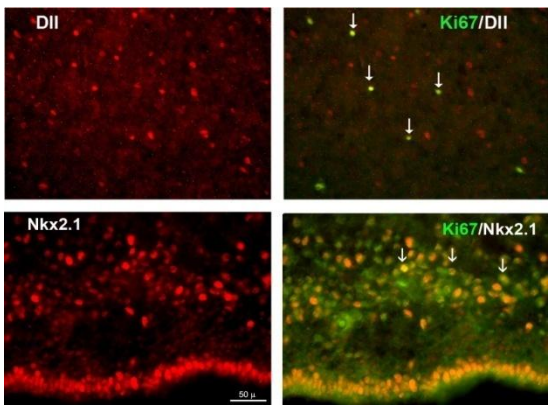


Fig. 4. At mid-term numerous Dlx$^+$ (Dll antibody) and Nkx2.1$^+$ cells (red) are seen on cryosections of the neocortical SVZ and some cells are proliferating (co-labeled with Ki67$^+$, green)- arrows.

Experiment 1: Cells expressing ventral transcription factors (TF). In contrast to results in rodents, our preliminary results show that cells expressing ventral TF (Dlx, Nkx2.1) proliferate in dorsal SVZ (Fig.4). Notably, Nkx2.1$^+$ cells are also found in the human cortical plate at mid-term (Fig.5), confirming our previous results (Zecevic et al., 2005).

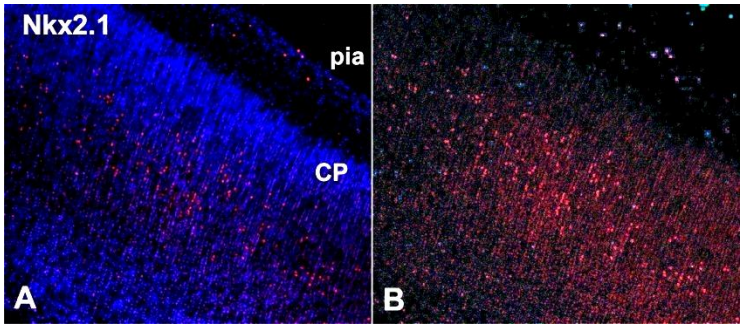


Fig.5. Nkx2.1$^+$ cells (red) at 20gw cerebral cortex in deep layers of the cortical plate (CP). Blue-nuclear stain.

Western Blot analysis on human fetal forebrain confirmed that antibodies used for ventral TF are working appropriately. Unexpectedly, similar quantities of these proteins are found in ventral and dorsal regions (Fig.6B).

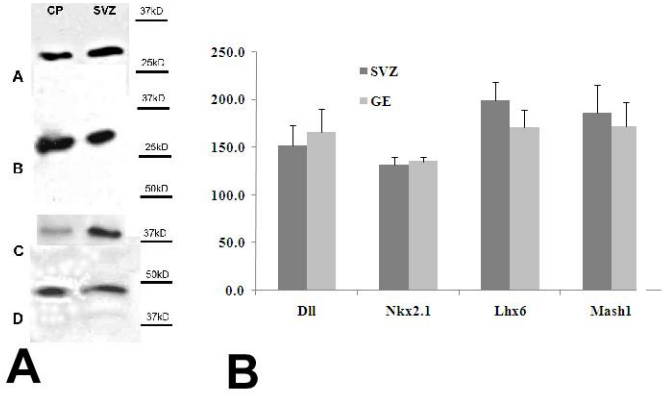


Fig 6 ***A.*** *Western Blots at 21 gw confirms antibody specificity in human brain. A) rabbit polyclonal anti-CalR, B) mouse CB, C) rabbit monoclonal anti-Nkx2.1 (D) polyclonal anti-Lhx6 in the CP and the SVZ. Each of the antibodies labeled a single band of predicted molecular weight. Protein loading: 20 μg/well.* ***B.*** *Quantities of ventral TF in GE and SVZ of four fetal brains (19-22 gw) do not show significant differences between the two regions.*

Distribution of Nkx2.1, Dlx, Lhx6 and Mash1 immunoreactive cells will be determined in a systematic way in the human telencephalon, from 5-22 gw (similar cases as in SA 1). To identify cell types that express these TF double-immunolabeling with NeuN and MAP2 (mature neurons), SMI31 and Tbr1 (projection neurons), CalR and GABA (interneurons) or PDGFRα (oligodendrocyte progenitors) will be used. The immunolabeled cells in the neocortex will be charted from frontal to occipital pole, using sagittal and coronal sections of the fetal brain.

*Experiment 2. Testing of promoters-reporters in human brain tissue. C*ell expressing ventral TF will be studied in cell and slice cultures by co-transfecting specific promoters-GFP: Lhx6-GFP and Nkx 2.1-GFP (Switch Gear Genomics, Menlo Park, CA). Retrovirus (CMV)-GFP or RFP (red fluorescent protein), will be used to mark all proliferating cells as a control of transfection efficacy. Dissociated neocortical SVZ and GE cell cultures at mid-term will be maintained for 7, 14 or 21 days in vitro (div) before being fixed and processed for immunolabeling in the same manner as described below for slices.

Electroporation of slices. Slices (200μm, n=20) cut from the cortical VZ/SVZ and cortico-striatal border at mid-term will be used to selectively label interneuron progenitors with the above cited promoters. Over 5-7 days, individual green cells will be visualized and recorded (Fig.7). Thereafter, slices will be fixed, frozen, resectioned, and double labeled with a variety of interneuron markers, markers of cell proliferation (Ki67, PH3, BrdU), and cell death (caspase3).

Experiment 3. Loss-and gain-of-function experiments. To address the role of Nkx2.1 on interneuron specification and migration, we will first transfect specific Nkx2.1 shRNAs to delete Nkx2.1 in cortical progenitors isolated from the SVZ. Gain-of-function experiments will be done by transfection of a full length construct for human GFP-Nkx2.1 (coding sequence is around 1KB from NCBI Database). Transfected cells will be co-labeled after 5-7div with CalR and GABA to quantify the number of these cells that differentiated into interneurons (please see SA1).

Anticipated results/interpretations

Our finding that Nkx2.1 and Dlx1 cells in the SVZ proliferate *in vitro* and, even more importantly, that immunolabeled proliferating cells were demonstrated on cryosections at mid-term (Fig.4) suggest that TF may be differentially regulated in primates. Moreover, Nkx2.1$^+$ cells were also distributed throughout the deep layers of the cortical plate (Fig. 5), which seems to be specific for human compared to rodents or even other primates. Proposed experiments will provide more details about molecular characteristics of Nkx2.1$^+$ cortical progenitors, and their temporo-spatial distribution not only at mid-term (20gw) but also in other gestational ages (11, 15 gw).

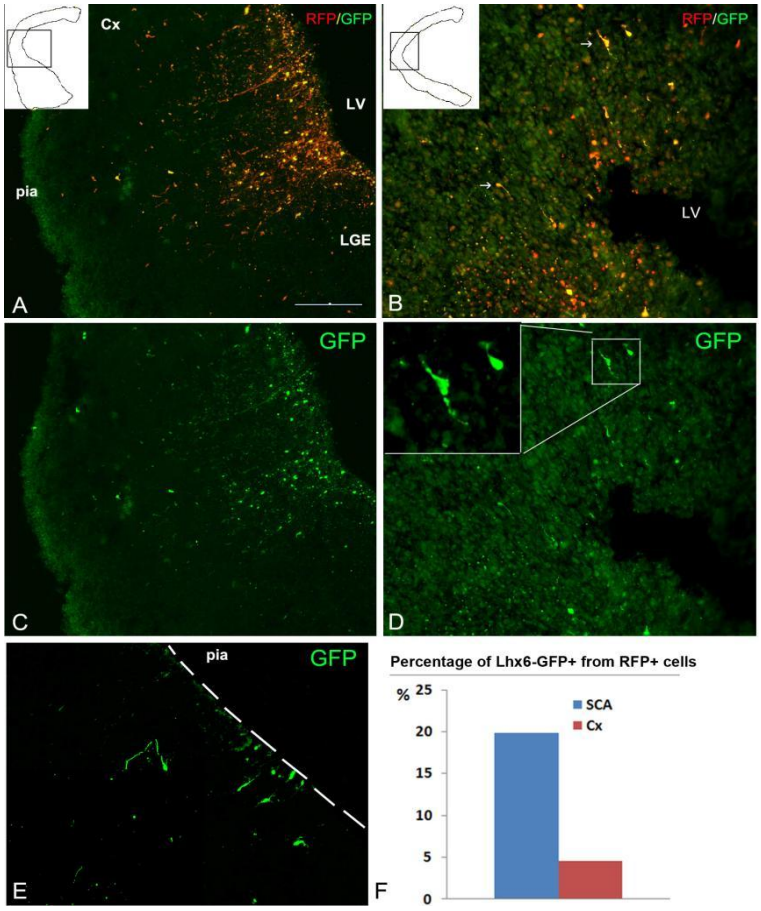


Fig.7. Lhx6$^+$ interneurons in human slice cultures from 16-21 gw. (A-D) Slices from 16-weeks-old forebrain containing striato-cortical angle (A, C) or dorso-lateral cortex only (B, D), transfected with RFP under general promotor (red) and Lhx6-GFP (GFP-green) after 4div. Insets on A and B- Neurolucida drawings of whole slices. LGE – lateral ganglionic eminence; Cx – cortex; LV – lateral ventricle. (E). Slice from a 16-weeks-old cortex after 7div. (F) Percentage of Lhx6-GFP cells from all transfected (RFP) cells in the cortex (Cx) and striato-cortical angle (SCA).

We expect to resolve whether proliferating Nkx2.1, Dlx and Mash1 cells in the cortical SVZ could be labeled with accepted interneuron markers. An alternative explanation could be that these cells migrated earlier from the GE and continued to proliferate dorsally. This explanation, however, would not agree with reports in mice, where cells migrating from the GE to neocortex do not proliferate (Polleux et al. 2002; Xu et al., 2004, 2005). Moreover, Nkx2.1 in these cells is down-regulated before the beginning of migration (Nobrega-Pereira et al., 2008). Thus, either in humans Nkx2.1 down-regulation is not a prerequisite for their tangential migration to the neocortex, or more likely, a second population of Nkx2.1$^+$ progenitors generates cortical interneurons locally, in the neocortical SVZ at mid-term. A small proportion of Dlx1/2 and Nkx2.1$^+$ cells can represent oligodendrocyte progenitors (Marshall and Goldman, 2002; Rakic and Zecevic, 2003b). This possibility will be tested by co-labeling with an oligodendrocyte progenitor marker, PDGFRα, that we successfully used before (Jakovcevski and Zecevic, 2005). We will compare our results on the expression of ventral TF in the human fetal brain to embryonic mouse brain (E16-18). This will serve to illustrate if our methods (and antibodies) produce comparable results. Preliminary results obtained on E16 mouse demonstrated adequate immunoreactions to Nkx2.1antibody, as previously reported (e.g., Anderson et al., 1997; 2001).

Testing promoters-reporters in human brain tissue

The second sub aim will test specific promoter-reporters known to be specific for cortical interneurons in rodents. In human tissue, however, this data is missing, although this information will be useful as a baseline for studying induced human stem cells (please see Dr. S. Anderson's letter).

Our preliminary results with electroporation of Lhx6-GFP on fetal brain slices (Fig. 7) are encouraging and were obtained in collaboration with Dr. S. Anderson's laboratory (Du et al., 2008). We are now doing these studies in our lab. We are currently inserting commercially available human Nkx 2.1 promoter (Switch Gear Genomics, Menlo Park, CA) into a Cre plasmid that will allow us to follow Nkx2.1 progeny.
In all described experiments constructs will be first tested in dispersed neocortical cell cultures (GE cultures will be done also for comparison), and subsequently in slice cultures. Imaging transfected (green) cells on slice cultures using time lapse photography will demonstrate whether they have interneuron morphology, what type of divisions they use, and modes of their migration. We performed initial experiments of genetically labeled cells with time lapse photography (Fig.8). Nkx2.1 loss-of function experiments in cultures of cortical VZ/SVZ cells will be particularly interesting to better understand the role of this TF for dorsal cortical progenitors, since these progenitors are not present in mice. If transfection of shRNA Nkx2.1 results in a lower number of CalR and GABA interneurons, we will proceed with experiments to over express Nkx2.1, which we anticipate might increase cortical interneuron number. In the future we plan to address the role of Mash1 and Dlx in a similar manner. We have expertise with similar experiments (Mo and Zecevic, 2008, SA 3), and we do not expect major problems. However, studies on molecular profiling of human interneurons could have a number of potential problems listed below.

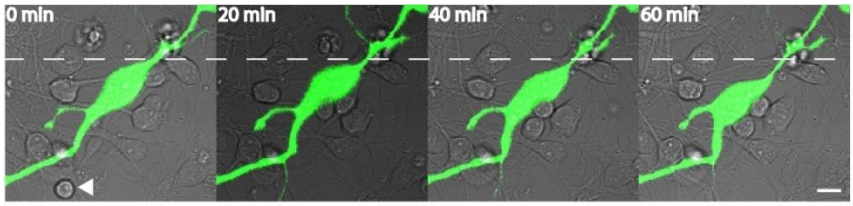


Fig.8. BLBP-Cre LoxP-YFP labeled cell (green) is visibly moving in respect to dotted line over a 60 min. period in this culture.

Potential problems: First, gene transfer relies on *in vitro* studies, that are often difficult to interpret, but are nevertheless necessary as the only experimental approach with human brain tissue. *In vitro* studies are also often used with animal models which can serve as a reference. Second, it is questionable how well transfected/infected cells will survive and differentiate in slices under *in vitro* conditions. Our preliminary results, obtained after five days survival are very encouraging (Fig. 7, Zecevic et al., 2010, Filipovic and Zecevic, 2009), but more experiments are needed to establish the optimal procedure. Survival, however, is not a problem with cell culture, where we have good survival for at least 3 weeks (Mo and Zecevic, 2009). Never the less we will cautiously interpret results of *in vitro* experiments, and always combine them with results on frozen sections of the same fetal brain. The slice experiments and fixed section analyses will be useful to support whether cells that grow from the dissociated cultures are likely to be indicative of the *in vivo* potential of those progenitors. The third limitation is the availability of fresh human fetal tissue that is either earlier or later than mid-gestation. While the age of human tissue available for our studies is clearly beyond our control, we acknowledge that this limits our developmental analysis. Mid-gestation, however, is the period when upper cortical neurons are generated and will allow us to test the veracity of our hypotheses. It should be noted that this is not a limitation for the proposed immunohistochemical studies, as we already have a well-characterized collection of fresh-frozen human brains that spans the intrauterine period from 5 to 24 g.w. Having available to us this unique collection and an opportunity to obtain fresh tissue, we are in a favorable position to perform the proposed studies. Dr. S. Anderson from Weill Cornell Medical College (NY) and Dr. N. Sestan from Yale Medical School (New Haven, CT) will advise us on genetic manipulation procedures (electroporation, overexpression, promoter-reporter constructs) as they use these methods more frequently (see attached letters).

Specific Aim 3: **Do radial glia and/or intermediate progenitors contribute to human cortical interneuron population?**

Hypothesis: Radial glia (RG) and intermediate progenitors (IPCs) in the human fetal SVZ generate a subtype of cortical interneurons, in addition to projection neurons.

Experimental Design*:* Single and double immunolabeling of frozen sections will be used to determine the distribution and proportion of IPCs in the cortical SVZ over the first half of gestation. Genetic labeling of RG from the cortical SVZ will be utilized for cell fate mapping.

Immunopanning. To isolate neural progenitor cells, we will use immunopanning with a surface marker LeX as described in our paper (Mo et al., 2007) or minicolumns (Miltenyi Biotec, Inc., Auburn,CA).

Experiment 1. Intermediate progenitors in human SVZ at mid-term. Distribution and density of IPC cells will be assessed by using Tbr2 antibody on frozen sections of the human fetal brain at different stages (same cases as used in Specific Aim 1). Their percentage from all cells in the cortical SVZ will be quantified. Double and triple labeling experiments in various combinations using antibody to Tbr2, interneuron markers (GABA, Dlx etc.) and antibodies for cell cycle, Ki67, PH3 will be used at mid-term to better characterize these progenitors in human outer SVZ. Preliminary results show that IPCs can be co-labeled with GABA, and that they proliferate in the SVZ at mid-term (Fig.9).

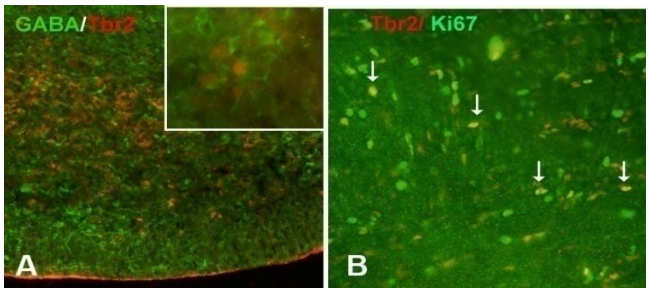


Fig.9. A) GABAergic cells (green) in the mid-term neocortical SVZ are double-labeled with Tbr2 . Inset shows higher magnification. B) Intermediate progenitors(Tbr2$^+$) are proliferating (Ki67$^+$) in the SVZ (arrows).

Experiment 2. Genetic manipulation of dorsal cortical progenitors. To permanently label RG/IPCs we will co-transfect them with BLBP-Cre and YFP-loxp plasmids. Two and three weeks after transfection cell cultures will be fixed and immunolabeled with markers of cortical interneurons and projection neurons to quantify the percentage of different neuronal types generated from transfected cells. Our preliminary results show that a fraction of genetically labeled RG cells differentiated into GABAergic cells (Fig.10).

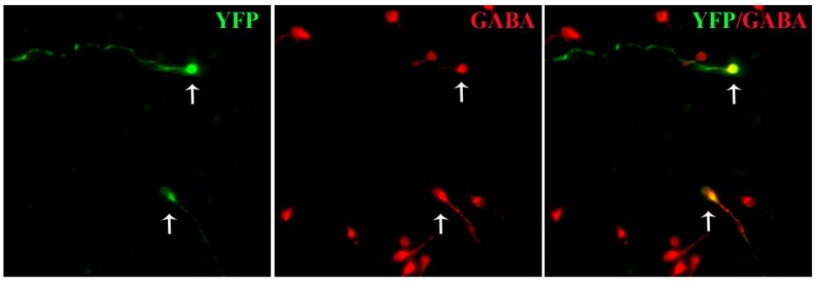
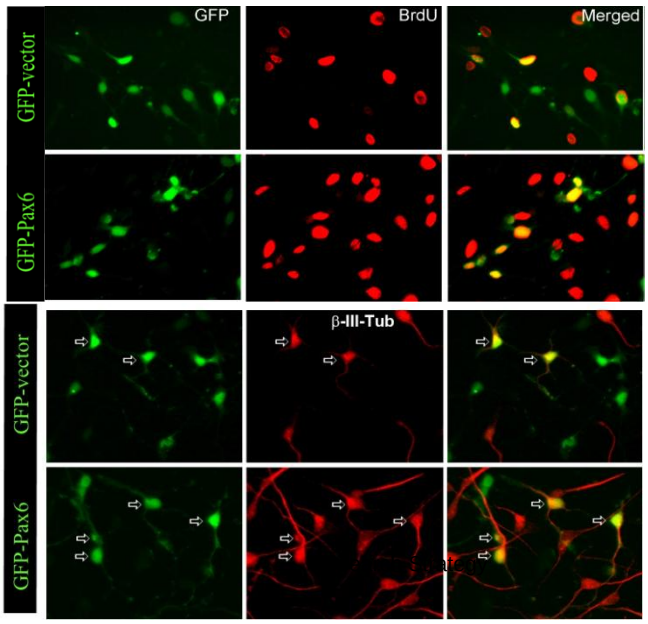
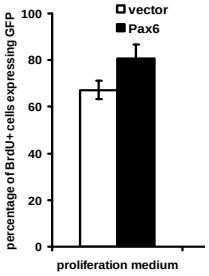


Fig.10. Neocortical SVZ cells co-transfected with BLBP-Cre LoxP-YFP (green). After 7div these cells are co-labeled with GABA (arrows).

The role of Pax6 in fate determination of RG and IPCs will be studied by loss-and-gain of function experiments. Preliminary gain-of-function experiments demonstrate that RG infected with the retrovirus Pax6-GFP increase Pax6 signal *(Metamorph program),* proliferate more, and produce more β-III-tubulin neurons in comparison to control infected cells (Fig. 11). We will repeat these experiments using various interneuron markers to document the effect of increased Pax6 on neuron-type specification (pyramidal vs. interneurons).





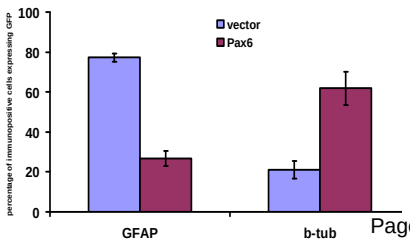


Fig.11. Lex$^+$ RG cells transfected with control vector and Pax6-GFP (green). Upper picture: Pax6-GFP transfected cells incorporate BrdU (red) significantly more than control transfected ones (graph). Lower picture: They also differentiate in more β-III-tubulin$^+$ neurons than into GFAP$^+$ astroglia, in contrast to controls (lower graph).

Anticipated results/interpretations

We expect to determine the distribution and prevalence of IPCs in the human cortical VZ/SVZ from 7 gw to mid-term. The percentage of dividing $Tbr2^+$ cells from all SVZ cells will reveal the importance of this cell type in late corticogenesis, when the upper cortical layers are formed. Preliminary immunolabeling results suggest that IPCs could generate cortical interneurons (Fig. 9). We however need additional *in vitro* results, which will allow us to follow the progeny of genetically labeled RG/IPCs cells and determine whether and which subtypes of cortical interneurons are generated from RG/IPCs progenitors. So far our preliminary results point to $CalR^+$ interneurons, but further confirmation is needed. We expect to answer whether regulation through the TF Pax6 is cell-subtype specific. We reported that knocking down Pax6 reduces the number of generated $CalR^+$ cells (Mo and Zecevic, 2008), but it is not clear how other interneuron subtypes could be affected, nor how the over-expression of Pax6 will affect interneuron generation. Controls will be run to demonstrate that only a gene of interest is knock-down, and not other genes (scrambled RNA, immunoreactions, RT-PCR, WB). Caspase 3 labeling will determine the role of cell death in this experimental setting, but from our previous results we do not expect problems (Filipovic and Zecevic, 2008).

Progress Report

In the current funding period from 2006 to 2010, we addressed all three specific aims. The results are published in 7 papers, 1 review, and 1 chapter; 1 paper is in press and 2 are in preparation (uploaded separately in Progress Report Publication List). Although the majority of experiments proposed were successfully finished, we made some adjustments by adding experiments about the role of Pax6 instead of Notch and Numb distribution, as initially planned (SA1). Our interest has shifted to progenitor cells of human cortical interneurons, which is the main topic of the new application. A number of results show important species-specific differences in interneuron development in primates relative to rodents. Thus, more detailed knowledge about generation of this important cell type in human is necessary. I summarize the work done so far in the original three specific aims:

Specific Aim 1: Do multiple progenitor cells contribute to the human cerebral cortex?

We demonstrated in more than one way that human cerebral cortex develops from a complex progenitor pool. Using single and double immunolabeling on cryosections of increasing developmental ages we first demonstrated several classes of cortical neuron progenitors in human (Howard et al., 2006; 2008). Next, we confirmed this finding more directly with *in vitro* studies (Mo et al., 2007). The progeny of RG progenitor cells isolated from the fetal cortical VZ/SVZ and genetically labeled (BLBP-Cre/Floxed-YFP) was studied with a combination of complementary experimental techniques (clonal analysis, immunofluorescence, and patch clamp recordings). Human fetal RG cells are heterogeneous cell population that generates mainly astrocytes, but also a small number of cortical neurons (Mo et al., 2007). Simultaneously, however, we identified another progenitor subtype, a neuron-restricted progenitor, in the human cortical SVZ, with double immunolabeling on cryosections (Howard et al., 2006; 2008) as well as *in vitro* (Mo et al., 2007). The significance of these results is that they demonstrate that diverse populations of cortical progenitor cells contribute to cortical development in the human, including multipotent RG and neuron-restricted progenitors. In collaboration with Dr. Antic, we provided the first physiological characterization in the human brain of neurons derived from RG cells. A subset of human RG progeny has a detectable transient inward sodium current sensitive to TTX, a whole indicator of neurons (Mo et al., 2007). We demonstrated in brain slices that one third of cortical plate neurons were able to generate sodium action potentials upon direct current injection, whereas only the subplate (SP) neurons were able to fire repetitive action potentials at 16gw. This finding clearly establishes that SP neurons are functionally the most mature neurons in the human fetal cortex. These results yield the first recording of developing human fetal cortical neurons with preserved morphologies in the natural surrounding tissue (Moore et al., 2009).

Specific Aim 2: Lineage analysis of radial glia cells

We established heterogeneity of cortical RG cells by double immune labeling of acute cell cultures (Howard et al., 2006), and further confirmed this heterogeneity by clonal analysis of neurospheres formed *in vitro* (Mo et al., 2007). Genetic labeling of RG with BLBP-Cre/Floxed-YFP demonstrated not only that RG generate all three neural cell classes, astrocytes, neurons, and oligodendrocites (Mo and Zecevic, 2009), but also that RG cells have a potential to generate both projection neurons ($SMI31^+$) and interneurons ($CalR^+$) *in vitro* (Mo et al., 2007, Mo and Zecevic, 2008). These studies will continue in the new SA 3.

To study neurogenetic determinants of human RG, we focused on the role of transcription factor Pax6. Loss-of-function experiment, using shRNA Pax6 *in vitro,* successfully abolished Pax6 protein expression, and decreased cell proliferation and neurogenesis from knock-out cells (Mo and Zecevic, 2008). This study is the first report on the neurogenic role of Pax6 in human, similar to earlier reports in rodents (Goetz et al., 1998). However, in human Pax6 was expressed by more cell types and in more forebrain regions than in rodents. This study also suggested that Pax6 may regulate the genesis of a subpopulation of cortical $CalR^+$ interneurons (Mo and Zecevic, 2008). Differences in Pax6 distribution and role suggest that developmental regulation by transcription factors may differ in primates and non-primate mammals. This idea will be further studied in new SA 2 and 3.

In a collaborative study with Dr.M.Goetz laboratory, we reported that transcription factor AP2γ is expressed in basal progenitors destined for upper cortical layers, both in mice and primates, including humans. In addition, the expression of AP2γ has been demonstrated to be regulated by Pax6 (Pinto et al., 2009).

Specific Aim 3: Environmental influences and fate of cortical progenitor cells

After experiments proposed in this SA, our conclusion is that microenvironment can influence fate determination of cortical progenitor cells. This is based on *in vitro* studies, where we first isolated Lex^+ progenitors from cortical VZ/SVZ at 14 gw and 20 gw human fetal brains. Next, we genetically labeled these cells with the retrovirus NIT-GFP, and transplanted these green cells into cell cultures of cortical VZ/SVZ or GE of the same age fetal brain. These experiments demonstrated that more $CalR^+$ cells were generated from transplanted cells in the GE than in cortical VZ/SVZ cell cultures, implying the effect of local environment. In these cultures different concentrations of EGF and FGF2 were measured (Mo et al., 2007). We expanded this finding to explore the effect of these growth factors on interneuron generation (Yu et al., in preparation).

In another set of experiments, we reported that *in vitro* RG can generate $O4^+$ oligodendrocyte (OL) progenitors if cultured in OL permissive medium. The differentiation from RG to $O4^+$ cells was enhanced by the addition of Sonic hedgehog (SHH) and reduced by the SHH inhibitor-cyclopamine, suggesting the role of SHH-signaling in this process (Mo and Zecevic, 2009).

E. HUMAN SUBJECTS

1. 1. Number of fetal brains were collected and frozen or paraffin embedded over more then 10 years period in Yugoslavia (app. from 1980-1993). This tissue was collected according to Yugoslav laws and Ethics Committee and following International Conventions. These studies were approved as a part of two International Projects. Frozen and paraffin sections represent a unique collection of different developmental stages and are still being studied with new immunocytochemical markers and in situ hybridization probes. More then 10 papers have been published using this material. However I do not collect material abroad any more.

2. I have been collaborating with The Albert Einstein Brain Bank and have been receiving brain tissue from them since 1995. This tissue is obtained from autopsies done after medical abortions (with the consent of parents) and shipped on ice in buffer, fixative solution or tissue culture solution. Some results in the grant application, such as the ones from immunohistochemistry, came from the tissue obtained in this way. For other experiments, such as slice preparations, we have to drive to The Albert Einstein, wait for tissue, put it immediately in oxygenated HEPES-buffered MEM solution on ice, and drive back (app. 2 h.). The same protocol will be used in our future experiments.

3. In January 2006 based on Human subject research-Determination form, it has been determined that the brain tissue that we collect from autopsies done after medical abortions (with the consent of parents) does not require IRB involvement. The identity of the patient is not known to us, and procedure is not done to benefit our research.

4. Included is a letter from Dr.Brad Poulos from the Albert Einstein University.

F. VERTEBRATE ANIMALS

1. Proposed use of animals: Each pregnant mouse will provide approximately 8 offspring that we can use for our immunohistochemical and electrophysiological experiments. While it is difficult to ascribe exactly the number of mice needed for any given experiment, we have requested only 2 pregnant mice per year. We also requested 3 cages of neonatal animals. This number is conservative, and proposed based on statistical analysis to ensure that the minimal number of animals are used yet still obtain statistically meaningful results, as well as on past experience in similar studies and reviews of related use in the literature.

2. Justification for choice of species and number to be used: There is no computer or in vitro system that can be used to model development of the nervous system and the maturation of neuronal function. Mice are a suitable species to compare with humans, and check possible differences. Even when the developmental process differs, the species-related difference has provided important insights toward understanding the human conditions. Mice were selected for these reasons: (1) They are not short in supply, economical to maintain and are thus an ideal species to use in this proposal. (2) Data to be acquired in this proposal will be used in the future as base line for studies on transgenic mice.

3. Veterinary care: Veterinary care is available at the University of Connecticut Health Center. The University of Connecticut Health Center is committed to the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training. This commitment is reflected by the Animal Care and Use Program's continuous full accreditation since 1977 by the American Association for Accreditation of Laboratory Animal Care. The CLAC facilities include standard and containment animal holding rooms, a clinical laboratory, operating suite, treatment room, necropsy room, procedure rooms, cage wash areas, and training room. Some important services provided by CLAC include veterinary medical care and diagnostic services, basic animal husbandry, specialized technical support, animal procurement, investigator consultations, and investigator training programs.

4. Procedures for ensuring that discomfort, distress, pain and injury will be limited: There is no pain or discomfort associated with anesthesia and immediate subsequent decapitation.

5. Method of euthanasia: Adult mice: overdose of sodium pentobarbital anesthesia. Fetal mice: cooling on ice followed by decapitation. Neonatal mice (up to 6 days): cooling on ice followed by perfusion with 4% paraformaldehyde. In all cases, the regimen of decapitation is swift, produced no apparent discomfort and death is instantaneous.

Principal Investigator/Program Director (Last, First, Middle): Zecevic, Nada

References Cited:

Anderson SA., Eisenstat DD, Shi L, Rubenstein JL. (1997) Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. Science 278: 474-476.

Anderson S.A., Marin O., Horn C., Jennings K., Rubenstein J.L.R. (2001) Distinct cortical migrations from the medial and lateral ganglionic eminences. *Development* 128, 353-363.

.

Baraban SC, Tallent MK. (2004) Interneuron diversity series: interneuronal neuropeptides—endogenous regulators of neuronal excitability.*Trends Neurosci.,* 27:135--142.

Bayatti N, Moss JA, Sun L, Ambrose P, Ward JFH, Lindsay S, Clowry GJ (2008) A Molecular Neuroanatomical Study of the Developing Human Neocortex from 8 to 17 Postconceptional Weeks Revealing the Early Differentiation of the Subplate and Subventricular Zone. *Cer Cortex* 18 (7):1536-48.

Butt S.J.B., Sousa V.H., Fuccillo M.V., Hjerling-Leffler J., Miyoshi G., Kimura S., Fishell G. (2008) The Requirement of Nkx2-1 in the Temporal Specification of Cortical Interneuron Subtypes. *Neuron,* 59: 722,-732.

Bystron I., Rakic P., Molnar Z., Blakemore C. (2006) The first neurons of the human cerebral cortex. *Nat Neurosci*.; 9(7): 880-6.

DeFelipe J. (1999) Chandelier cells and epilepsy. *Brain* 122; 1807-1822.

DeFelipe J., Ballesteros-Yanez I., Inda M.C., Munoz A. (2006) Double-bouquet cells in the monkey and human cerebral cortex with special reference to areas 17 and 18. *Prog Brain Res*; 154: 15-32.

Du, T., Xu, Q., Ocbina, P.J., and Anderson, S.A. (2008). NKX2-1 specifies cortical interneuron fate by activating Lhx6. *Development* 135: 1559–1567.

Fertuzinhos S., Krsnik Z., Kawasawa Y.I., Rasin M.R., Kwan K.Y., Chen J.G., Judas M., Hayashi M., Sestan N. (2009) Selective Depletion of Molecularly Defined Cortical Interneurons in Human Holoprosencephaly with Severe Striatal Hypoplasia. *Cer Cortex* 19(9): 2196-207

Filipovic and Zecevic, 2008 The effect of CXCL1 on human fetal oligodendrocyte progenitor cells.GLIA 56 (1): 1- 15.

Gleeson J. G., Walsh C. A. (2000) Neuronal migration disorders: from genetic diseases to developmental mechanisms. *TINS* 23: 352-359.

Gotz, M., Stoykova, A., and Gruss, P. (1998) Pax6 controls radial glia differentiation in the cerebral cortex. *Neuron* 21: 1031-1044.

Hansen D, Lui J, Parker PRL, Kriegstein AR. (2010) Neurogenic radial glia in the outer subventricular zone of human neocortex. *Nature* 464 (7288): 554-561.

Haydar TF, Wang F, Schwartz ML, Rakic P. 2000. Differential modulation of proliferation in the neocortical ventricular and subventricular zones. *J. Neurosci* 20: 5764-5774.

Haubensak W., Attardo, A., Denk W., Huttner, W. B. (2004) Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: A major site of neurogenesis. *Proc. Natl. Acad. Sci*. 101(9): 3196-3201.

Hevner R.F. (2006) From Radial Glia to Pyramidal-Projection Neuron. *Molecular Neurobiology* 33: 33-43.

Principal Investigator/Program Director (Last, First, Middle): Zecevic, Nada

Hill R.S., Walsh C.A. (2005) Molecular insights into human brain evolution. *Nature*, 437 (7055): 64-7.

Howard B., Chen Y., Zecevic N. (2006) Cortical Progenitor cells in the developing human telencephalon. *GLIA.* 53, 57-66.

Howard B., Mo Z., Filipovic R., Moore AR, Antic SD and Zecevic N. (2008) Radial glia cells in the developing human brain . *The Neuroscientist* . doi:10.1177/1073858407313512

Jakovcevski I., Zecevic N. (2005) Olig transcription factors are expressed in oligdendrocyte and neuronal cells in human fetal CNS. *J.Neurosci*. 25(44): 10064-10073.

Jakovcevski I., Filipovic R., Mo Zh., Rakic S., Zecevic N. (2009) Oligodendrocyte development and the onset of myelination in the human fetal brain. Frontiers in Neuroanatomy, doi: 10.3389/neuro.05.005.2009

Kowalczyk T., Pontious A., Englund C., Daza R.A.M., Bedogni F., Hodge R., Attardo A., Bell C., Huttner W.B., Hevner R.F. (2009) Intermediate Neuronal Progenitors (Basal Progenitors) Produce Pyramidal-Projection Neurons for All Layers of Cerebral Cortex. *Cer Cortex* 19: 2439-2450.

Letinic K., Zoncu R., Rakic P. (2002) Origin of GABAergic neurons in the human neocortex. *Nature*, 417: 645-649.

Levitt P. (2003). Genetic disruption of cortical interneuron development causes region- and GABA cell type-specific deficits, epilepsy, and behavioral dysfunction. *J. Neurosci.* 23: 622–631..

Lewis D.A., Hashimoto T., Volk D.W. (2005) Cortical inhibitory neurons and schizophrenia. *Nat Rev Neurosci* 6, 312-324.

Lukaszewicz A., Savatier P, Cortay V, Giroud P, Huissoud C, Berland M, Kennedy H, Dehay C. 2006. The concerted modulation of proliferation and migration contributes to the specification of the cytoarchitecture and dimensions of cortical areas. *Cereb. Cortex 16* (Suppl. 1): 26-34.

Marin O., Rubenstein J.L.R. (2001) A long remarkable journey: tangential migration in the telencephalon. *Nat Rev Neurosc.* 2: 780-90.

Marshall CA, Goldman JE. 2002. Subpallial Dlx2-expressing cells give rise to astrocytes and oligodendrocytes in the cerebral cortex and white matter. *J Neurosci.,* 22: 9821–9830.

Mo Z., Moore A., Filipovic R., Ogawa Y., Kazuhiro I., Antic S., Zecevic N. (2007) Human cortical neurons originate from radial glia and neuron-restricted progenitors. *J.Neurosc*., 27(15):4132- 4145.

Mo Z., Zecevic N. (2008) Is Pax6 critical for neurogenesis in the human fetal brain? *Cer Cortex,* 18: 1455-1465.

Mo Z., Zecevic N. (2009) Human fetal Radial glia cells generate oligodendrocytes *in vitro. GLIA* 57:490–498. (cover page)

Molnár Z., Métin C., Stoykova A., Tarabykin V., Price D.J., Francis F., Meyer G., Dehay C., Kennedy H. (2006) Comparative aspects of cerebral cortical development. *Eur J Neurosci*., 23: 921-934.

Molyneaux B.J., Arlotta P., Menezes J.R.L., Macklis J.D. (2007) Neuronal subtype specification in the cerebral cortex. *Nature* 8(6): 427-437.

Moore A, Filipovic R, Mo Z., Rasband M, Zecevic N. Antic S. (2009) Electrical excitability of early

Principal Investigator/Program Director (Last, First, Middle): Zecevic, Nada

neurons in the human cerebral cortex during the second trimester of gestation. *Cer Cortex* 206 (19):1795-1805.

Mueller F, O'Rahilly R. (1990) The human brain at stages 21-23 with particular reference to the cerebral cortical plate and to the development of the cerebellum. *Anat. Embryol.* 182: 375-400.

Nobrega-Pereira S., Kessaris N., Du T., Kimura S., Anderson S.A., Marin O. (2008) Postmitotic Nkx2-1 Controls the Migration of Telencephalic Interneurons by Direct Repression of Guidance Receptors. *Neuron* 59(9), 733-745.

Noctor S. C., Flint A. C., Weissman T. A., Dammerman R. S., Kriegstein A. R. (2001) Neurons derived from radial glial cells establish radial units in neocortex. *Nature* 409: 714-720.

Noctor S. C., Martinez-Cerdeno V., Ivic L., Kriegstein A. R. (2004) Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat Neurosci* 7: 136-144.

Osumi N., Shinohara H., Numayama-tsurita K., Maekawa, M. (2008) Concise Review:Pax6 transcription factor contributes to both embryonic and adult neurogenesis as a multifunctional regulator. *Stem Cells* 26:1663-1672.

Owens DF and Kriegstein AR 2002. Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 3:715-727.

Parnavelas J.G., Anderson S.A., Lavdas A.A., Grigoriou M., Pachnis V., Rubenstein J.L. (2000) The contribution of the ganglionic eminence to the neuronal cell types of the cerebral cortex. *Novartis Found Symp*. 2000; 228, 129-139.

Petanjek Z., Berger B., Esclapez M. (2009) Origins of Cortical GABAergic Neurons in the Cynomolgus Monkey. *Cer Cortex* 19(2), 249-262.

Pinto[1], L. Daniela Drechsel[1,2], Marie-Theres Schmid[1], Jovica Ninkovic[1], Martin Irmler[3], Monika S. Brill[1,4], Laura Restani[5], Laura Gianfranceschi[5], Chiara Cerri[5], Susanne N. Weber[6], Victor Tarabykin[7], Kristin Baer[8], Francois Guillemot[2], Johannes Beckers[3,9], Nada Zecevic[10], Colette Dehay[11], Matteo Caleo[5], Hubert Schorle[6] and Magdalena Götz (2009) AP2gama regulates basal progenitor fate in a region- and layer-specific manner in the developing cortex. *Nature Neurosci*. 12(10): 1229-1237.

PolleuxF, Whitford KL, Dijkhuizen2 PA, Vitalis T and Ghosh A. (2002) Control of cortical interneuron migration by neurotrophins and PI3-kinase Signaling. Development 129: 3147-3160.

Rakic P. (2009) Evolution of the neocortex: a perspective from developmental biology. *Nat Rev Neurosci* 10 (10): 724-35.

Rakic S. and Zecevic N (2003a) Emerging complexity of cortical layer I in humans. *Cer.Cortex* 13, 1072-1083.

Rakic and Zecevic (2003b) Early oligodendrocyte progenitor cells in the human fetal telencephalon. *GLIA*, 41:117-127.

Smart I. H. M., Dehay C., Giroud P., Berland M., Kennedy H. (2002) Unique morphological features of the proliferative zones and postmitotic compartments of the neural epithelium giving rise to striate and extra striate cortex in the monkey. *Cer Cortex* 12: 37-53.

Simoni and Yu 2006 Preparation of organotypic hippocampal slice cultures: interface method. *Nature Proto*

1(3):1439-1445.

Stoppini LA, Buchs D, Muller A. 1991. A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods* 37: 173-182.

Weir et al. (2007) Characterizing the cancer genome in lung adenocarcinoma. *Nature* (6) 450 (7171):893-8.

Wonders C, Anderson SA. (2006) The origin and specification of cortical interneurons. *Nat Rev Neurosci.* 7(9): 687-696.

Xu Q., Cobos I., De La Cruz E., Rubenstein J.L., Anderson S.A. (2004). Origins of Cortical Interneuron Subtypes. *J Neuroscience* 24(11), 2612-2622.

Yu,X., Jakovcevski, I, Mo,Z.,Zecevic, N. (2009) Cortical origin of calretinin-expressing neurons in the human fetal forebrain. *Abstract for SFN 2009* Oct.

Zecevic,N. (1998) Synaptogenesis in Layer I of the Human Cerebral Cortex in the First Half of Gestation. *Cer Cortex*, 8(3): 245-252.

Zecevic N. 2004. Specific characteristics of radial glia in the human fetal telencephalon. *Glia* 48(1): 27-35.

Zecevic N*, Chen Y, Filipovic R. (2005) Contributions of Cortical Subventricular Zone to the Development of the Human Cerebral Cortex, *J.Comp.Neurol*. 491: 109-122.

Zecevic N., Rakic P. (2001) Development of Layer I neurons in the Primate Cerebral Cortex. *J Neurosci* 21(15): 5607-5619.

Zecevic N, Milosevic A. (1997) Initial development of gamma-aminobutyric acid immunoreactivity in the human cerebral cortex. *J Com Neurol* 380: 495-506.

Zecevic N, Hu F, Jakovcevski I (2010) Cortical interneurons in the developing human neocortex. *J Neurobiology* (in press).

Albert Einstein College of Medicine
OF YESHIVA UNIVERSITY

Science at the heart of medicine

Human Fetal Tissue Repository

Bradford K. Poulos, Ph.D., C.S. Med.
Director, hFTR

Jack and Pearl Resnick Campus
1300 Morris Park Ave.
Bronx, NY 10461
718.430.3478 fax 718.430.3631
hFTR@aecom.yu.edu

May 24, 2010

Dr.Nada Zecevic
Dept.Neuroscience
University of Connecticut Health Ctr.
Farmington, CT 06030-3401

Dear Dr. Zecevic,

We will be happy to continue providing you with brain tissue from the Human Fetal Tissue Repository at the Albert Einstein College of Medicine for use in your research and will be able to supply you with this tissue twice a month.

Your research is important and will further advance knowledge of the human brain and its development.

Sincerely,

Bradford K. Poulos, PhD, CSMed
Director, hFTR

Weill Cornell Medical College

NewYork-Presbyterian Hospital
Weill Cornell Medical Center

Stewart A. Anderson MD
Professor, Dept. of Psychiatry
Dept. of Neurology and Neuroscience
Graduate Program in Neuroscience

1300 York Ave, Box 244, Rm Lc921
New York, NY 10065
Tel: 212 746-3921; Fax 6-8529
SAA2007@med.cornell.edu

Dr. Nada Zecevic 5/25/2010
RE: Letter of collaboration

Dear Nada,
I am very excited about your preliminary results from electroporating slices from human embryonic forebrain with the Lhx6-GFP promoter reporter construct we provided you. These experiments, and indeed the entire theme of your grant, are of tremendous relevance on many levels. For my lab in particular, better understanding of normal processes in human cortical interneuron generation will be invaluable to our efforts to derive functional interneurons from human ES and Induced Pluripotent stem cells. We are testing a variety of human promoter-reporter constructs for labeling putative interneuron progenitors for use in isolating these cells from stem cell-derived cultures. I am happy to provide you with reagents, further slice electroporation training, and any advice on experimental design or interpretation that might be helpful for your project.

Sincerely,

Stewart Anderson

Yale University

Nenad Sestan, M.D., Ph.D.
Associate Professor
Department of Neurobiology
Kavli Institute for Neuroscience
School of Medicine
P.O. Box 208001
New Haven, Connecticut 06520-8001

Campus address:
Sterling Hall of Medicine C-323C
333 Cedar Street
Telephone: 203-737-2190
Fax: 203-785-5263
Email: nenad.sestan@yale.edu
Web: www.sestanlab.org

May 24, 2010

Nada Zecevic, M.D., Ph.D.
Department of Neuroscience
University of Connecticut Health Center
Farmington, CT 06030

Dear Nada,

This is to affirm my intention to collaborate with you on your work studying the development of the human cerebral cortex. As you know, this is an area of great interest to us. Our continued collaboration will insure optimal use of our resources and our effort, to facilitate advances in the genetic manipulations of human neural cells in vitro..

I will be glad to provide expertise and technical help in manipulating NKX2.1, DLX or TBR2 expression in human neural cells. My laboratory has considerable experience with generating promoter constructs, and using viral and non-viral methods of gene expression in mouse and human brain tissue. I believe that your research on human cortical development is important and clinically relevant, and I will be please to advise you on particular aspects of molecular genetics. I enjoy our interactions, and look forward to many more.

I wish you the best of luck on your proposal.

Best regards,

Nenad Sestan, M.D., Ph.D.
Associate Professor of Neurobiology

PHS 398 Checklist

OMB Number: 0925-0001

1. Application Type:

From SF 424 (R&R) Cover Page. The responses provided on the R&R cover page are repeated here for your reference, as you answer the questions that are specific to the PHS398.

* Type of Application:

☐ New ☒ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

Federal Identifier: NS041489

2. Change of Investigator / Change of Institution Questions

☐ Change of principal investigator / program director

Name of former principal investigator / program director:

Prefix:
* First Name:
Middle Name:
* Last Name:
Suffix:

☐ Change of Grantee Institution

* Name of former institution:

3. Inventions and Patents (For renewal applications only)

* Inventions and Patents: Yes ☐ No ☒

If the answer is "Yes" then please answer the following:

* Previously Reported: Yes ☐ No ☐

4. * Program Income

Is program income anticipated during the periods for which the grant support is requested?

☐ Yes ☒ No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

5. * Disclosure Permission Statement

If this application does not result in an award, is the Government permitted to disclose the title of your proposed project, and the name, address, telephone number and e-mail address of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information (e.g., possible collaborations, investment)?

☒ Yes ☐ No