

AD _____

Award Number: W81XWH05-01-0061

TITLE: U.S. Army Battlefield Exercise and Combat Related Spinal Cord Injury
Research: Neuroprotection and Repair After Spinal Cord Injury

PRINCIPAL INVESTIGATOR: W.Dalton Dietrich, Ph.D.

CONTRACTING ORGANIZATION: University of Miami, Miller School of Medicine
Miami, FL 33136

REPORT DATE: March 2009

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.				
1. REPORT DATE 1 March 2009		2. REPORT TYPE Final Addendum		3. DATES COVERED 6 Nov 2004 – 7 Feb 2009
4. TITLE AND SUBTITLE U.S. Army Battlefield Exercise and Combat Related Spinal Cord Injury Research: Neuroprotection and Repair After Spinal Cord Injury			5a. CONTRACT NUMBER	
			5b. GRANT NUMBER W81XWH05-01-0061	
			5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) W. Dalton Dietrich, Ph.D.; Co-PI's: John L. Bixby, Ph.D.; Robert Keane, Ph.D.; Vance P. Lemmon, Ph.D.; Daniel J. Liebl, Ph.D.; Damien Pearse, Ph.D.; Pantelis Tsoulfas, M.D. E-Mail: ddietrich@med.miami.edu			5d. PROJECT NUMBER	
			5e. TASK NUMBER	
			5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Miami, Miller School of Medicine Miami, FL 33136			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)	
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				
13. SUPPLEMENTARY NOTES				
14. ABSTRACT The purpose of this research program is to use a multidisciplinary approach to investigate the pathophysiology of spinal cord injury (SCI) with the major goal of developing therapies targeted at both the acute and more chronic injury setting. We are testing the overall hypothesis that high content screening (HCS) of available libraries containing 100's to 1000's of chemicals, small molecules, and small interfering RNA's (siRNA's) will lead to the identification of novel strategies to reduce cell death and improve axonal regeneration following SCI. In this period of funding, we have made significant progress in this overall goal. In Specific Aim #1, the identification of molecules from HCS screening that inhibit excitotoxic brain cell death in primary neurons has been conducted. One of these compounds BMP7 has now been translated into a clinically relevant model of SCI and shown beneficial effects in preserving neuronal survival. Other studies have assessed the cell-signaling cascades in which BMP7 may influence. In Specific Aim #2, a variety of EphA4 dependent receptor functions have been evaluated using a various of approaches. Candidate small molecular drug screening procedures have resulted in several exciting compounds that reduce the amount of EphA4 induced apoptosis. Some of these candidate molecules have now been translated into relevant models including EphA4-mediated cell death in primary oligodendrocytes. In Specific Aim #3, the compounds and siRNA's capable of producing mononuclear phagocyte inactivation have been evaluated. An in vitro system to evaluate microglial cell activation has been developed and the BIOMOL compound library which includes more than 400 drugs have been screened. The in vivo testing of one of these agents for therapeutic efficacy is now being conducted in a clinically relevant model of spinal cord injury. In Aim #4, a screening approach to target specific kinase inhibitors to block astrocytic or oligodendrocyte differentiation of fetal neuroprecursors has been initiated. The identification of several inhibitors that influence the differentiation of neural precursors along the astrocytic and oligodendrocyte lineages have been accomplished, and these studies are now being tested in a clinically relevant model of spinal cord injury. In Specific Aim #5, axonal growth and hippocampal neurons is being investigated using HCS approaches specifically targeting kinase and phosphatase overexpression. Also, shRNA's to knock down these molecules and study their effects on axon growth has also been evaluated. Our core facilities that support these individual projects are established and providing the infrastructure and expertise to support the HCS methodologies as well as a successful translation of these findings to clinically relevant models of SCI.				
15. SUBJECT TERMS inflammation, apoptosis, spinal cord injury (SCI), trauma, high content screening (HCS), chemical libraries, small interfering siRNA's				
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 44
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U		
				19b. TELEPHONE NUMBER (include area code)

Table of Contents

Page

Specific Aim 1 – WD Dietrich, PhD & RW Keane, PhD

Introduction.....	4
Body.....	4-5
Key Research Accomplishments.....	6
Reportable Outcomes.....	N/A
Conclusion.....	6
References.....	N/A
Appendices.....	N/A

Specific Aim 2 – D. Liebl, PhD

Introduction.....	7
Body.....	7-11
Key Research Accomplishments.....	11
Reportable Outcomes.....	11
Conclusion.....	12
References.....	12
Appendices.....	N/A

Specific Aim 3 – D. Pearse, PhD

Introduction.....	13
Body.....	13-14
Key Research Accomplishments.....	15
Reportable Outcomes.....	15
Conclusion.....	15
References.....	16
Appendices.....	N/A

Specific Aim 4 – P. Tsoulfas, MD

Introduction.....	17
Body.....	17-20
Key Research Accomplishments.....	20-21
Reportable Outcomes.....	21
Conclusion.....	21-22
References.....	22
Appendices.....	N/A

Specific Aim 5 – V. Lemmon, PhD & J. Bixby, PhD

Introduction.....	23
Body.....	23-27
Key Research Accomplishments.....	28
Reportable Outcomes.....	28-31
Conclusion.....	32
References.....	32
Appendices.....	32-

Specific Aim 1. (WD Dietrich, PhD & RW Keane, PhD)

To inhibit protein expression in cell death and inflammatory pathways by screening a chemical library for compounds to inhibit cell death.

Introduction

Central nervous system (CNS) destruction in spinal cord injury (SCI) is caused by a complex series of cellular and molecular events termed secondary injury of which inflammation and apoptosis play crucial roles in destruction of the injured tissue. Thus, inhibition of these signaling pathways is a promising therapeutic target for the acute treatment of neurological trauma and disease. Our current experimental studies examine whether various molecules prevent production of proinflammatory and proapoptotic signaling thus leading to functional improvement after SCI. Hypothesis: Compounds identified by high content screening will inhibit cell death and inflammatory cascades leading to improved histopathological and behavioral outcomes after SCI.

Research/experiments done

During the funding period we have identified two molecules from high content screening that inhibit excitotoxic-induced cell death of primary neurons. We have tested one molecule, BMP7 in a clinically relevant model of SCI in the rat and show beneficial therapeutic effects by preserving neuronal viability. Adult male Sprague-Dawley rats received a moderate contusion injury (3 Kdyn of force) by dropping a 10 g weight that displaced the spinal cord by 0.95 mm using the circular flap tip of the impactor of the Electromagnetic SCI Device (Ohio State University). Animals were injected intravenously and intraperitoneally with 1 µg BMP7 (Curis Inc.) at 20 min after SCI. In addition, animals received a daily injection of 1 µg BMP7 intraperitoneally for 2 more days. The control group consisted of animals injected with saline and sham animals. All treatments were performed in a double-blind manner. BMP7 activates activin receptors that initiate the Smad signaling pathway and the p38 MAPK signaling pathways. To determine whether SCI and BMP7 administration induced alterations in Smad and p39 MAPK signaling pathways, spinal cord lysates from injured, BMP7 treated, and sham-operated animals were analyzed by immunoblotting procedures. Figure 1 shows that BMP 7 treatment caused a decrease in the levels of activin 1 receptor, but an increase in the levels of phosphorylated p38 MAPK after SCI. In contrast, BMP7 administration did not significantly alter expression of Smad signaling proteins (not shown). Thus, it appears that BMP7 treatment after SCI induces p38-MAPK signaling, but does not significantly induce the Smad signaling pathway.

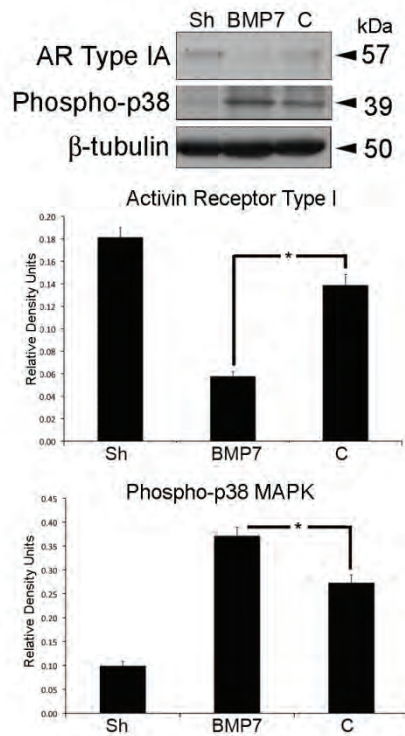


Figure 1. BMP7 delivery after SCI stimulates phosphorylation of p38-MAPK and decreases the levels of the activin receptor type I. Representative immunoblot analysis of spinal cord lysates of sham (Sh), BMP7 treated (BMP7) and injured untreated (C) animals at 3 d after trauma. Spinal cord lysates were immunoblotted with antibodies against the activin receptor type I and phospho-p38. Densitometric analysis indicates a significant decrease in the levels of the activin receptor following BMP7 treatment after injury and elevated levels of phospho-p38 after trauma, with BMP7 treated animals presenting higher levels of p38 phosphorylation. β -tubulin was used as internal standard and control for protein loading. N=3. Data are presented as mean $\pm$ SEM. * $p < 0.05$.

Next, we tested if BMP7 delivery after SCI increased neuronal viability. Paraffin-embedded, 10 μ m transverse sections, taken every 500 μ m apart from the injury epicenter for 1.5 mm rostral and caudal (7 sections analyzed) were stained with the neuronal antibody NeuN (Chemicon) using diaminobenzidine (DAB) as the chromogen. The total number of NeuN immunoreactive cells per spinal cord was quantified using unbiased stereological procedures. Accordingly, sections collected in the systematic random manner were analyzed by an observer blinded to the treatments using StereoInvestigator software (MicroBrightfield, Inc., Colchester, VT) and microscopy. Areas of interest were analyzed and determined by the physical fractionator method in which the physical dissector probe was applied to estimate the neuronal number in the enclosed volume corresponding to the area of the ventral horn. Immunoreactive cells were those that had degrees of staining greater than background. The number of preserved neurons after injury in BMP7 treated animals (BMP7) was significantly greater than the number of neurons remaining in the ventral horn of the spinal cord of untreated injured animals (C). N=4 Data are presented as mean $\pm$ SEM. * $p < 0.05$ compared to untreated, C.

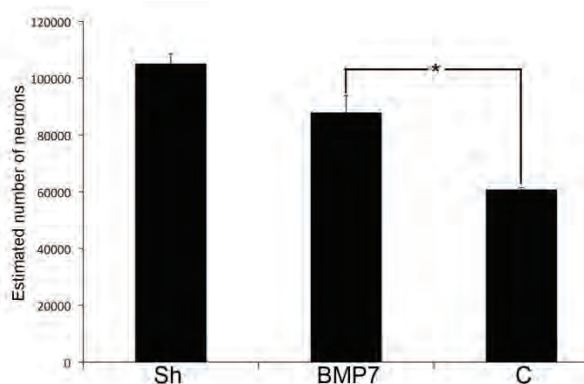


Figure 2. BMP7 treatment preserves neurons following SCI. After SCI, a person blinded to the treatment protocols assessed the number of NeuN positive cells (neurons) in the ventral horn of the spinal cord by unbiased stereological procedures.

We have also indentified a candidate inhibitor of excitotoxicity Ro 25-6891 of neurons in culture and have initiated studies to test efficacy after SCI.

Key Research Accomplished. From the screen of the chemical library, we have successfully identified two compounds that prevent excitotoxic cell death. We tested efficacy of BMP7 in a clinically relevant model of SCI in the rat and have shown beneficial therapeutic effects in preserving neurons. Current studies are underway to evaluated efficacy to a second putative candidate compound to inhibit excitotoxic cell death after SCI.

Conclusions and Future Directions.

In the next phase of work we determine whether Ro25-6981, a candidate inhibitor of excitotoxicity prevent cell death in the injured spinal cord thus leading to improvements in tissue sparing and functional outcomes. Additionally, we will continue high content screening for molecules and siRNA that inhibit apoptotic and inflammatory cell death processes. Uncovering cell death-specific expression changes will ultimately contribute to the design of novel and targeted therapies for human SCI and CNS diseases.

Specific Aim 2 (D. Liebl, PhD)

Introduction: Spinal cord injury (SCI) results in an initial traumatic insult and a secondary response that includes reactive astrogliosis, invasion of foreign cells, and apoptotic cell death. Programmed cell death has been found to occur as early as a few hours after injury and progress for several weeks. In particular, oligodendrocytes, the principle myelinating cell of the central nervous system (CNS), are thought to be extremely sensitive to early apoptosis following injury. However, little is known of the factors or events that mediate the early apoptotic response. Our studies implicate Eph receptors in mediating caspase-dependent apoptosis after SCI. We hypothesize that these receptors regulate important axonal guidance decisions in the developing spinal cord; however, in the injured adult spinal cord they function as “dependency receptor”. Dependency receptors are defined by dependence on their respective ligand, and during periods of ligand-receptor dissociation, these receptors can undergo cleavage and induce caspase-dependent apoptosis. Our current studies will screen numerous small molecules and siRNAs to identify signal transduction pathways and determine whether they function to inhibit Eph-mediated apoptosis, which in turn may lead to the development of CNS protective strategies to improve recovery.

Body:

Developing the *in vitro* dependency model. To begin evaluate EphA4 dependent receptor functions, stable EphA4 expressing NIH3T3 (EphA4-3T3) cells were generated and examined for expression and activation. Immunostaining of the V5-tagged showed strong expression in this stable cell line, and application of soluble ligand (i.e. ephrinB3) induced clustering as compared to vehicle controls (Fig. 1a). These findings demonstrate surface expression of EphA4 in the stable cell line, a prerequisite for EphA4 activation. Western blot analysis of EphA4 expression and phosphorylation demonstrates significant greater membrane expression in the EphA4-3T3 expressing cells as compared to vector-3T3 cells when immunoprecipitated with either anti-V5 or anti-EphA4 antibodies (Fig. 1b). Over-expression of EphA4 resulted in increased EphA4 tyrosine phosphorylation as compared to pcDNA3.1-V5 only cultures, which may result from auto-phosphorylation and/or ephrin stimulation. It is well known that over-expressing receptor tyrosine kinases lead to auto-phosphorylation (1-6), however, it is noteworthy that our previous studies demonstrate that tyrosine phosphorylation is not a requirement for inhibiting dependence receptor cleavage and subsequent cell death (7). Furthermore, there is a trend towards increased levels of EphA4 tyrosine phosphorylation in the ephrinB3-stimulated as compared to ephrinB1-stimulated cells, although immunoprecipitation is thought to be a semi-quantitative method of analysis.

To begin screening the small molecule drug (SMD) library, cells were dissociated and replated in 96-well plates in serum-containing media (see schematic (Fig. 2a)). After a 24 hrs stabilization period, media was replaced with serum-free media for 48 hrs and examined using a live/dead assay on the KineticScan Reader. Cell undergoing cell death were labeled with the Sytox stain (red) and compared to total Hoechst (blue) labeling (Fig. 2b). Vector-3T3 cells grown in serum-free media for 48 hrs induced a significant increase in cell death as compared to serum-containing media as measured by Sytox-positive cells (Fig. 2b, 2c). The over-expression of EphA4 augmented cell death, which was blocked by addition of soluble ephrinB3 (Fig. 2b, 2c). These findings support the dependence receptor functions for EphA4 and ephrinB3 in our assay. To determine the optimal incubation period for stress-induced cell

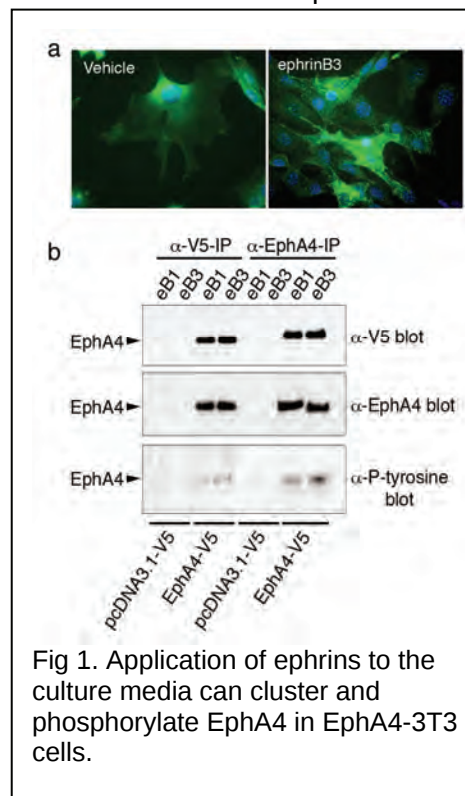
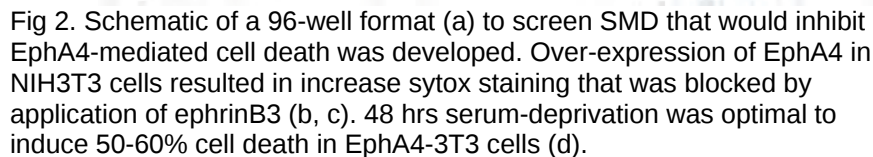


Fig 1. Application of ephrins to the culture media can cluster and phosphorylate EphA4 in EphA4-3T3 cells.

shown), although 48 hrs was considered optimal since it represents a moderate degree of cell death that can be significantly reversed and is not excessive in serum-free conditions (Fig 2d). Cell 'seeding' concentrations were also examined to optimize consistency. Cell concentration is important due to the need to seed significant numbers of cells to provide an accurate assessment of cell death, but minimize cell-cell contact over the experimental period. This is important since many cell lines express endogenous ephrins that can potentially interact and activate EphA4 and subsequently reduce the amount of EphA4 induced apoptosis. We observed a seeding concentration of 5000

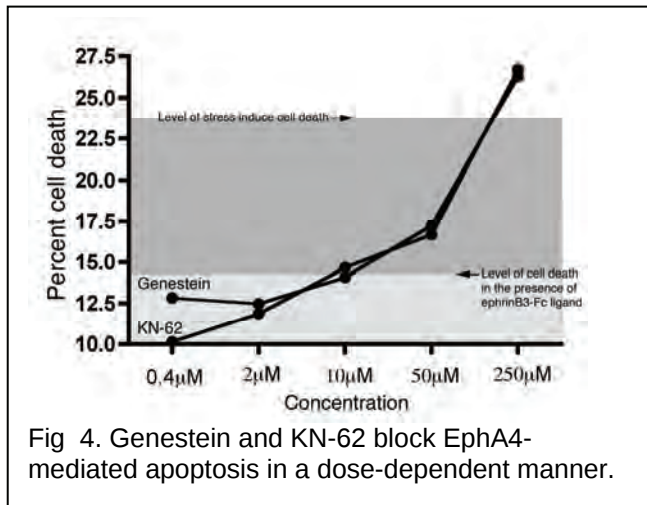


43 compounds induce >1.25 fold survival
22 compounds induce >2 fold survival

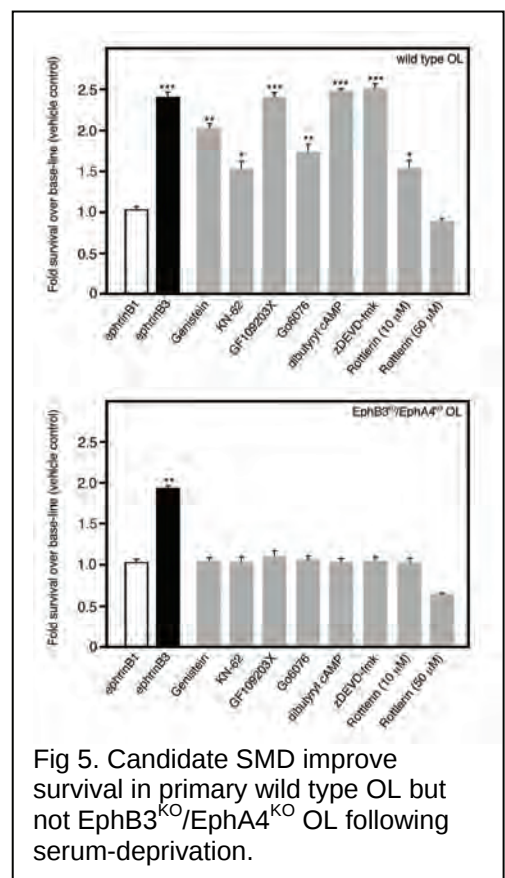
Compound	Change in survival (Arb units)	Category
Control	1.0	
etomidate	0.5	
Gesatriene	2.8	Tyrosine Kinase/ Broad spectrum Kinase
Staurosporine	2.8	Tyrosine Kinase/ Broad spectrum Kinase
IC5252-a	4.2	Tyrosine Kinase/ Broad spectrum Kinase
Tyrophostin 9	1.8	Tyrosine Kinase/ Broad spectrum Kinase
Tyrophostin-XG 925	2.2	Tyrosine Kinase/ Broad spectrum Kinase
Indomethacin	5.8	Tyrosine Kinase/ Broad spectrum Kinase
Thalidomide	6.5	Tyrosine Kinase/ Broad spectrum Kinase
KV102	6.3	Cam/CamK
Phenylethylbenzamide	1.5	Cam/CamK
GF-109203X	2.3	Cam/CamK
Ged978	6.8	PKC
Rotenone	1.5	PKC
Cathecolamin C	1.8	PKC
Cathecolamine	2.4	PKC
Exoporphyrin	3.0	PKC
Exoporphyrin	2.9	PKC
PCA-8246	2.5	PKC
H-89	1.9	PKA
Dibutyryl cAMP	1.7	PKA
U0124	1.6	MapK/Erk Signaling
ZM36272	1.7	MapK/Erk Signaling
Splatinoyl	9.0	MapK/Erk Signaling
RHC-80201	2.3	MapK/Erk Signaling
RK-682	2.0	Transcription/translation Modifiers
Swainsoneine	5.8	Transcription/translation Modifiers
Propidium Iodide	2.3	Cell Cycle
FK-506	6.8	Cell Cycle
NSC-93597	2.0	Transmitters
PRIMA-1	4.2	Transmitters
6 / ADT11 HB	2.1	Transmitters
Propafenone (S-)	2.1	Transmitters
Yohimbine	1.5	Ion Channels
Kavain	2.1	Ion Channels
Bacardi	1.8	Ion Channels
Diazoxide	1.8	Ion Channels
Diltiazem	1.8	Ion Channels
AA-504	2.1	Ion Channels
BAPTA-AM	1.8	Ion Channels
Nigamycin	2.3	Ion Channels
Wiskostatin	2.0	Ion Channels
Cyclopent	3.1	other



Screening small molecule library. We next initiated a screen of the small molecule kinase library (Biomol International), where each molecule was examined in triplicate in both vector-3T3 and EphA4-3T3 cells. Vector-3T3 controls were examined to determine whether there was a general non-EphA4-mediated effect on survival or toxic effects. All experiments were compared to EphA4-3T3 cells serum-deprived in the presence of ephrinB3 (positive control). We identified approximately 50 candidate SMD that can significantly block EphA4-mediated cell death (representatives are shown in Fig 3). From this analysis, candidate SMD have fallen into several group classifications (including tyrosine kinase inhibitors, CamKinase inhibitors, PKC inhibitors, etc). Concentration for each SMD employed in the screen was based on previous studies in the literature describing reaction specificity. To confirm optimal concentration, each of the candidate SMD were dose-dependently tested. For example, genestein and KN-62 demonstrate a dose-dependent improvement in their ability to inhibit cell death with optimal concentration at 2 and 0.4 μ M, respectively. Optimal concentrations were employed to screen primary oligodendrocyte (OL) cultures (see below).



Screening candidate molecules in primary OL cultures. To begin translating our findings to physiological relevant models, we established an *in vitro* model of Eph-mediated cell death in primary oligodendrocytes (OL) harvested from the adult mouse spinal cord. We found that OL precursors, pro-OL, and mature OL all express both EphB3 and EphA4 receptors (not shown). If mature OL are serum-deprived for 48 hrs then approximately 50% of the OL undergo apoptotic cell death (not shown). To evaluate SMD, we compared cultures incubated in the presence of an ephrinB1 (negative control), ephrinB3 (positive control), and SMD as compared to vehicle control. A value of “1” indicates no change from vehicle control, whereas increased values reflex improved survival and decreased values reflex cell death (Fig. 5). We examined 7 candidate SMD for their ability to promote OL survival as compared to ephrinB1 and ephrinB3, and found that all 7 candidates promoted survival between 1.5- and 2.5-fold as shown by live Sytox labeling similar to the application of ephrinB3. When EphB3^{KO}/EphA4^{KO} OL cultures were employed neither ephrinB3 nor the 7 SMD showed significant improvement in survival, supporting the hypothesis that these compounds are specific to Eph-mediated apoptosis (Fig. 5). One exception was the application of 50 μ M Rottlerin, which showed a toxic effect in both wild type and EphB3^{KO}/EphA4^{KO} OL cultures. This likely resulted from the non-specific nature of pharmacological kinase inhibitors at high concentrations where multiple kinase are effected that lead to toxic conditions. In addition, these same 7 SMD were tested in OL cultures and evaluated for the number of OL expressing cleaved caspase-3, which we found were similar to our findings with Sytox (not shown). Together, our studies demonstrate that genestein, KN-62, GF109203X, Go6976, dibutyryl cAMP,



Rottlerin, and zDEVD-fmk all block Eph-mediated apoptosis in cultured OL, and support further analysis in an *in vivo* SCI model.

Testing SMD candidates in a murine SCI model. We next evaluated candidate SMD in an *in vivo* murine SCI model using PLP-GFP mice (gift from Wendy Macklin) and a moderate T8 contusion injury (Fig. 6a, b). These transgenic mice express green fluorescent protein (GFP) specifically in OL, which were colabeled with either TUNEL or cleaved caspase-3 antibodies (Fig. 6b) to determine the percentage of apoptotic OL at 3- and 7-days post-injury. Figure 6c shows that infusion of 500 μg/ml ephrinB3 or 100 μM GF109203X for either 3 or 7 days resulted in a ~2-fold increase in OL survival as compared to ephrinB1 and PBS controls, respectively. Additional studies will be performed to determine whether increase OL survival correlates with increase locomotor behavior and white matter sparing.

siRNA screen of EphA4-mediated apoptosis in NIH 3T3 cells.

We also initiated a secondary genetic approach to determine whether gene candidates (identified in the SMD screen) could also be inhibited using siRNA knockdown technology. These screens will provide additional data on a separate and more extensive library, but also provide direct and specific evidence for the involvement of kinase genes. Our initial studies developed the siRNA approach, where we screened multiple hybridization buffers and siRNA concentrations.

We identified optimal buffers and siRNA concentrations for control GAPDH and cyclophilin protein knockdown (>80%). The SMD screen using the CamKII inhibitors (KN-62) and PKC inhibitors (GF109203X and Go6976) suggested that these might be two important families to evaluate using the siRNA approach. We found

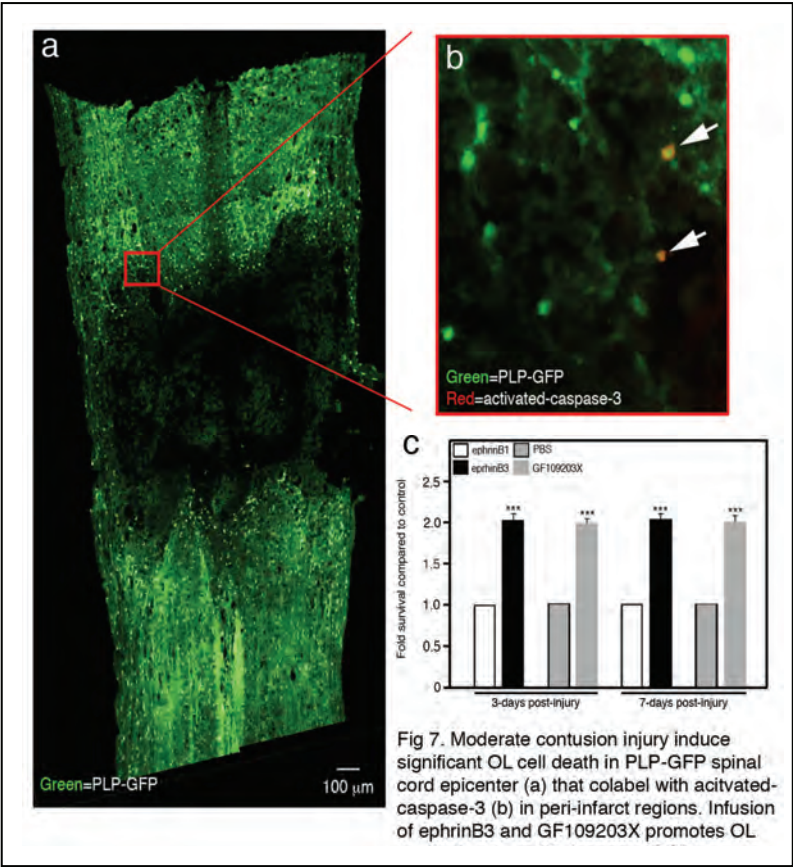


Fig 7. Moderate contusion injury induce significant OL cell death in PLP-GFP spinal cord epicenter (a) that colabel with acitvated-caspase-3 (b) in peri-infarct regions. Infusion of ephrinB3 and GF109203X promotes OL

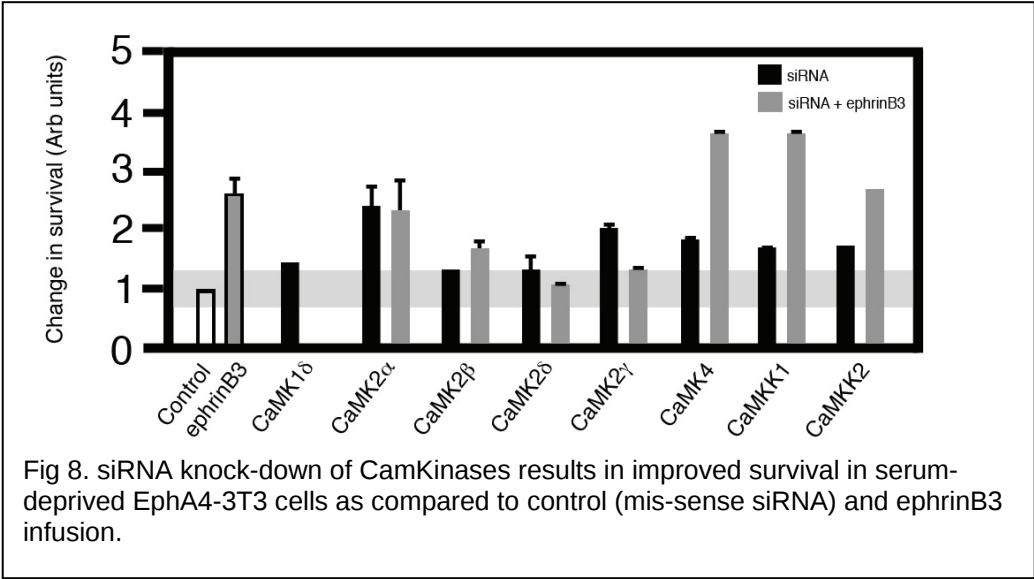


Fig 8. siRNA knock-down of CamKinases results in improved survival in serum-deprived EphA4-3T3 cells as compared to control (mis-sense siRNA) and ephrinB3 infusion.

that knockdown of CamK2α, CamK2γ, CamK4, CamKK1 and CamKK2 call all improve cell survival,

however, only CamK2 α showed no additional response when treated in combination with ephrinB3. This supports the role of CamK2 α as a signal intermediate in the Eph-mediated cell death. Interestingly, knockdown of CamK2 β , CamK2 δ and CamK2 γ all inhibited the ephrinB3-mediated improvement in survival suggesting that these genes may also be important candidates to further evaluation. We will continue to examine other candidate genes identified in the SMD screen (i.e. PKC) to determine specificity within the gene family. These findings suggest that individual CamKinase genes may be involved in Eph signaling as it relates to Eph-mediated apoptosis, and supports a more targeted approach to blocking dependence receptor cell death.

Key research accomplishments:

- 1) Generated an EphA4 receptor stable mouse NIH3T3 cell line.
- 2) Established the EphA4-mediated apoptosis model in NIH3T3 cells for small molecule and siRNA approaches.
- 3) Identified the optimal transfection buffer to minimize toxicity and maximize gene transfection and protein knockdown.
- 4) Verified RNAi model in NIH3T3 cells using three control proteins (i.e. GAPDH, cyclophilin, and laminin).
- 5) Initiated small-molecule screening of EphA4-mediated apoptosis to identify pharmacological inhibitors.
- 6) Identified numerous inhibitors and activators of Eph-mediated apoptosis.
- 7) Performed dose-dependent screen on a small set of interesting small molecule candidates.
- 8) Examined small molecule candidates using primary mouse OL cells.
- 9) Initiated screening using *in vivo* SCI models.
- 10) Initiated screening using primary non-human primate OL cells.

Reportable outcomes:

- 1) Developed EphA4-3T3 stable cell line to model dependence receptor cell death.
- 2) Furne C, Ricard J, Cabrera JB, Pays L, Mehlen P, Bethea JR, Liebl DJ. ephrinB3 is an anti-apoptotic ligand that inhibits the dependence receptor functions of EphA4 receptors during adult neurogenesis. *BBA-Molecular Cellular Research*, 1793(2):231-238 (2009).
- 3) Hendrick-Theus M, Baumann G, Liebl DJ. Essential role for EphB3 receptor in subventricular neurogenesis following traumatic brain injury. *Revision* (2009).
- 4) Barerras H, Runko E, Nellersa C, Shi Y, Bethea J, Bixby J, Lemmon V, Liebl DJ. High-Content Screen of Pro-Apoptotic EphA4 Dependence Receptor Functions using Small Molecule Drug Libraries. *In preparation* (2009).
- 5) Hendrick-Theus M, Ricard J, Runko E, Liebl DJ. Eph receptors are new pro-apoptotic dependence receptors during adult neurogenesis and following CNS injury. *Keystone Symposia* (2009).
- 6) Liebl DJ. Eph receptors are new pro-apoptotic dependence receptors during adult neurogenesis and following CNS injury 7th International Symposium on Experimental Spinal Cord Repair and Regeneration (2009).
- 7) Runko E, Ricard J, Liebl DJ. Eph receptors mediate apoptosis following spinal cord injury. *Cold Spring Harbor* (2008).
- 8) Hendrick-Theus M, Riard J, Liebl DJ. Pro-apoptotic Function for Eph Receptors during Adult Neurogenesis and following CNS Injury. *Society for Neurotrauma* (2008).
- 9) Hendrick-Theus M, Riard J, Liebl DJ. Pro-apoptotic Function for Eph Receptors during Adult Neurogenesis and following CNS Injury. *Christopher and Daina Reeve Foundation Symposia* (2008).
- 10) Ricard J, Salinas J, Liebl DJ. EphrinB3 and its receptor EphA4 control adult subventricular zone neurogenesis *International Society for Development Neuroscience*. (2008).

Conclusion: We have determined that high-content screening in EphA4-3T3 cells is a validate model for screening and identifying SMD candidates that can inhibit dependence receptor induced cell death. These SMD candidates were confirmed for their anti-apoptotic functions in primary OL cultures derived from the adult murine spinal cord and *in vivo* SCI models. These findings suggest that several of these candidate SMD may be important in protecting OL from early apoptosis following SCI, and support this strategies for the development of clinical trials.

References:

- [1] P.P. Di Fiore, J.H. Pierce, M.H. Kraus, O. Segatto, C.R. King and S.A. Aaronson, erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells, *Science* 237 (1987), pp. 178–182.
- [2] A.M. Hinsby, J.V. Olsen, K.L. Bennett and M. Mann, Signaling initiated by overexpression of the fibroblast growth factor receptor-1 investigated by mass spectrometry, *Mol. Cell. Proteomics* 2 (2003), pp. 29–36.
- [3] N. Jones, S.H. Chen, C. Sturk, Z. Master, J. Tran, R.S. Kerbel and D.J. Dumont, A unique autophosphorylation site on Tie-2/Tek mediates Dok-R phosphotyrosine binding domain binding and function, *Mol. Cell. Biol.* 23 (2003), pp. 2658–2668
- [4] N. Jones and D.J. Dumont, The Tek/Tie-2 receptor signals through a novel Dok-related docking protein, *Dok-R*, *Oncogene* 17 (1998), pp. 1097–1108.
- [5] N. Jones, Z. Master, J. Jones, D. Bouchard, Y. Gunji, H. Sasaki, R. Daly, K. Alitalo and D.J. Dumont, Identification of Tek/Tie-2 binding partners. Binding to a multifunctional docking site mediates cell survival and migration, *J. Biol. Chem.* 274 (1999), pp. 30896–30905.
- [6] X. Wei, S. Ni and P.H. Correll, Uncoupling ligand-dependent and -independent mechanisms for mitogen-activated protein kinase activation by the murine Ron receptor tyrosine kinase, *J. Biol. Chem.* 280 (2005), pp. 35098–35107.
- [7] Furne C, Ricard J, Cabrera JB, Pays L, Mehlen P, Bethea JR, Liebl DJ. ephrinB3 is an anti-apoptotic ligand that inhibits the dependence receptor functions of EphA4 receptors during adult nerurogenesis. *BBA-Molecular Cellular Research*, 1793(2):231-238 (2009).

Specific Aim 3. (D. Pearse, PhD)

Screening of compounds/siRNA capable of producing mononuclear phagocyte inactivation

(i) Introduction. Injury to the spinal cord produces activation of both resident (microglia) and systemic (macrophage) immune cell populations, collectively termed 'mononuclear phagocytes'. Within 3 days of injury, the epicenter of the injured spinal cord is dominated by large numbers of rounded, phagocytic and fully activated mononuclear phagocytes. These activated immune cells, within the injured CNS, are thought to exacerbate the initial insult by secreting cytotoxic neurotoxins and cytokines, tissue degrading proteases and free oxygen species (Schubert et al., 1998; Cross & Woodroffe, 1999; Flavin & Zhao, 2001; Kingham & Pocock, 2001). Indeed, this phase of mononuclear phagocyte activation does coincide with a period of cell death and tissue loss subsequent to the initial mechanical trauma termed the 'secondary injury' (Bartholdi and Schwab, 1997). Furthermore, prevention of mononuclear phagocyte activation (Popovich et al., 2002) or the inhibition of the pro-inflammatory molecules produced by activated mononuclear phagocytes (Bethea and Dietrich, 2002; Pearse et al., 2003) has been demonstrated to reduce secondary tissue loss after SCI. Although several targets have been identified that have shown promise in ameliorating mononuclear phagocyte activation and subsequent tissue damage after spinal cord injury, successful use of a compound clinically in SCI has been elusive.

The current investigations utilized a high content screening (HCS) system, with cultured (activated) microglia to identify compounds capable of reverting SCI-activated mononuclear phagocytes (Pearse et al., 2004) from selected chemical libraries or genes that are involved in potentiating activation through the application of small interference RNA (siRNA) technology. Subsequently, 'hit' compounds or genes, those capable of inactivating microglia in HCS, were investigated for their ability to reduce microglia activations well as improve anatomical and functional recovery in a contusive SCI model. Not only are aberrant immune activation and subsequent cytokine dysregulation pivotal events in SCI, but also in many neurological disorders and thus the identification of novel targets for reverting activated mononuclear phagocytes will have, additionally, wide therapeutic applicability for many diseases beyond SCI.

(ii) Body:

Specific statement of work. To identify compounds or novel genes (siRNA) capable of reverting injury-activated mononuclear phagocytes.

Establish an in vitro system to evaluate microglial cell activation. We chose for these investigations, based upon immunophenotypical characteristics, a microglial cell line that behaves similarly to CNS injury-derived microglial cells and which could be employed in the large numbers required for High Content Screening (HCS) analysis. For activation of microglial cells we employed a dose range of the following known physiological and bacterial activators: TNF-alpha, glutamate, peroxynitrite, lipopolysaccharide from either E. Coli or Salmonella as well as leupeptin. We found that all agents were effective in activating close to 100% of the cells even at the smallest

doses of the compounds that were tested as evidenced by altered morphology and ED1 staining. We chose to use TNF-alpha, the most physiologically-relevant stimulus for our activation model. Furthermore, we showed that a positive control for inactivating microglia, aspirin, was able to work in this system.

Transfer microglial cell culture to a 96-well plate format. In order to apply the cell culture system to HCS, the culture conditions needed to be amended such that the microglial cells could be grown on specific 96-well plates. Using a variety of plate coatings we determined the PLL could be used to allow adherence on 96-well plates without inadvertently activating the microglia.

Screen the Biomol compound library (>400 drugs) for molecules than can prevent microglial cell activation. Using HCS we screened the Biomol drug library for compounds that were able to inactivate microglia. Our screen outcome involved looking for reductions in microglial conversion to activated phagocytes (ED1) and their ability to reduce pro-inflammatory cytokines (COX2) in a simultaneous evaluation procedure using immunocytochemistry. Drugs that reduced activation more than 60% of controls (decreased ED1 and Cox-2 staining, more so than aspirin) were identified as positive 'hits'. A total of 58 drugs were identified.

Complete protocols for delivery of gene/siRNA plasmids to microglial cells. In addition to pharmacological therapeutics we also aimed to screen for putative molecular therapeutics. We were able to show that we could transduce activated microglia with high efficiency (>95%) using a pmaxGFP expression plasmid. Such techniques could also be used to transfect siRNA plasmids. We demonstrated with positive control tests using a tnf-alpha siRNA plasmid that we could knockdown tnf-alpha mRNA and produce a phenotypic response in activated microglia that is characteristic of inactivation (as shown previously in Pearse et al., 2004, Eur J Neurosci) and as measured using the Cellomics device.

Re-confirm the efficacy of positive hit compounds on CNS injury-activated microglia. Using activated, primary microglia extracted at 3 days after SCI from the injury site, we were able to demonstrate that 56 of the 58 compounds were not toxic to microglia (did not induce cell death across a concentration range) and were able to inactivate the cells.

Test a HCS identified positive hit compound for anatomical and functional efficacy in a model of CNS injury, a contusive SCI. From our 56 'hit' compounds, we identified a canabanoid receptor agonist that was then taken forward to evaluate its therapeutic efficacy in a SCI model, its ability to revert microglial activation as well as improve histological and functional outcome will be assessed. Although we did not see an alteration in microglial cell activation, significant changes in cytoprotection were observed. Further testing of the 'hit' compounds is planned.

(iii) Key Research Accomplishments.

1. The development of an in vitro model system for investigating microglial cell activation.
 - a. The successful culture of microglial cells in 96-well plate format
 - b. The formulation of techniques for high efficiency transduction of microglial cells with plasmids encoding genes or siRNA.
 - c. The development of an assay system [with positive and negative controls] for drug candidate evaluation using HCS.
2. The application of HCS to the model system to screen drug and gene libraries for putative therapies for microglial cell inactivation.
3. The identification of 56 novel compounds (from a >400 compound drug library) using HCS that are able to prevent microglial cell activation.
4. The in vivo testing of one of these agents for therapeutic efficacy in an in vivo, clinically-relevant rodent model of SCI.

(iv) Reportable Outcomes.

1. Puzis, L., Odashima, K. and Pearce, D.D., Gene delivery strategies for targeting microglia cells, Immunology 2007: The Meeting of the American Association of Immunologists, 2007.
2. Puzis, L., García-Castillo, D., Tuesta, L.M., Iorgulescu, J.B., Pearce, D.D., The use of a high content screening approach to identify pharmacological agents capable of reversing microglial cell activation, 9th International Congress on Cell Biology, 2008.
3. Puzis, L., Garcia-Castillo, D., Tuesta, L.M. and Pearce, D.D., Identification of novel compounds that reverse microglial cell activation through use of a high content screening approach, International Society for Cellular Therapy, 2008.
4. Puzis, L., García-Castillo, D., Tuesta, L.M., Shi Y, Iorgulescu, J.B., Bixby J, Lemmon V, Pearce, D.D., The use of a high content screening approach on small molecule drug libraries to identify agents capable of reversing microglial cell activation. In preparation, 2009.

(v) Conclusions. We have been able to successfully develop a cell culture model system to perform HCS of both drug and gene/siRNA libraries so as to identify putative therapeutic agents capable of inactivating microglial cells. In almost all neurodegenerative diseases and after CNS injury, microglial cell activation plays a central role in pathological progression. Furthermore, we have developed an approach by which HCS 'hit' compounds can be rapidly confirmed in primary cells and tested for therapeutic efficacy in a CNS injury model. Both the HCS model and the approach can be used to screen the ever-growing number of chemical and genetic libraries for novel therapeutic agents and by using our developed pre-clinical translation approach, move these agents towards use in clinical trials for not only CNS injury, but for a myriad of neurodegenerative diseases.

(vi) References.

- Bartholdi, D. & Schwab, M.E. (1997) Expression of pro-inflammatory cytokine and chemokine mRNA upon experimental spinal cord injury in mouse: an in situ hybridization study. *Eur. J. Neurosci.*, 9, 1422–1438.
- Bethea JR, Dietrich WD. Targeting the host inflammatory response in traumatic spinal cord injury. *Curr Opin Neurol.* 2002 Jun;15(3):355-60.
- Cross, A.K. & Woodroffe, M.N. (1999) Chemokine modulation of matrix metalloproteinase and TIMP production in adult rat brain microglia and a human microglial cell line in vitro. *Glia*, 28, 183–189.
- Flavin, M.P. & Zhao, G. (2001) Tissue plasminogen activator protects hippocampal neurons from oxygen-glucose deprivation injury. *J. Neurosci. Res.*, 63, 388–394.
- Kingham, P.J. & Pocock, J.M. (2001) Microglial secreted cathepsin B induces neuronal apoptosis. *J. Neurochem.*, 76, 1475–1484.
- Pearse DD, Chatzipanteli K, Marcillo AE, Bunge MB, Dietrich WD. Comparison of iNOS inhibition by antisense and pharmacological inhibitors after spinal cord injury. *J Neuropathol Exp Neurol.* 2003 Nov;62(11):1096-107.
- Pearse DD, Pereira FC, Stolyarova A, Barakat DJ, Bunge MB. Inhibition of tumour necrosis factor- α by antisense targeting produces immunophenotypical and morphological changes in injury-activated microglia and macrophages. *Eur J Neurosci.* 2004 Dec;20(12):3387-96.
- Popovich PG, Guan Z, McGaughy V, Fisher L, Hickey WF, Basso DM. The neuropathological and behavioral consequences of intraspinal microglial/macrophage activation. *J Neuropathol Exp Neurol.* 2002 Jul;61(7):623-33.
- Schubert, P., Ogata, T., Miyazaki, H., Marchini, C., Ferroni, S. & Rudolphi, K. (1998) Pathological immuno-reactions of glial cells in Alzheimer's disease and possible sites of interference. *J. Neural Transm. Suppl.*, 54, 167–174.

List of Personnel.

Dr. Damien D. Pearse PI
Dr. Leopold Puzis
Dr. Amanpreet Singh

Specific Aim 4. (P. Tsoulfas M.D)

i. Introduction:

Multipotential neural precursor cells can generate both neurons and glia in adult mammals. Neural precursors can be mobilized and differentiate, in diverse physiological and pathological conditions, into distinct neural lineages (1). In spinal cord after injury neural precursors seem to contribute to the formation of the glial scar (2), which forms a barrier for regenerating axons. Mobilizing and directing these cells to undergo neuronal or oligodendrocytic maturation, instead of astrocytic differentiation, might provide a more favorable microenvironment for regenerating axons. We proposed to block the differentiation of these endogenous precursors towards the astrocytic lineage by using inhibitors of serine/threonine and tyrosine kinases (3). These proteins are known to be at the intersection of most cellular processes like proliferation, transcription, translation, cell migration and development. We focused our investigation on neural precursors using in vitro assays that mimic the developmental processes of astrocytic differentiation.

ii. Body:

Milestones:

- A. Use of kinase inhibitors to block the astrocytic or oligodendrocytic differentiation of fetal neural precursors that have received instructive signals.
- B. Use of candidate molecules on neurons to ensure that will not affect neurite growth.

A. We completed the screening with the kinase inhibitors. We used 66 selective inhibitors for the differentiation of neural progenitor assays. For the inhibition of astrocytic and oligodendrocytic differentiation, the cells were exposed to small molecular weight molecules at 2 different concentrations in the presence of cardiotrophin-1 (CT-1) or triiodothyronine (T3), respectively. Positive hits were screened further using a larger scale of dilutions. We did identify two inhibitors that block the differentiation of neural precursors towards the astrocytic lineage (see below and figure 5 for further details). At the beginning we proposed to use 96 well dishes to be able to screen with a High Content Screening Microscope. This approach had many difficulties due to edge effects and cellular growth that was random instead being clonally expanded. For this reason we did our assays in individual dishes. We devoted considerable amount of our time to this approach because was labor intensive and we could not meet our initial estimate of using at least 400 different inhibitors. In the future growing these precursors as neurospheres might provide a better way of screening large numbers of small molecules. A large number of inhibitors had various biological effects on the neural precursor cells (Figure 1, 2, 3 and 4) For example. (Hypericin, Sphingosine, and 5 iodotubercidin) had toxic effects or had a direct influence on cellular survival pathways, resulting in cell death (Figure 1 and 2). Hence, we were unable to assess the differentiation of these inhibitors along different lineages. Roscovitine, a known cell cycle inhibitor reduced the proliferation on cells treated with CT-1 without affecting the expression of the cell lineage marker GFAP (Figure 1).

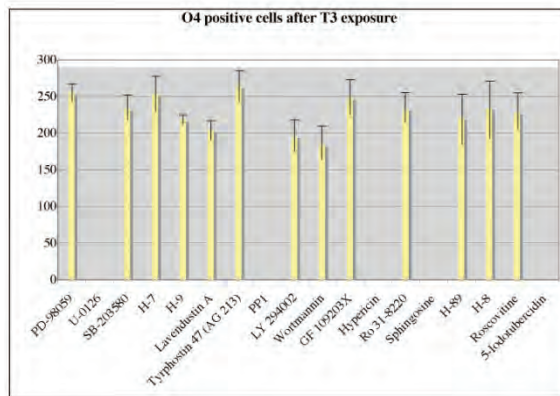


Figure 1: Kinase inhibitors screen for oligodendrocytic differentiation of neural precursors in the presence of T3. Neural precursors from rat fetal brain were grown semi-clonal for few days in the presence of FGF2 and 2-3 days later switched to T3 the presence of inhibitors at a concentration of 10 micromolar and let grow for 6 days. Cells were stained with an antibody that recognizes an early antigen (O4) of oligodendrocytes. Cells counts for each inhibitor are indicated in

the Yaxis.

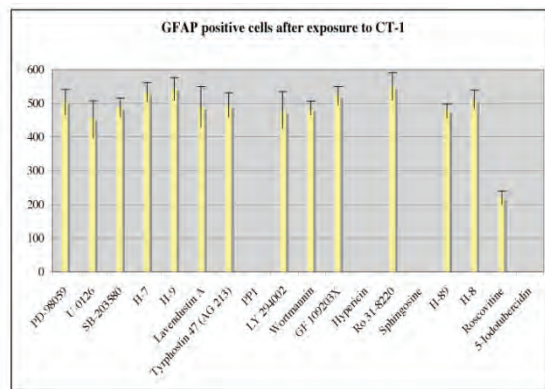


Figure 2: Kinase inhibitors screen for astrocytic differentiation of neural precursors in the presence of CT-1. Neural precursors from rat fetal brain were grown semi-clonal for few days in the presence of FGF2 and 2-3 days later switched to CT-1 in the presence of inhibitors at a concentration of 10 micromolar and let grow for 6 days. Cells were stained with an antibody that recognizes the astrocytic cell lineage marker GFAP. Cells counts for each inhibitor are indicated in the Yaxis.

Other compounds like the Y27632 an inhibitor for Rho-kinase (ROCK), has a direct effect on the morphology of the cells without affecting their differentiation as assessed by cell lineage markers (Figure 3).

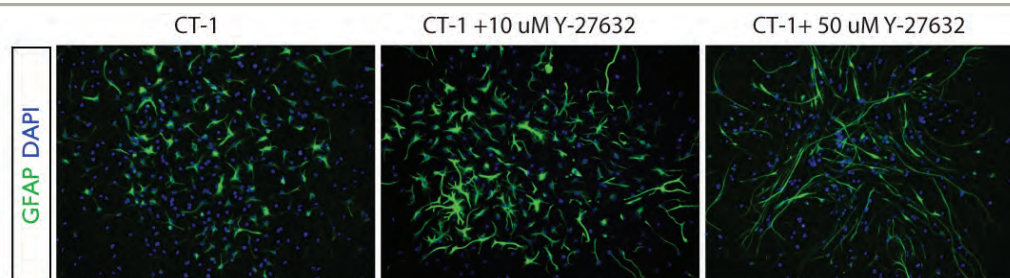


Figure 3: A Rho-Kinase (ROCK) inhibitor alters the morphology of cells that express the astrocytic lineage marker GFAP: The presence of Y27632 changes the morphology of cells exposed to CT-1. Cells were stained with an antibody that recognizes GFAP (green) and the nuclei were revealed with DAPI (blue)

The inhibitor for the MEK/MAPK pathway U2106 blocks the differentiation towards the oligodendrocytic lineage and enhances the expression of GFAP. This is specific to the T3 treatment and not with CT-1 (Figure 1 and 2). This phenomenon is interesting and

could provide important clues about the role of T3 in the generation and maturation of oligodendrocytes during development and after birth. However, this compound cannot be used in our models of spinal cord injury because blocks the differentiation of precursor cells towards the oligodendrocytic lineage and therefore might impede myelination after spinal cord injury.

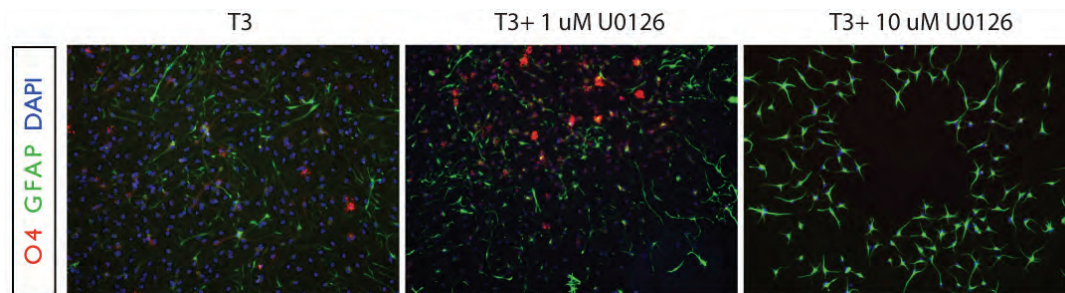


Figure 4: The MEK kinase inhibitor U0126 suppress oligodendrocytic differentiation and changes the morphology of cells that express GFAP: The continuous presence of U0126 in cells treated with T3 inhibit the cell differentiation towards the oligodendrocytic lineage and change the morphology of cells exposed to CT-1. Cells were stained with an antibody that recognizes O4 in red GFAP in green and the nuclei were revealed with DAPI (blue)

Out of the 66 compounds we identified the myosin light chain inhibitors ML-7 and ML-9 as the lead candidate to block the differentiation of neural precursors towards the astrocytic lineage (Figure 5). The results with these inhibitors have been consistent thru multiple experiments and they work at low concentrations.

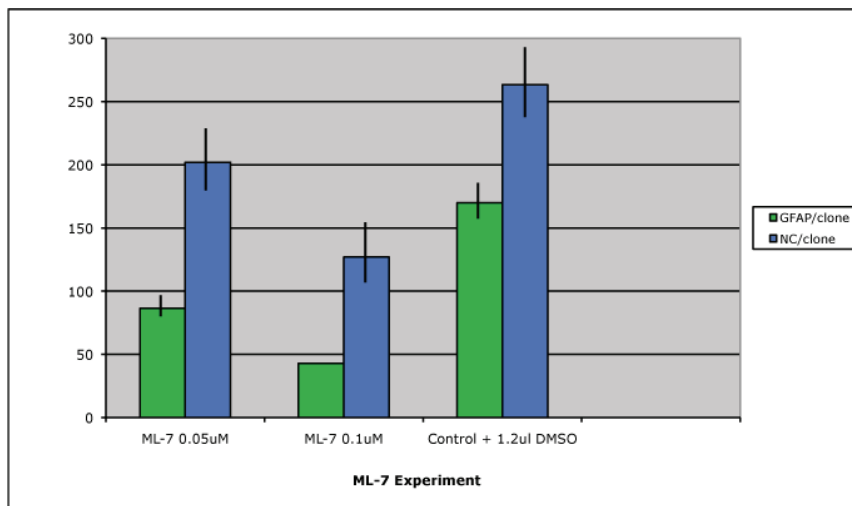


Figure 5: Myosin light chain inhibitor ML-7 inhibits astrocytic differentiation. Neural presursors from rat fetal brain were grown semi-clonal for few days in the presence of FGF2 and 2-3 days later switched to CT-1 in the presence of ML-7 at a concentration of 0.05 and 0.1 micromolar and let grow for 6 days before fixing and staining. Control samples were treated only with DMSO. Cells were stained with an antibody (GFAP) that recognizes astrocytes. In blue is the number of all the cells counted and in green are the cells positive to GFAP.

B. Having ML-7 and 9 as the lead candidates from our screen was essential to show that these compounds did not influence neural morphology and in particular did not affect neurite outgrowth. For this reason we performed a second assay to see whether the neurite outgrowth of CNS neurons was affected. This is important because we want to use some of these molecules in a spinal cord injury model to modify the glial scar and promote the regeneration of supraspinal neurons. If these molecules affect the neurite growth then might have negative effects on the regeneration of axons. We tested these compounds in vitro using hippocampal neurons. Was surprising to find that the MLCK inhibitors had small positive influence on the neurite outgrowth of the Hippocampal neurons. Collectively these results make these small molecules excellent candidates to test in as spinal cord injury model.

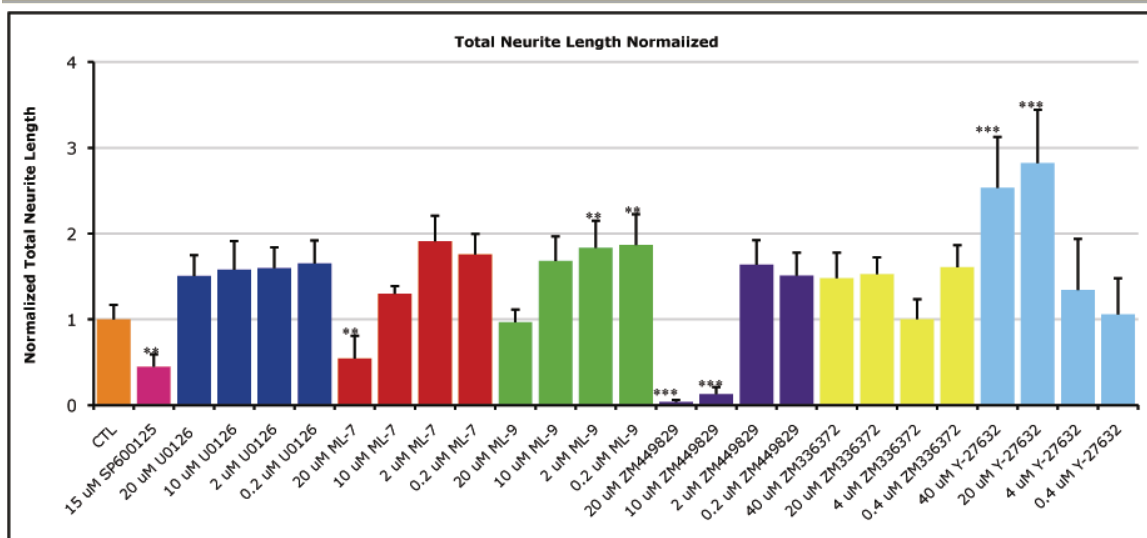


Figure 6. **Measuring total neurite growth in hippocampal neurons with different hit compounds.** Hippocampal neurons at embryonic age 17 were exposed to various concentrations of hit compounds. Three days later were fixed, stained and counted. The results show that the MLCK inhibitors (ML-7 and ML-9) and rhoA inhibitor (Y27632) increase the total neurite length at specific concentrations. Control samples were DMSO (CTL) and an inhibitor of neurite outgrowth SP00265 an inhibitor of jnk kinases. U0126 is a selective MEK inhibitor and ZM449829 and ZM336372 are selective raf inhibitors

iii. Key Research Achievements

- 1) Functional testing of 66 unique genes in neural precursors with phenotypic analysis of over 100,000 of neural precursors.
- 2) Development of the assay and conditions for testing small molecules in vitro using neural precursor cells

- 3) Screening in primary CNS neurons with the positive hits to identify inhibitors for testing in spinal cord injury models.
- 4) Identification of several inhibitors that influence the differentiation of neural precursors along the astrocytic and oligodendrocytic lineages
- 5) Identification of a lead compound for testing in spinal cord injury.

iv. Reportable Outcomes

Invited Presentations related to this project:

2007

Symposium on ALS and stem cell therapies (Miami)

Tokyo University (Dept of Biology), Japan

Keynote speaker at the Japanese Society for Transplantation and Tissue Engineering in Musculoskeletal System. Hamamatsu (Japan) October 2007.

Dental and Medical school (Tokyo), Japan

2008

Cajal Institute (Madrid, Spain), October 9, 2008

Abstracts related to this project:

J. Darr, M. Arenas-Valdez, Y. Wang, T. Okamoto, P. Tsoulfas "Mitogenic Factors Maintain Proliferation of Neural Progenitors Through the Regulation of Cks1b Transcription" Society for Cell Biology San Francisco CA, 2008

Manuscripts in preparation:

Arenas-Valdez, Y. Wang, P. Tsoulfas. Identifying novel pathways for neural stem cell differentiation.

v. Conclusions:

These experiments allowed us to identify new signaling pathways that influence the differentiation of neural precursors. The involvement of some of these pathways were previously unknown to this field. This will enable us to further dissect the involvement of these pathways to cell biological processes. However, the most important finding from

these experiments is that we discovered an inhibitor that blocks neural stem cell differentiation towards the astrocytic lineage and promotes the neurite outgrowth of CNS neurons. Therefore we are at the point where we will be able to use this small molecule in an animal model of spinal cord injury.

vi. References:

- 1) Goldman S.A. Directed mobilization of endogenous neural progenitor cells: the intersection of stem cell biology and gene therapy. *Curr Opin Mol Ther.* 2004: 65:466-472.
- 2) Logan A. and Berry M. Cellular and molecular determinants of glial scar formation. *Adv Exp Med Biol.* 2002:513:115-158.
- 3) Johnson S.A, and Hunter T. Kinomics: methods for deciphering the kinome. *Nat Methods.* 2005 2:17-25.

Specific Aim 5. (V. Lemmon, PhD & J. Bixby, PhD)

i. Introduction:

It is well established that CNS axons have difficulty growing through injured regions of the CNS and this is a major barrier to regeneration after stroke, Traumatic Brain Injury (TBI) or Spinal Cord Injury (SCI). Two major factors are thought to account for this. The first is that the injured region contains components, such as myelin and chondroitin sulfate proteoglycans, that inhibit regeneration. The second is that mature neurons, through changes in gene expression, no longer express proteins needed to extend regenerating axons and make connections with appropriate targets. We proposed to alter the expression of major classes of proteins known to regulate intracellular pathways that influence gene expression and cytoskeletal reorganization. These are the kinases and phosphatases and their adaptor molecules. We proposed to do this in Central Nervous System (CNS) neurons, such as hippocampal neurons, using overexpression and RNA silencing

ii. Body:

Milestones:

- A. Use kinase and phosphatase overexpression on axon growth in hippocampal neurons
- B. Use shRNAs to knock down kinases and phosphatases; study effects on axon growth in hippocampal neurons
- C. Use bioinformatics approaches to analyze data from A and B, creating cluster maps of genes that give similar phenotypes in neuronal differentiation and survival.

A. We completed a large overexpression screen of kinases and phosphatases in hippocampal neurons. We screened over 800 different cDNAs from our library, which represented 492 unique genes. Of these 45% were protein kinases, 20% were other kinases (lipids, nucleotides, etc), 12% were protein phosphatases, 14% were other phosphatases and 9% were proteins that interact with kinases and phosphatases, such as adaptors. The screening was done with a High Content Screening Microscope. Each experiment would test 84 genes. Measurements would be made from about 75-400 neurons per gene, totaling 165,000 transfected neurons. Over 40 parameters concerning shape, length, branching and intensity from different cellular compartments were made for each cell. In the screen genes were tested at least twice. This very large data set allowed us to identify hits for subsequent rescreening and analysis. It also allowed us to undertake a bioinformatic analysis to look for gene families and signaling pathways that are critical in neurite growth.

B. RNAi approaches in neurons are challenging. Although a number of papers have reported knockdown in neurons, the readouts have typically involved measurements of changes in mRNA levels and not protein. We use the more rigorous criterion of knock-down of protein since it is the loss of protein that is required before there can be a

phenotypic change in the test cells. We have tested a number of methods and targets in cell lines and primary neurons and found that, while it is relatively easy to knock down proteins in cell lines, it is much more difficult in neurons. Our observations have been confirmed in a dramatic way by the Yokota laboratory (FEBS Letters (2009) 583:213). They made transgenic animals expressing shRNAs to different targets. While complete knockdown is observed in virtually all tissues, it has not been observed for the same targets in the CNS. We completed a mini-screen using shRNAs to targets in the JNK pathway and identified targets important in axon growth. A manuscript describing this work is in preparation.

C. Significant work was devoted to developing methods and tools for normalizing data across wells, plates and experiments done on different days. Part of this effort involved established the optimal controls for interpreting the screen and building tools for assessing the statistical significance of the results. Perhaps the most effort in this project has been devoted to the development of methods for mapping the results of the phenotypic screen onto other data sets, such as gene families and signaling pathways.

To fully establish controls used in normalization we evaluated several candidate controls to use as normalization standards, and concluded we should use empty vector (a control for DNA introduction) and vector encoding a neutral protein, red fluorescent protein (a control for CMV based overexpression of a protein). We also used as positive and negative controls two transcription factors that we discovered in another screen that have potent effects in terms of promoting or inhibiting axon growth. When these two controls behaved as expected we were assured the overall assay was performing as expected. Normalization was done by exporting data assembled by Spotfire Decision Site software into a program written in "R". Statistical significance was evaluated using a t-test in R, followed by a correction for multiple comparisons (Benjamini & Hochberg, 1995). Data was then reimported into Spotfire for visualization and integration with sequence information and various ontologies about molecular interactions and pathways.

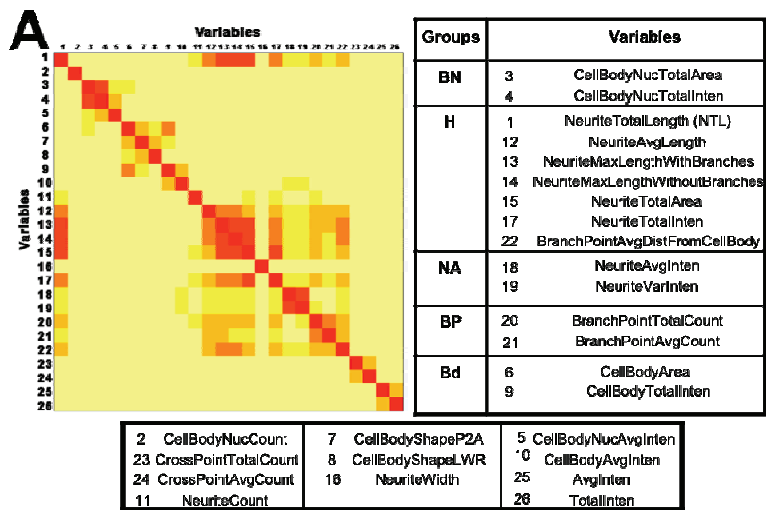


Figure 1: Morphological Parameters Reduced Corelated A) Clusters of correlated parameters were established based on the correlation coefficient between pairs. Two variables with a correlation coefficient higher than 0.8 were put into the same cluster.

An interesting and important observation comes from overexpressing different genes and then correlating the results from different parameters (Figure 1). Many different measurements of neurite extension, such as total neurite length or length of longest neurite, were highly correlated. However, these two did not correlate with neurite branching or neurite number (the number of neurites emerging from the soma). This suggests that the molecular processes that control axon and dendrite extension are different than those that determine axon/dendrite initiation and also branching.

Hierarchical clustering revealed clusters of genes that produced similar phenotypes (Figure 2). Examples include cells with a single long neurite (left inset), multiple short neurites and no long neurite (presumed axon, center inset), or no neurites (right inset). This analysis extends the information from the correlation analysis and provides information about groups of genes that control particular aspects of neuronal differentiation.

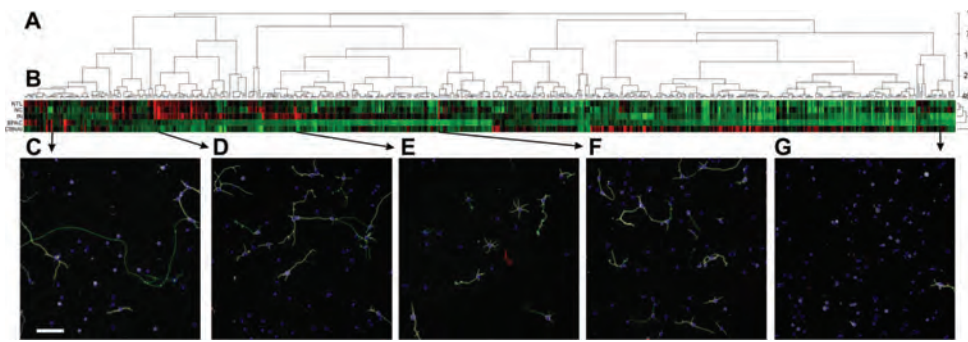


Figure 2: Kinase and Phosphatase Overexpression in Hippocampal Neurons.

Hippocampal neurons growing on laminin for 48 hours usually produce one axon, 4-5 dendrites, and have total neurite lengths around $>200\ \mu\text{m}$. Overexpression of many genes coding for kinases or phosphatases was able to significantly perturb neuronal morphology. A Hierarchical cluster (maximum likelihood, correlation) was done on 468 genes (homologs averaged) with the parameters neurite total length, neurite count, % with neurites, branch point average count, and nuclear intensity. The dendrogram (A) and heat map (B) from the clustering show the wide range of results (red = increase from control, green = decrease). C-G, representative images from the screen. Arrows under heatmap indicate region where the image was sampled. C) Neurons with few, long neurites. D) Neurons with a high number of primary neurites, average branching, and relatively long neurites E) Neurons with many short primary neurites. F) Neurons with low branching but longer neurite lengths. G) Inhibited neurite growth. Scale bar $100\ \mu\text{m}$.

An alternative way of examining the data is to map quantitative information about different phenotypic parameters onto dendrograms of the genes based on protein sequence. This approach allows identification of gene families that regulate different aspects of neuronal differentiation. First the sequences of all the proteins tested were assembled and dendrograms generated. For kinases, only the kinase domain was used for the analysis. For other proteins the complete sequence was used. A Spotfire plug-in was made that allowed easy mapping of the phenotypic data onto the dendrograms (an example of the method is shown in Figure 3).

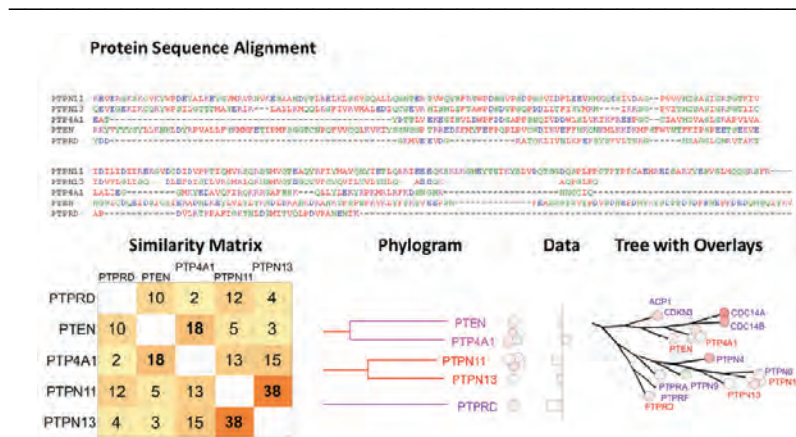


Figure 3: Construction of Phylogenetic Tree with Functional Overlays.

Peptide sequences from the library were obtained based on their accession #s, and then aligned with ClustalW2. A) Example of 5 phosphatase genes, with alignment results. B) During alignment, a similarity matrix is

produced, the first of which is pictured, with the score for PTPN11 vs. PTPN13 the highest. This high score causes the nodes to be joined. A new group is analyzed next, where there are only four nodes, PTPRD, PTEN, PTP4A1, and the combination of PTPN11+13. In this similarity matrix, PTEN and PTP4A1 have the highest score, cause them to be joined. This is repeated and a phylogram is produced. Then, for a particular parameter, we overlay our functional data (normalized and averaged) as colored markers onto the final radial tree.

An example of mapping data about neurite branching onto the dendrograms of gene families is shown in Figure 4. Among the protein kinases it is possible to identify families that promote branching (green) and inhibit branching (red).



Figure 4: Families of Kinases and Phosphatases Perturb Neurite Branching of Hippocampal Neurons on Laminin. The cDNAs screened were aligned by amino acid sequence using ClustalW2, resulting in the identification of families of proteins. The branch lengths in the trees represent the evolutionary distance between genes. Protein kinases were

further subdivided into 7 families (based on kinase domain sequences). Circular markers were overlaid atop the trees, with the color of the marker indicating either no change in neurite length from control (white), an increase (green) or decrease (red). Positive and negative controls are known regulators of neurite growth, which were included in the screen.

To gain additional insight into the data the genes can be clustered using information from Gene Ontology (GO) annotations regarding molecular function, etc (Figure 5). The statistical significance of the various clusters can be tested using "bootstrap" approaches.

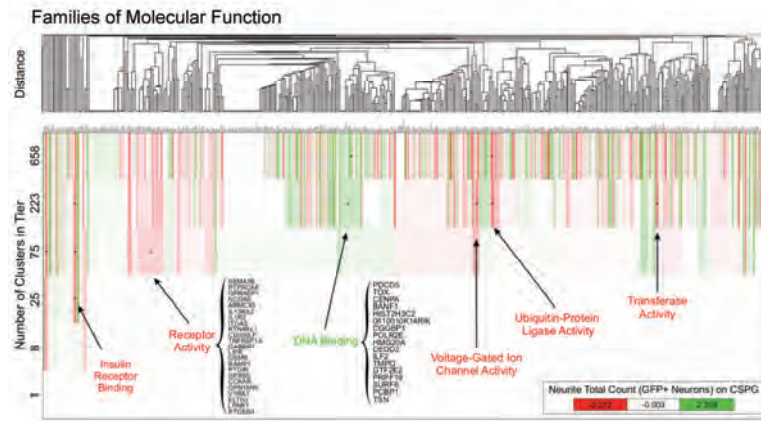


Figure 5: Various Molecular Function Gene Ontologies Correlate with Neurite Growth on CSPG.

At the root of the tree (bottom tier of heatmap, "1"), all the genes are in one large cluster, and their average phenotypic change from control is zero, so they are colored in white. Moving towards the top, families of genes are represented by a rectangle,

colored by relative change from control in neurite count (red = decrease, green = increase, legend on bottom right). Asterisks indicate significance (by bootstrap sampling). Several significant ontologies are marked with their member genes listed. The marked ontology is not the only ontology in the family, but is the most prevalent or defining term.

A second clustering approach that may be more likely to yield critical information about genes that regulate regeneration is to cluster by pathway ontologies instead of sequence homologies. We used pathway information from the Kyoto Encyclopedia of Genes and Genomes (KEGG). A pathway that was particularly inhibitory to neurite growth was the pathway that regulates the formation of tight junctions (Figure 6).

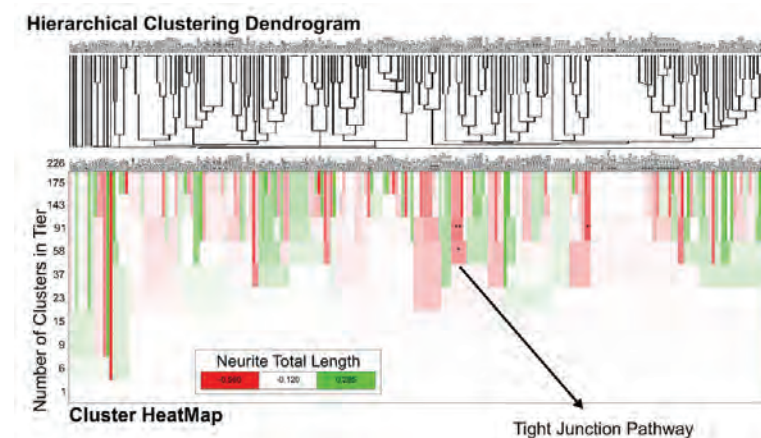


Figure 6. Pathway Annotations for 225 Screened Genes.

In the bottom row (1), all genes are in 1 large cluster. Moving up the tree, clusters are represented by rectangles, colored by relative change from control (red= decrease; green=increase). Each row is labeled with the number of clusters in that tier. Asterisks, significant differences. Four genes

implicated in the tight junction pathway formed a group that significantly decrease neurite outgrowth of hippocampal neurons.

iii. Key Research Achievements

- 1) Functional testing of 491 unique genes in primary hippocampal neurons with phenotypic analysis of over 150,000 neurons
- 2) Development of techniques for normalization of multiple parameters describing neuronal differentiation across plates and across experiments. Development of statistical methods to analyze normalized data to determine which genes significantly alter differentiation.
- 3) Development of methods to compile dendrograms of genes bases on sequence.
- 4) Development of methods to cluster phenotypic data with different ontologies, such as gene ontologies describing molecular function or pathway information.
- 5) The identification of specific genes that enhance or inhibit neurite growth that were not previously associated with neuronal differentiation.
- 6) The identification of molecular processes and pathways that regulate different aspects of neuronal differentiation, such as neurite extension, neurite branching and neurite formation.

iv. Reportable Outcomes

2004 through 2009

Manuscripts:

1. Buchser WJ, Pardinas JR, Shi Y, Bixby JL, Lemmon VP. 96-well electroporation method for transfection of mammalian central neurons. *Biotechniques*. (2006) 41:619-24.
2. Smith RP, Buchser WJ, Lemmon MB, Pardinas J, Bixby JL, Lemmon VP. EST Express: PHP/MySQL based automated annotation of ESTs from expression libraries. *BMC Bioinformatics* (2008) 10:186

Manuscripts in preparation:

1. WJ Buchser, T Slepak, O Gutierrez-Arenas, JL Bixby, VP Lemmon. Perturbation of Hippocampal Neurite Outgrowth after Overexpression of Kinase and Phosphatase Genes.
2. WJ Buchser, T Slepak, O Gutierrez-Arenas, JL Bixby, VP Lemmon. A System's Biology Approach to Discovering Significant Families of Genes which Effect Neuronal Morphology.

3. AA Oliva, Atkins CM, JL Bixby, V Lemmon. Regulation of axon formation by the JNK pathway
4. AA Oliva, Atkins CM, JL Bixby, V Lemmon. Axon-specific roles of JNK1, JNK2, JIP1 and JIP3

Abstracts:

1. John L. Bixby, Tania Slepak, William Buchser, Omar Gutierrez-Arenas, and Vance Lemmon. A phenotypic screen identifies kinases and phosphatases regulating neurite outgrowth from primary neurons. Omics Meets Cell Biology, Keystone, CO, 2009.
2. J Bixby and V. Lemmon. HCA of primary neurons to identify kinases and phosphatases regulating neurite outgrowth. Cambridge Healthtech Institute, High Content Analysis, San Francisco, CA 2009
3. V. Lemmon; W. Buchser; Y. Shi; T. Slepak; O. Gutierrez Arenas; J. Bixby. Validation and Visualization of Data from Phenotypic Screens of Primary Neurons. HUGO High content Screening Workshop. Singapore, 2008.
4. V. Lemmon. Integrating a LIMS for High Content Screening into an Academic Lab. Thermo Fisher Bioinformatics World, Las Vegas, NV, 2008.
5. Buchser W, Shi Y, Gonzalez D, Blackmore M, Slepak T, Bixby J, Lemmon VP. From High Content Screening to Scoring Hits: Standard Reporting for Screening Primary Neurons. Cambridge Healthtech Institute, High Content Analysis, San Francisco, CA 2008. Award for Outstanding Poster.
6. Buchser W, Smith RP, Pardinas JR, Bixby JL, Lemmon VP. Phenotypic Screening of cDNAs to Discover Regeneration Associated Genes in Mammalian Neurons. Reeve-Irvine Symposia and Roman Reed Research Meeting. University of California Irvine. 2007
7. Buchser W, Smith R, Pardinas J, Bixby J, Lemmon VP. Phenotypic Screening of cDNAs to Discover Regeneration Associated Genes in Mammalian Neurons. Cambridge Healthtech Institute, High Content Analysis, San Francisco, CA 2007
8. Oliva AA, Jr., Atkins CM, Bhatti S, Salcedo N, Lemmon VP. Inhibition of axon formation by the c-Jun N-terminal kinase (JNK) pathway agonist, anisomycin. Soc for Neurosci, Atlanta, 2006
9. Lemmon VP. Hunting for Regeneration Associated Genes using Subtraction cDNA Libraries and High-Content-Screening, National Neurotrauma. St. Louis, MO, 2006
10. Lemmon VP, Buchser WJ, Pardinas JR, Smith RP, Tapanes-Castillo A, Li F, Shi Y, Bixby JL. Hunting for Regeneration Associated Genes using Subtraction cDNA libraries and High-Content-Screening. 21st Century COE International Symposium on Global. Renaissance by Green Energy Revolution, Nagaoka, Japan, 2006.
11. Buchser WJ, Shi Y, Vila C, Smith R, Pardinas J, Bixby J, Lemmon VP. High-throughput gene overexpression in primary neurons by plasmid electroporation. Cambridge Healthtech Institute, High Content Analysis, San Francisco, CA 2006.

Invited Presentations:

John Bixby

2004:

Florida Inst. of Technology
Sils Conference on Neuromuscular Synapse Formation (Switzerland)
University of Louisville

2005:

University of North Texas
University of Vermont (Keynote Speaker),
Amgen Inc.

2006:

Cambridge University (UK)
Sils Conference on Neuromuscular Synapse Formation (CH)
Max-Planck-Institut for Neurobiology (DE)
High Content Analysis Conference, San Francisco, CA
Pediatric Critical Care Scientist Development Program Retreat, Deer Valley, UT

2007:

Cornell University
Spelman College
Eli Lilly (Indianapolis)

2008:

University of Erlangen-Nuremberg
Ludwig-Maximilians-University (Munich)
Conference on Neuromuscular Synapse Formation (Guarda, CH)
UC Irvine
University of Puerto Rico
8th Intl. Conference on Protein Phosphatases, Maebashi, Japan

2009:

High Content Analysis Conference, San Francisco, CA

Vance Lemmon

2004:

Dept. of Neurosurgery, Osaka National Hospital, Osaka, Japan
Dept. of Cell Biology, Nara Institute of Science and Technology, Nara, Japan
Dept of Dynamic Pathology, Kyoto Prefectural Univ., Kyoto, Japan
Dept of Clinical Pharmacy and Pharmacology, Kagoshima Univ., Kagoshima, Japan
Division of Mol. and Cell. Biology, Univ of Nigata, Nigata, Japan
Dept. of BioEngineering, Nagaoka Institute of Technology, Nagaoka, Japan
Fishberg Research Center for Neurobiology, Mt Sinai School of Medicine, NY, NY
Dept. of Biomedical Sciences/VetMed, Iowa State University, Ames, IA

2005:

American College of Neuropsychopharmacology, Kona, Hawaii
Cell Biology, Sloan Kettering Research Institute, NY, NY

2006:

High Content Analysis Europe, Vienna, Austria
Symposium on New Approaches, National Neurotrauma Meeting, St Louis, MO
Dept. of Biological Sciences, Univ. of Delaware, Newark, DE
Dept. of BioEngineering, Nagaoka Inst. of Tech., Nagaoka, Japan
The 21st Century COE Symposium, Nagaoka Inst. of Tech., Nagaoka, Japan
Dept of Physiology, Keio University, Tokyo, Japan

2007:

Dept of Neuroscience, Medical Univ. of S.C. , Charleston, S.C.
Dept of Biochemistry, Univ. of Zurich, Zurich, Switzerland
Cancer Center, Univ. of Heidelberg, Heidelberg, Germany
Abbott, Mannheim, Germany
Novartis, Basel Switzerland
Dept of Physiology, Univ. of Conn. Storres, Conn

2008:

Human Genome Organization, HCS Workshop, Singapore
Thermo Informatics World, Las Vegas, Keynote Address
High Content Analysis, San Francisco, Ca, Two invited lectures

2009:

Cambridge Centre for Brain Repair Spring School, Cambridge, England
South East Nerve Net: Keynote Address. Jacksonville Fl
Dept. of Biology, University of North Florida, Jacksonville Fl

Funding Applied For:

The methodologies developed with the support of this DOD funding have been used in proposals submitted to the NIH, DOD and private agencies. Successful applications included:

NIH/CHHD HD057632
P.I. Vance Lemmon
Novel Gene Targets for CNS Axonal Regeneration
07-12 direct costs

C. Neilson Foundation
P.I. Vance Lemmon
Identification of Corticospinal Tract Regeneration Associated Genes
07-09 direct costs

C. Neilsen Foundation
The Role of NF-kappaB in Neuronal Growth and Regeneration
P.I.: John Bethea
Co-Investigator: Vance Lemmon
06-08 direct costs

Wilson Foundation
P.I. Vance Lemmon
Identification and Testing of Regeneration Associated Genes
07-08 direct costs

v. Conclusions

The research performed over the past five years has lead to three major accomplishments. The first is the development of the first 96 well based system for economical electroporation of primary neurons, enabling high content screening to be applied to genomic studies of nerve regeneration, plasticity and neuroprotection. The second was the development of robust analytical systems with underlying software to normalize and analyze HCS data of neurons. Of particular note was the ability to automatically annotate genes from a variety of databases. The third and most significant in the context of this 5 year funding period, was the identification of specific genes, gene families and signaling pathways that enhance or inhibit axon regeneration.

vi. References

Benjamini, Y. Hochberg, Y. Controlling the false discover rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society* (1995) 57:289-300.

Buchser WJ, Pardinas JR, Shi Y, Bixby JL, Lemmon VP. 96-well electroporation method for transfection of mammalian central neurons. *Biotechniques*. (2006) 41:619-24.

Smith RP, Buchser WJ, Lemmon MB, Pardinas J, Bixby JL, Lemmon VP. EST Express: PHP/MySQL based automated annotation of ESTs from expression libraries. *BMC Bioinformatics* (2008) 10:186

Hiroki Sasaguri, Tasuku Mitani, Masayuki Anzai, Takayuki Kubodera, Yuki Saito, Hiromi Yamada, Hidehiro Mizusawa, Takanori Yokota. Silencing efficiency differs among tissues and endogenous microRNA pathway is preserved in short hairpin RNA transgenic mice. *FEBS Letters* (2009) 583:213–218

vii. Appendices

Buchser WJ, Pardinas JR, Shi Y, Bixby JL, Lemmon VP. 96-well electroporation method for transfection of mammalian central neurons. *Biotechniques*. (2006) 41:619-24.

Smith RP, Buchser WJ, Lemmon MB, Pardinas J, Bixby JL, Lemmon VP. EST Express: PHP/MySQL based automated annotation of ESTs from expression libraries. *BMC Bioinformatics* (2008) 10:186

Software

Open Access

EST Express: PHP/MySQL based automated annotation of ESTs from expression libraries

Robin P Smith^{1,2}, William J Buchser^{1,2}, Marcus B Lemmon¹,
Jose R Pardinás^{1,5}, John L Bixby^{1,2,3} and Vance P Lemmon*^{1,2,4}

Address: ¹The Miami Project to Cure Paralysis, University of Miami Miller School of Medicine, Miami, USA, ²Neuroscience Program, University of Miami Miller School of Medicine, Miami, USA, ³Department of Pharmacology, University of Miami Miller School of Medicine, Miami, USA, ⁴Department of Neurological Surgery, University of Miami Miller School of Medicine, Miami, USA and ⁵Egea Biosciences, La Jolla, USA

Email: Robin P Smith - rsmith@med.miami.edu; William J Buchser - w.buchser@umiami.edu; Marcus B Lemmon - citron1984@gmail.com; Jose R Pardinás - jpardina@cntus.jnj.com; John L Bixby - jbixby@miami.edu; Vance P Lemmon* - vlemmon@miami.edu

* Corresponding author

Published: 10 April 2008

Received: 10 January 2008

BMC Bioinformatics 2008, 9:186 doi:10.1186/1471-2105-9-186

Accepted: 10 April 2008

This article is available from: <http://www.biomedcentral.com/1471-2105/9/186>

© 2008 Smith et al; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Background: Several biological techniques result in the acquisition of functional sets of cDNAs that must be sequenced and analyzed. The emergence of redundant databases such as UniGene and centralized annotation engines such as Entrez Gene has allowed the development of software that can analyze a great number of sequences in a matter of seconds.

Results: We have developed "EST Express", a suite of analytical tools that identify and annotate ESTs originating from specific mRNA populations. The software consists of a user-friendly GUI powered by PHP and MySQL that allows for online collaboration between researchers and continuity with UniGene, Entrez Gene and RefSeq. Two key features of the software include a novel, simplified Entrez Gene parser and tools to manage cDNA library sequencing projects. We have tested the software on a large data set (2,016 samples) produced by subtractive hybridization.

Conclusion: EST Express is an open-source, cross-platform web server application that imports sequences from cDNA libraries, such as those generated through subtractive hybridization or yeast two-hybrid screens. It then provides several layers of annotation based on Entrez Gene and RefSeq to allow the user to highlight useful genes and manage cDNA library projects.

Background

The growing trend towards high-throughput science has generated a wealth of sequence information. In many instances specific subsets of mRNAs are isolated with the goal of determining differences in expression between different populations of cells. Although microarrays have been used extensively to gauge relative expression levels, many applications such as subtractive hybridization and yeast two-hybrid libraries require that an mRNA transcript simply be present for inferences to be made. To assist in

the analysis of expressed sequence tags [1] and data from other types of sequencing projects, we have designed EST Express, a web-based software suite that accepts EST sequences and gene lists and performs analyses to ascertain the identity and function of genes expressed in a sample population.

Implementation

Software Design

EST Express uses PHP to generate dynamic HTML and Javascript. A MySQL database records sequence and analysis information in 13 relational tables. UniGene, Entrez Gene and RefSeq updates are downloaded from the NCBI FTP server through a PHP script and saved in a local folder or parsed. Several dependency modules are required for installation, including Crossmatch [2,3], NCBI's BLAST distribution [4], and the JPGraph PHP graphics library [5]. Although EST Express is designed to be run as a web server application, it can be used in standalone mode (i.e. with no connection to the internet) if a web server application is available. Setup requires the installation of two modules (BLAST and Cross_match) and the configuration of a centralized PHP settings file, but is relatively straightforward.

Data Analysis and Reports

Data Pipeline

EST Express accepts base calls and Phred scores in FASTA format, which it then parses and screens for user provided contaminating vector sequence using Crossmatch [2,3] (See Figure 1). Phred scores are then used to define a window within the sequence that is suitable for BLASTing. Sequences without high (>20) Phred scores are designated low sequence reads, and those with predominantly vector sequence are designated vector-only. The remaining sequences are then subjected to a similarity search against a local copy of the UniGene database using BLASTN. The top cluster from each BLAST result is stored and linked to the sample sequence. The "gene2unigene" conversion table produced by NCBI [6] is then used to link UniGene clusters with the Entrez Gene database for further annotation. To simplify the annotations of those identifiers that

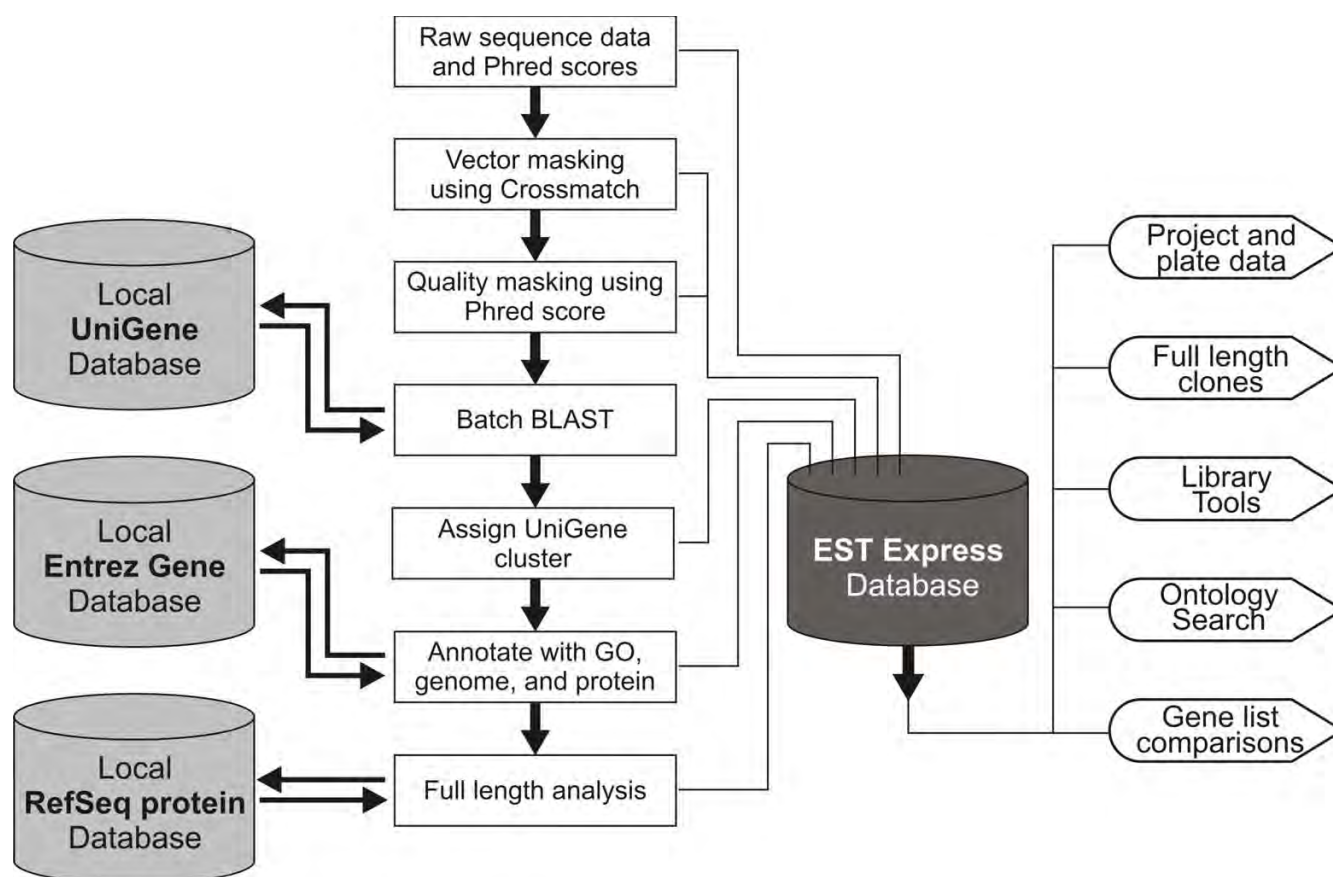


Figure 1

Data Pipeline. Raw sequence data is imported into EST Express along with phred scores, where it is then screened for contaminating vector sequences and masked for quality. Good quality sequences are then batch BLASTed against a local UniGene database and the top hit is assigned to each sample. A local copy of the Entrez Gene database is then linked to the UniGene identifier and used to annotate each sequence with a description, Gene Ontology identifiers, RefSeq mRNA and protein links, and genomic context. Oligo(dT)-primed sequences can then be analyzed for full-length status using a local copy of the RefSeq protein database and the Entrez Gene cross references. The user interface then provides several ways to browse and visualize the results from the pipeline.

have many-to-many relationships, EST Express builds a second table named "unigeneprefs" which selects the best Entrez Gene ID for each UniGene entry based on the relative degree of annotations (e.g. descriptive naming, mRNA link, protein link). Other analyses listed below are then performed on the combined data and linked back to the sample.

Data Representation

Sequences imported into EST Express are represented as "samples" (Figure 2a) and linked to different analyses through unique identifiers. Each sample is, in turn, part of a "plate", which encompasses all samples that were part of the original imported sequence file. Each plate then belongs to an overall "project" (Figure 2b), which possesses functional characteristics that make it distinct. This structure was adopted because of the nature of sequencing

projects – often 96 or 384 well plates are sequenced in succession as part of a larger project. Analyses such as batch BLAST can be performed on individual plates or on an entire project.

Sample Identification

Once samples have been loaded into a project, the underlying goal is to assign them a UniGene cluster and a resulting Entrez Gene ID, which provides access to the vast collection of annotations available through the Entrez Gene database. Because this requires that a UniGene cluster database be available, the EST Express framework is most relevant for projects involving model organisms (of which there were 74 at the time of writing). Sequences from non model organisms can also be identified provided they have sufficient sequence similarity with those of a model organism.

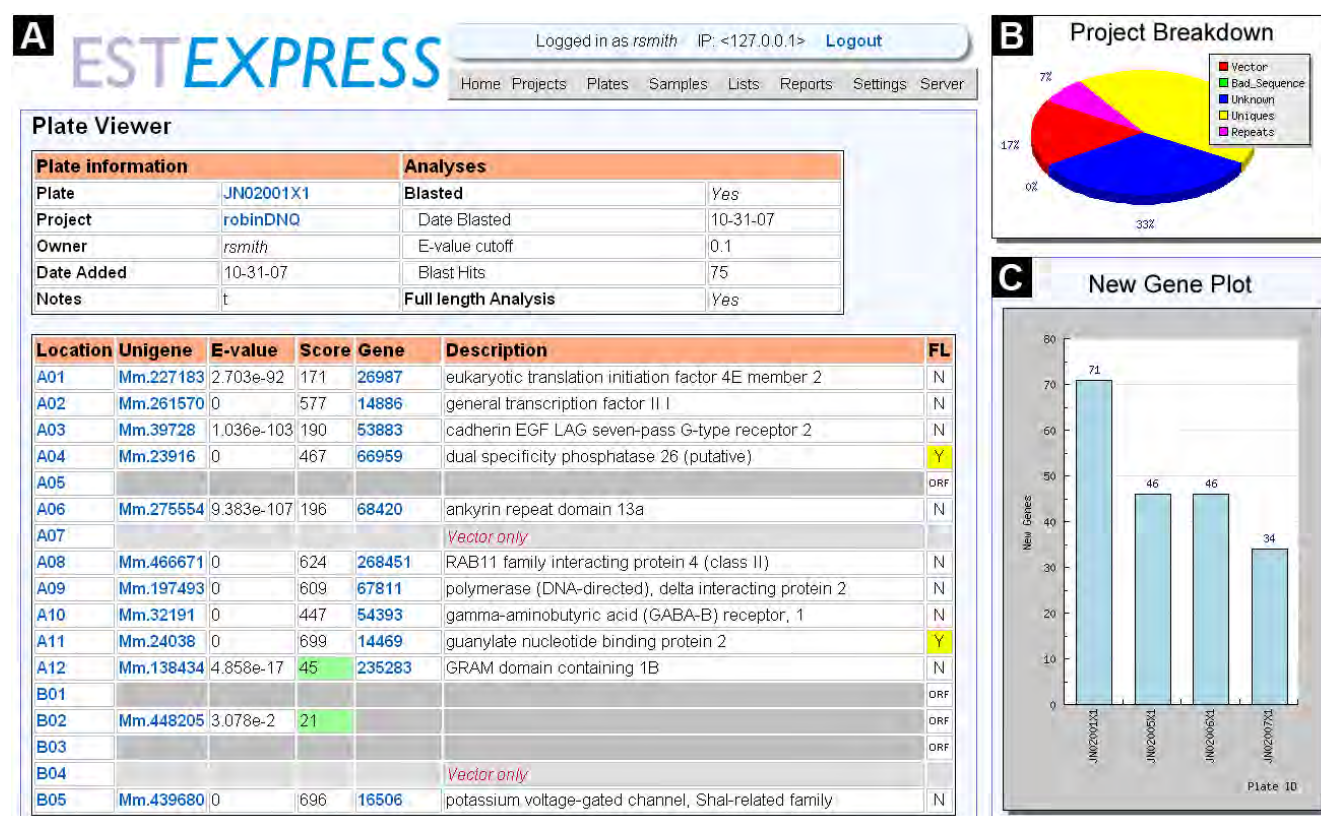


Figure 2

Screenshots from EST Express. A: Screenshot of the "Plate Viewer" page showing details for plate JN02001X1 owned by user "rsmith" in project "robinDNQ". For each matched sample in the plate a UniGene identifier is listed, along with the BLAST score and Entrez Gene and full-length annotations. B: Capture from the "Project Viewer" page showing a graphical breakdown of ESTs within a project. "Vector" refers to sequences designated vector-only. "Bad_sequence" refers to sequences with low quality sequence reads. "Unknown" refers to samples that are neither vector-only nor low quality, but do not match against the UniGene database. "Uniques" refers to the number of unique UniGene clusters in the project and "Repeats" refers to additional instances of those unique clusters. C: Capture from the "New Gene" library tool, showing the number of new unique UniGene clusters found with each successive round of sequencing. Further rounds of sequencing produce progressively fewer unique clusters. Both B and C were produced dynamically using the JGGraph PHP graphics library.

Entrez Gene Annotations

The Entrez Gene database [7] is a central depot for gene-specific information. EST Express makes full use of the annotations contained within, linking UniGene cluster IDs to Entrez Gene IDs. Because of the large size of the Entrez Gene database (>600MB for the Mus_musculus version alone) there is considerable interest in developing utilities that can parse the provided ASN.1 files into a useable format [8]. Many of the Entrez Gene annotations, however, can also be found in flat text files [6], which are much easier to parse. Four of these files (gene_info, gene2unigene, gene2go and gene2refseq) are downloaded by EST Express and combined into a single MySQL table within minutes. Users can then search annotations that match to samples using the search tool.

Full-length Analysis

In many cases it is desirable to know whether a library clone contains the full open reading frame for the gene in question. This allows for selected full-length clones to be re-arrayed and used in a variety of expression studies. EST Express can carry out such an analysis for Oligo(dT)-primed cDNAs that have sequence reads from the 5' end. Once a sample sequence has been identified, the corresponding RefSeq protein ID is extracted from the Entrez Gene table and matched against a locally downloaded copy of the RefSeq protein database. The EST is then translated into three different frames and matched against the first 10 amino acids of the protein sequence. Using this comparison, each annotated sequence is assigned "full-length" or "not full-length" status. Samples that are not annotated with a RefSeq protein identifier are examined for long open reading frames, the results of which are stored and can be queried for further analysis.

Library Tools

EST Express offers two tools that enable the user to assess the content of the source library being sequenced. The first tool generates a graph of the number of novel UniGene clusters found in each successive sequenced plate added to a project (Figure 2c). This feature is a useful indicator of library complexity as well as of how many sequences the user can expect to obtain. The second tool reports the number of times each UniGene cluster has been found within a project. This is a useful measure for subtracted libraries because cDNAs sampled more frequently correspond to transcripts that are enriched in the tester mRNA pool.

Gene Lists

Thus far, no individual technique provides complete information about the genes that are at work in a system. It is therefore often useful to compare lists of genes for commonalities or differences. EST Express allows the user to generate a list of sample IDs, UniGene clusters or Entrez

Gene IDs from a project or plate based on specific criteria. Lists of identifiers may also be uploaded as a text file originating from another experiment (e.g. microarray, mass spectrometry). Once a list is created it can be compared against one or more lists, the results of which can be saved as a new list. Each list can then be exported with full Entrez Gene annotations to an Excel spreadsheet for further analysis.

Results and Discussion

Evaluation with subtracted library sequences

EST Express has been successfully implemented and used to identify and annotate 4 separate libraries containing over 2,500 samples. Of these four libraries, the largest is the "subtracted" library generated through subtractive hybridization of tissue specific genes. For this library, 21 plates containing 2,016 samples were analyzed, resulting in 1,068 unique cDNAs (See Figure 3a). Of the 2,016 samples, 192 were vector-only sequences and 107 were low quality sequence reads. Of the 1,068 unique cDNAs, 914 matched Entrez Gene entries. Selection of appropriate Entrez Gene identifiers based on RefSeq links proved efficacious: only 23 sequences match Entrez Gene identifiers without a RefSeq link, allowing full-length analysis of 83% of samples returning a BLAST hit (Figure 3b). Of

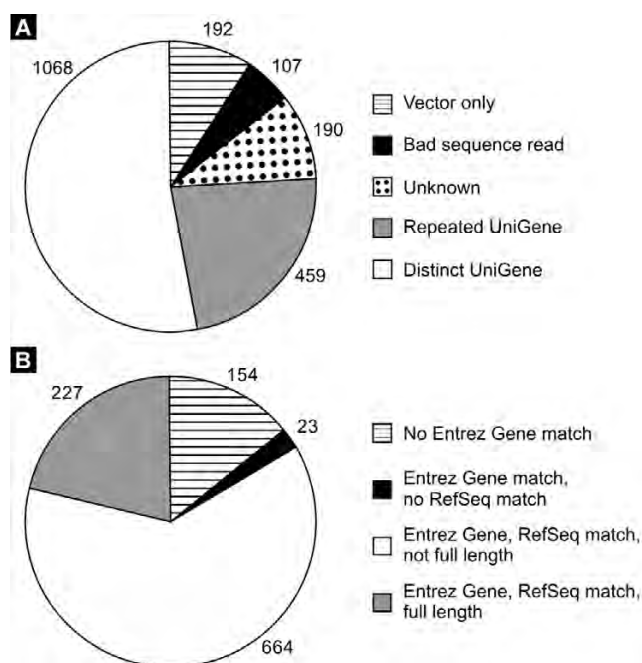


Figure 3
Results of analyses on the subtracted data set. A: Distribution of identifications made by EST Express for all 2,016 samples. B: Distribution of associations made for 1,068 distinct UniGene entries.

those samples that were analyzed, 227 were found to be full-length.

Comparison to related software packages

EST Express is similar in broad terms to other sequence pipeline software packages, including PipeOnline 2.0 [9], ESTAP [10], EST-PAGE [11] and ESTIMA [12]. However, there are several key differences that make EST Express an attractive alternative to the bioinformatics community.

EST Express is written entirely in PHP, an open source scripting language that is platform independent and extremely popular amongst web developers. All four of the packages listed above are Perl based and could not be installed on Windows based server without modifications. EST Express uses the MySQL database platform for storage of sequence data and analyses. MySQL is also open source and freely available under the GPL, contrasting with the commercial package Oracle, which is employed by ESTAP [10] and ESTIMA [11]. Unlike PipeOnline 2.0 [9], EST Express is also freely available for download and installation, and is distributed with explicit instructions for both Linux and Windows based machines.

The central difference between EST Express and these other packages is that it was designed for a post genome world in which researchers have the ability to generate specialized expression libraries and require a pipeline for identifying the mRNAs within. EST Express is unique in that it has a built-in support for identifying full-length cDNAs, diagnostic tools for gauging the complexity of the cDNA library, gene list tools for comparisons with microarray data and convergence of annotations through the use of the relatively recent Entrez Gene database [7].

Potential applications

Although EST Express was primarily developed to analyze libraries generated by subtractive hybridization, it could be employed in any number of applications, some of which are outlined below:

- a) Generic libraries in which the host organism has an established UniGene cluster database.
- b) Libraries generated through subtractive hybridization of two or more mRNA populations
- c) Screened yeast two-hybrid prey libraries
- d) Comparison of gene lists generated on different platforms
- e) Annotation of custom gene lists with terms from the Entrez Gene database

Conclusion

We have developed a valuable new tool named EST Express for the identification, annotation and analysis of cDNA library sequences. EST Express is unique in that it is cross-platform, is freely available, makes full use of annotations from the Entrez Gene database and allows the user to assess the state of the cDNA library using diagnostic tools. EST Express is available under the GNU General Public License [13] and may be downloaded from its project website [14].

Availability and Requirements

- **Project name:** EST Express
- **Project home page:** <http://www.sourceforge.net/projects/estexpress>
- **Operating system(s):** Windows NT/2000/XP, Linux, potentially others
- **Programming language:** PHP/MySQL
- **Other requirements:** NCBI BLAST Toolkit, Crossmatch, JGraph library
- **License:** GNU General Public License [13]
- **Any restrictions to use by non-academics:** Licence required

List of Abbreviations

BLAST: Basic Local Alignment Search Tool; cDNA: Complementary Deoxyribonucleic Acid; EST: Expressed Sequence Tag; FTP: File Transfer Protocol. GPL: GNU General Public License; HTML: Hypertext Markup Language. ID: Identifying number; GUI: Graphical User Interface; mRNA: Messenger Ribonucleic Acid; MySQL: My Structured Query Language; PHP: PHP Hypertext Processor.

Authors' contributions

RS wrote the code for the software package, developed the project website and documentation, and prepared the manuscript. WB and ML participated in the testing and development of the software and contributed to the manuscript and software manual. JP, JB and VL provided insights on software development and testing and critically reviewed the manuscript.

Acknowledgements

This work was supported by the Miami Project to Cure Paralysis, the Buoniconti Fund, DOD W81XWH-05-1-0061, grant no. 2396 from the Paralyzed Veterans of America Research Foundation and NIH HD057632. W. Buchser is a recipient of Lois Pope LIFE Scholar award. V. Lemmon holds the Walter G. Ross Chair in Developmental Neuroscience at the University of Miami. The authors thank S. Khuri for critical comments on the manuscript.

References

1. Adams MD, Kelley JM, et al.: **"Complementary DNA sequencing: expressed sequence tags and human genome project"**. *Science* 1991, **252**(5013):1651-6.
2. Ewing B, Hillier L, et al.: **"Base-calling of automated sequencer traces using phred. I. Accuracy assessment"**. *Genome Res* 1998, **8**(3):175-85.
3. Ewing B, Green P: **"Base-calling of automated sequencer traces using phred. II. Error probabilities"**. *Genome Res* 1998, **8**(3):186-94.
4. Altschul SF, Gish W, et al.: **"Basic local alignment search tool"**. *J Mol Biol* 1990, **215**(3):403-10.
5. **JPGraph PHP Graphics Library** [<http://www.aditus.nu/jpggraph>]
6. **NCBI FTP site** [<ftp://ftp.ncbi.nih.gov/gene/DATA/gene2unigene>]
7. Maglott D, Ostell J, et al.: **"Entrez Gene: gene-centered information at NCBI"**. *Nucleic Acids Res* 2005:D54-8.
8. Liu M, Grigoriev A: **"Fast parsers for Entrez Gene"**. *Bioinformatics* 2005, **21**(14):3189-90.
9. Ayoubi P, Jin X, et al.: **"PipeOnline 2.0 automated EST processing and functional data sorting"**. *Nucleic Acids Res* 2002, **30**(21):4761-9.
10. Mao C, Cushman JC, et al.: **"ESTAP--an automated system for the analysis of EST data"**. *Bioinformatics* 2003, **19**(13):1720-2.
11. Matukumalli LK, Grefenstette JJ, et al.: **"EST-PAGE--managing and analyzing EST data"**. *Bioinformatics* 2004, **20**(2):286-8.
12. Kumar CG, LeDuc R, et al.: **"ESTIMA, a tool for EST management in a multi-project environment"**. *BMC Bioinformatics* 2004, **5**:176.
13. **GNU General Public License** [<http://www.gnu.org/copyleft/gpl.html>]
14. **EST Express Project Website** [<http://www.sourceforge.net/projects/estexpress>]

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp



96-Well electroporation method for transfection of mammalian central neurons

William J. Buchser, Jose R. Pardinas, Yan Shi, John L. Bixby, and Vance P. Lemmon

BioTechniques 41:619-624 (November 2006)
doi 10.2144/000112279

Manipulating gene expression in primary neurons has been a goal for many scientists for over 20 years. Vertebrate central nervous system neurons are classically difficult to transfect. Most lipid reagents are inefficient and toxic to the cells, and time-consuming methods such as viral infections are often required to obtain better efficiencies. We have developed an efficient method for the transfection of cerebellar granule neurons and hippocampal neurons with standard plasmid vectors. Using 96-well electroporation plates, square-wave pulses can introduce 96 different plasmids into neurons in a single step. The procedure results in greater than 20% transfection efficiencies and requires only simple solutions of nominal cost. In addition to enabling the rapid optimization of experimental protocols with multiple parameters, this procedure enables the use of high content screening methods to characterize neuronal phenotypes.

INTRODUCTION

The expression of proteins in cells using promoter driven cDNAs is a widely used approach for studying the function of proteins and analyzing molecular networks. Over the past 20 years, a number of approaches have been developed to allow transfection of cDNA containing plasmids into cells, especially cell lines. However, primary cells, such as neurons and T cells, have been resistant to most transfection methods, requiring the use of time-consuming and expensive viral-based methods. Neurons have been particularly challenging to transfect, with low efficiency of transfection up until the past few years (1). Recently, Amara (2) has introduced the nucleofection method, which achieves neuronal transfection efficiencies of >20% (3,4). A single cuvette is used in this method to electroporate a million or more neurons at a time. Each electroporation requires the use of an expensive proprietary reagent. For testing multiple genes, millions of neurons have to be harvested, and electroporations have to be performed one at a time. This presents two major problems for testing many genes. First, the repetitive action

of electroporating single samples, and then manually placing them correctly in their destination, lends itself to variability and error. Second, the extended time it takes to sequentially transfect each gene leaves the neurons in a toxic environment leading to even lower viabilities and efficiencies.

High content screening (HCS) uses automated acquisition of images of cells in multiwell plates combined with detailed quantitative image analysis to obtain multiple parameters about cell morphology and molecular expression. HCS is ideally suited for screening various kinds of libraries for their effects on cell proliferation, apoptosis, or other cell biological processes. It is ideal for quantitatively studying the morphology of neurons and how various agents alter process growth. The paucity of efficient methods for testing cDNAs in mammalian neurons using HCS has restricted the usefulness of HCS approaches to drug and compound libraries and inhibited genome style studies. To overcome this roadblock, we have sought to develop an inexpensive method that would allow multiple transfections at once—with identical conditions except for the gene to be transfected.

This electroporation approach involves a mixture of a minimal set of components, all at reasonable volumes and with small numbers of cells, such that experiments can be performed quickly with multichannel pipets or 96-well liquid handlers.

MATERIALS AND METHODS

Primary Neuron Culture

Postnatal day 8–11 mouse cerebella were prepared as described previously (5). Briefly, cerebella were harvested from ketamine-euthanized mice and minced with a razor blade. The cerebellar pieces were incubated in 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) for 20 min at 37°C, with occasional swirling. The trypsin was inactivated by adding horse serum to 10% and diluted with Hank's balanced salt solution (HBSS; 5.4 mM KCl, 0.44 mM KH₂PO₄, 131 mM NaCl, 4.2 mM NaHCO₃, 0.34 mM Na₂HPO₄, 10 mM HEPES). The cells were triturated sequentially with large- and small-bore flame-polished glass pipets in the presence of 1 mg/mL DNase I (Sigma, St. Louis, MO,

USA). Hoechst dye (Invitrogen) was added during this step. The cells were spun and resuspended in HBSS for counting. Centrifugation steps were all performed at 115× *g* for up to 7 min. Solutions and cells were kept at room temperature throughout the procedure. Preparations yielded >90% cerebellar granule neurons (CGNs).

Rat hippocampal slices were purchased from BrainBits LLC (Springfield, IL, USA), and dissociated neurons were prepared using a modified version of their protocol. Briefly, half hippocampal slices were incubated for 30 min in 2.5% trypsin and 100 μ L 30 mg/mL DNase and then rinsed four to five times with Hibernate-E, no Ca²⁺, media plus B27 (BrainBits LLC). Cells were triturated using flame-polished small- and large-bore glass pipets. After triturating, chunks were allowed to settle, and then the supernatant was transferred to new tubes, and the cells counted. Volumes were kept small (<2 mL) throughout the process. Preparations commonly yield >95% neurons.

Transfection

Typically, 100,000 neurons were resuspended in 100 μ L intracellular buffer (INB) solution (135 mM KCl, 0.2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 5 mM EGTA, pH 7.3), which results in about 3 nM free calcium. Cells (90–100 μ L) are added to a 96-well, 2-mm gap, electroporation plate (HT-P96-2; Harvard Apparatus/BTX, Holliston, MA, USA), and 1–5 μ g plasmid cDNA were also added to each well for a total volume of no more than 120 μ L. The plate is sealed with a 3M ScotchPad™ tape sheet (Qiagen, Valencia, CA, USA). All solutions were removed from the cold 10 min prior to transfection to allow them to come to room temperature. The electroporation plate was placed inside the Model HT-200 plate handler attached to an ECM 830 square wave pulse generator (Harvard Apparatus/BTX). This system was used to deliver pulses to the plate, one column at a time. For CGNs, one pulse was delivered with 340–350 V, 900 μ s pulse length. For hippocampal neurons, two pulses were delivered, each with 900 μ s pulse lengths and an

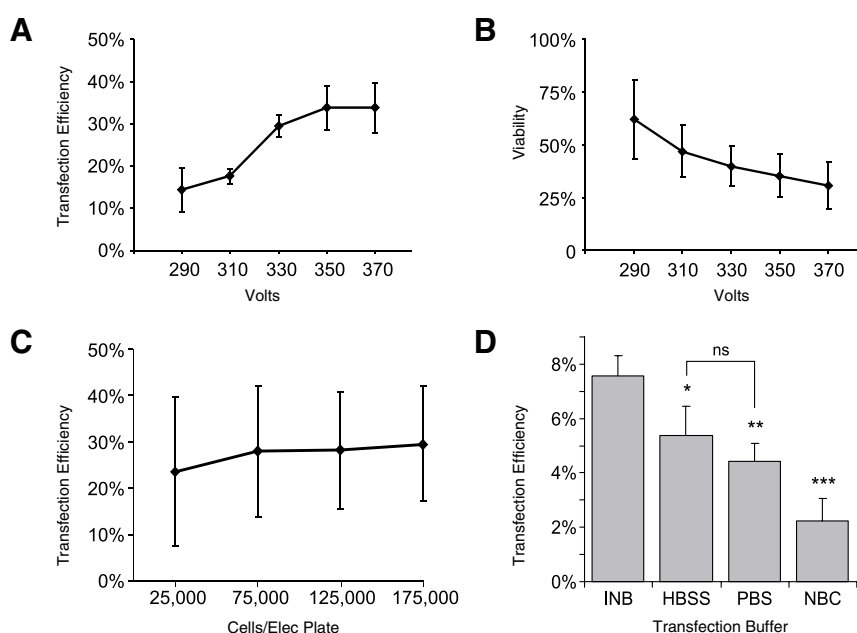


Figure 1. Electroporation in cerebellar granule cells. (A) Cerebellar cells are optimally transfected at voltages above 340 V, with an approximately 30% transfection efficiency. (B) The viability of the neurons in each transfection well decreases as the voltage increases, thus the lowest voltage that produces reasonable transfection efficiency is optimal. (C) The number of cells per well in the 96-well electroporation plate makes no significant difference in transfection efficiency for cell numbers examined. Standard deviations are large because data was averaged across the five voltages. (D) An intracellular buffer (INB) gave better transfection efficiency compared with Hank's balanced salt solution (HBSS), phosphate-buffered saline (PBS), and neurobasal complete (NBC) media. Data points in panels A, B, C, and D are presented as means $\pm$ standard deviation. Panels A and B, *n* = 4; panel C, *n* = 8; panel D, *n* = 3. ns, not significantly different.

approximate 2-s interval between. The first pulse was 140 V and the second 340 V.

Once transfection was complete, a fraction of the cells was immediately transferred to 96-well assay ViewPlates™ (Packard Instrument Company, Meriden, CT, USA), containing neurobasal complete (NBC) media (supplemented 1:50 with B27), 2 mM GlutaMAX™ media, 25 mM KCl, in Neurobasal™-A media (all from Invitrogen) preincubated to 37°C and 5% CO₂. Ten to twenty thousand cells were used per assay plate well, allowing up to six replicates per transfection condition. After plating, cells were placed in a 37°C incubator maintained at 5% CO₂.

Immunohistochemistry

Cells were left 2 days in vitro, then fixed, permeabilized, and stained. Briefly, fixation was performed for 45 min using 180 μ L/well of 4% paraformaldehyde, 0.01% glutaraldehyde in 0.1 M phosphate buffer,

pH 7.4. Cells were rinsed three times with phosphate-buffered saline (PBS) followed by a blocking solution (BS) of 0.2% gelatin and 0.03% Triton® X-100 in PBS. After blocking for 1 h, 100 μ L E7 mouse anti- β -tubulin primary antibody (diluted in BS) were added to each well and incubated overnight. The plate was washed three times with PBS and incubated with Alexa Fluor® 555 goat anti-mouse secondary antibody (Invitrogen) in BS. The fixation and staining procedures were performed using a liquid handling instrument (BioRobot® 3000; Qiagen), but could be done using multichannel pipets. Human L1 (see Figure 4) was stained with a monoclonal mouse anti-human L1 antibody—7B5. It was produced in the laboratory (3) and used 1/500 diluted in BS.

Microscopy and Analysis

The fixed and stained assay plate was analyzed by an HCS microscope, the KineticScan® Reader (Cellomics, Pittsburgh, PA, USA). This instrument

automatically took five images per well in three channels: (i) nuclei (Hoechst); (ii) neuron cell body and neurites (β -tubulin); and (iii) green fluorescent protein (GFP). A software suite built into the Cellomics package (Extended Neurite Outgrowth v1) processed the images and automatically identified neurons based on a valid nucleus and cell body. The program then quantified a variety of parameters and returned a report about each cell that satisfied predetermined thresholds and criteria. We used information about the number of cells, number of nuclei, length and number of neurites, and average fluorescence intensity in the cell body for further analysis.

Quantification

GFP transfection was determined first by finding the distribution of intensities in the green channel in wells in which GFP plasmids were not added. These intensities represented fluorescence signal that did not result from GFP. A threshold was set at 4 standard deviations above the mean in wells with no GFP, and cells with higher average intensities were considered GFP-expressing. GFP-expressing cells were then counted to determine transfection efficiency and were grouped to obtain average measures for each parameter studied (neurite length, tubulin intensity, cell body area, etc).

RESULTS

Transfection Efficiency

Optimization was first attempted in mouse CGNs, harvested from postnatal day 8–10 wild-type mice. Preparations from mouse cerebellum at this age yield a relatively homogenous population of neurons (6). Figure 1A demonstrates that increasing voltages gave higher transfection efficiency. However, increasing voltages resulted in lower viability (Figure 1B). Varying the number of cells in the 96-well electroporation plate did not significantly change either transfection efficiency (Figure 1C) or viability (data not shown) in CGNs. Of particular importance, it was possible to successfully electroporate very small numbers of neurons, only 25,000 per transfection well (see Supplementary Figure S1, B and C, available online at www.BioTechniques.com). Transfection efficiencies were also reliable across an entire 96-well plate (see Supplementary Figure S1, B and D). In addition, a calcium-free intracellular buffer (7) provided significantly better transfection efficiency than standard extracellular buffers or media (Figure 1D). Using conditions near 340 V, 100,000 cells, and INB buffer, 14 other independent experiments were performed. The transfection efficiency for these was an average of 26.8% with a standard deviation of 8.6% for the transfection of GFP in CGNs (see Supplementary Figure S1A). Hippocampal neurons were transfected with an average efficiency of $17.3\% \pm 3.2\%$ ($n = 4$).

The amount of plasmid DNA per transfection can affect expression level and transfection efficiency (see Supplementary Figure S2). For the plasmids we tested, 1–5 μ g DNA in 100 μ L transfection volume resulted in successful transfection.

Expression

The expression levels of the proteins encoded by various transfected plasmids were analyzed using a KineticsScan Reader HCS machine. GFP was expressed in CGNs and in hippocampal neurons (Figure 2). The GFP expression levels ranged from just

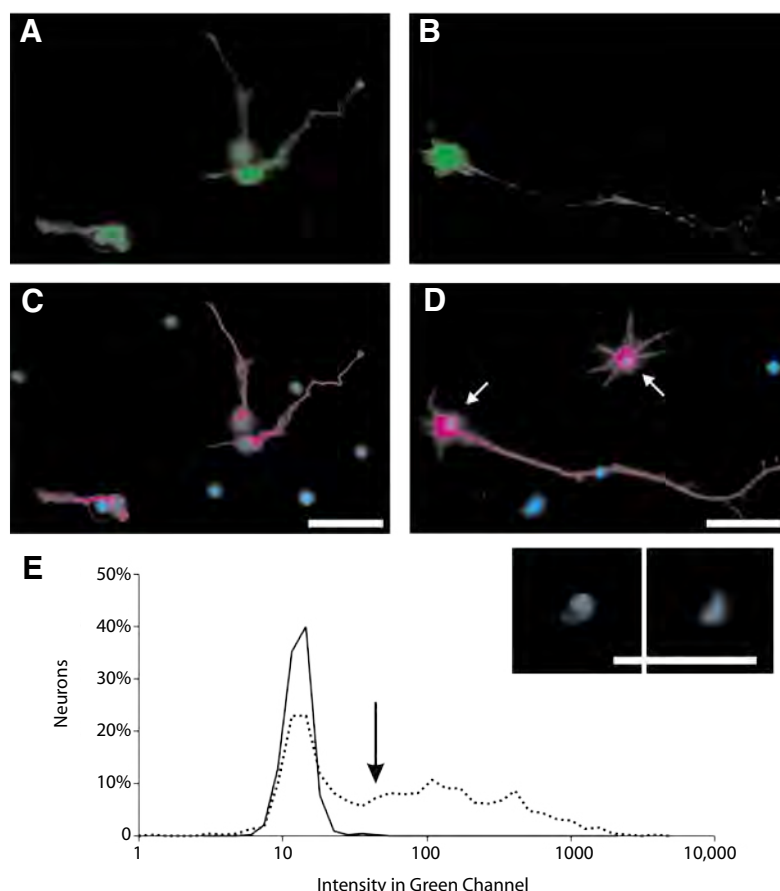


Figure 2. Expression of green fluorescent protein (GFP) in cerebellar granule neurons (CGNs) and hippocampal neurons. (A and C) Mouse CGNs 24 h after transfection. (B and D) Rat hippocampal neurons 48 h after transfection. (A and B) GFP fluorescence is displayed in green. (C and D) β -Tubulin staining in magenta, and nuclear staining (Hoechst) is in blue. An inset (below panel D) shows nuclear morphology for the neurons indicated by the arrows in panel D. Note that all β -tubulin positive neurons in panel A are expressing GFP. Dead neurons are nearby with distinct nuclear morphology and Hoechst intensity. Scale bar, 25 μ m. (E) Fluorescent intensity in the GFP (green) channel for control, nontransfected neurons (solid line), and GFP-transfected neurons (dotted line). Many neurons expressing GFP have intensities 2 orders of magnitude greater than the mean of the control intensities. The threshold for a neuron to be considered GFP positive is indicated by the arrow (see the Materials and Methods section).

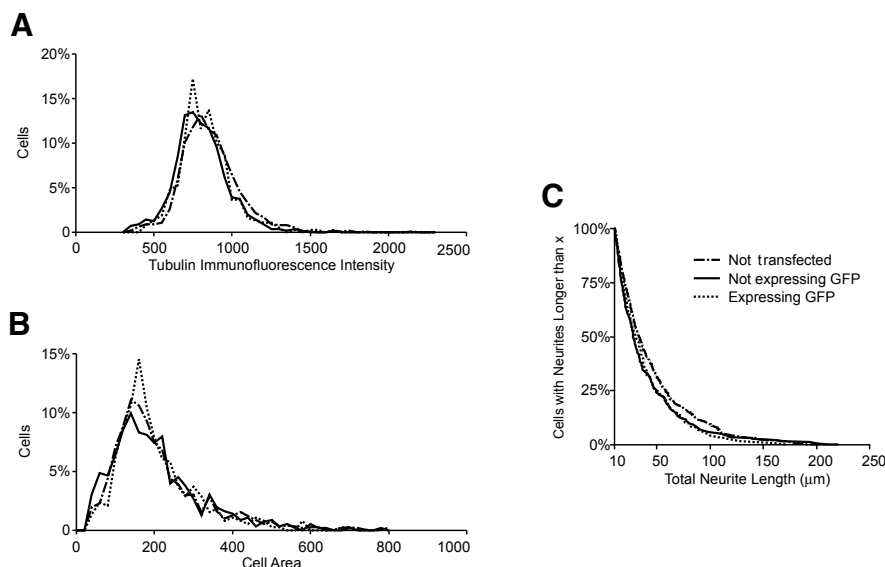


Figure 3. Viability and health of transfected cerebellar granule neurons (CGNs). Data are presented as standard histograms for (A) tubulin immunofluorescence and (B) cell body area. (C) Total neurite length profile is presented as a cumulative probability histogram. In all plots, dashed lines represent neurons that were electroporated without plasmid (not transfected, $n = 539$). Solid lines represent neurons that were electroporated with green fluorescent protein (GFP) cDNA, but were not expressing GFP after 48 h ($n = 263$). Dotted lines represent GFP-expressing neurons in the same wells ($n = 210$). Total neurite length was included only for values $>10 \mu\text{m}$. In the histograms, the y-axis reports percentage of total neurons, normalizing for the different number of cells in the three populations.

above background to more than 100 standard deviations from the nontransfected levels (Figure 2E). Expression of GFP in CGNs was seen in some cells at 6 h after transfection. By 10 h, about 6% of all the nuclei (live and dead) had GFP positive cell bodies. In addition to GFP, human L1CAM was transfected with transfection efficiency of $9.9\% \pm 5.4\%$ ($n = 12$), and GAP43-YFP was transfected with transfection efficiency of $29.8\% \pm 9.6\%$ ($n = 6$).

Viability

Because transfection often has adverse effects on neuronal viability, we evaluated parameters of cell health and viability in transfected neurons 1 or 2 days after plating (examples of data from entire 96-well plates are provided in Supplementary Figure S1, C and E). An average of $37.0\% \pm 16.3\%$ of the CGNs originally plated (18,000) remained alive after 2 days ($n = 14$ experiments). CGNs that were resuspended in INB but not electroporated had an average viability of $67.1\% \pm 9.7\%$ ($n = 4$ experiments). CGNs that were electroporated in INB, but with no cDNA had an average viability of $34.9\% \pm 8.7\%$

($n = 7$ experiments). Longer periods of incubation in the transfection buffer (INB) result in lower viability of cells. In our hands, if the time spent in INB is <15 min, the reduction in viability is minimal. Usually a 96-well plate can be set up within this time. Transfected cells were healthy with normal morphology and normal expression of proteins such as β -tubulin (Figure 3A). The nuclei of GFP-expressing cells had the same appearance as wild-type cultured neurons, by Hoechst staining, and were clearly not pyknotic (example Figure

2, C and D, inset). Distributions of cell areas (Figure 3B) and neurite length (Figure 3C) for GFP-expressing cells were similar to those for nonexpressing or nontransfected cells. Thus, the transfected cells were viable, healthy, and good candidates for study. After 6 days, we found that the number of GFP-expressing neurons stayed the same. The total number of neurons in the plate decreased (data not shown). Taking into account percent survival, transfection efficiency, and the optics of 96-well plates, we normally found 50–100 transfected cells to study in the central one-third of the well. These are generally sufficient numbers of neurons for studies in which single cells can be assayed.

Identifying Transfected Cells by Co-Expression

With 20%–40% transfection efficiency, it is important to identify the transfected neurons so they can be analyzed as a distinct population. One method for such identification is cotransfection of a test plasmid with a reporter such as GFP. By optimizing the ratio of the two genes (2:5; GFP reporter:gene of interest), 90% of GFP positive cells were found to co-express a second cDNA of interest (Figure 4). Transfection of two plasmids, such as L1 and GFP did not affect neurite length ($P = 0.08$), cell area ($P = 0.11$), or tubulin intensity ($P = 0.23$) compared with neurons expressing GFP alone (Kruskal-Wallis test, see Supplementary Figure S3). For many applications, such as screening libraries of genes, it is not

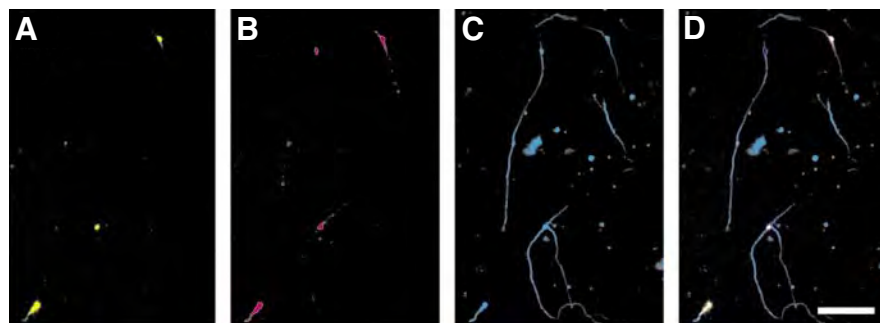


Figure 4. Co-expression of two plasmids. Green fluorescent protein (GFP) and the cell adhesion molecule L1 were cotransfected into cerebellar granule neurons (CGNs). The neurons were kept in culture for 48 h before being fixed and stained. (A) GFP fluorescence, (B) L1 immunofluorescence, and (C) β -tubulin immunofluorescence were used to determine transfection efficiencies. (D) A merge of the three channels in panels A, B, and C. Note that there are three cells expressing GFP and L1, and a fourth cell that is only expressing L1. Scale bar, $100 \mu\text{m}$.

practical to directly analyze expression of every experimental gene. Thus, by cotransfecting a reporter gene, the experimenter can ensure a high probability of co-expression and use reporter expression as a surrogate for expression of the experimental gene.

DISCUSSION

The introduction of HCS methods into academic and biotech laboratories has enabled the screening of drugs and compounds for effects on cell differentiation, cell signaling pathways, and various pathological conditions such as cancer. The extension of these methods to incorporate genomic approaches has been hindered in part by a lack of efficient and inexpensive ways to transfect primary cells, such as neurons, with hundreds of different DNAs. We have developed a 96-well-based electroporation method that overcomes this hurdle. In addition to cDNA plasmids, vector-based short hairpin RNA (shRNA) could also be used to knockdown gene expression. Thus, libraries of shRNAs that block the expression of genes throughout the genome could be screened with this method. In our hands, knockdown of gene expression with small interfering RNAs (siRNAs) in neurons requires different transfection parameters, which we have not yet optimized.

Buffers

Different minimal buffers were examined for use in electroporation. Extracellular buffers such as HBSS, PBS, L-15, and Neurobasal media were tested. HBSS and PBS worked but still failed to give reasonable transfection efficiencies. Next, an intracellular buffer was tested with a simple formulation based on those used in the electrodes for whole-cell patch clamping of neurons. This buffer turned out to be essential for the success of our system. Due to the large numbers of cDNAs that need to be tested in genome-wide or even smaller screens, it is significant that this methodology use a buffer produced inexpensively with off-the-shelf chemicals. The proprietary reagents often used for transfection are rendered unnecessary—drastically reducing the

cost of each transfection and making screens of large sets of genes possible for laboratories with modest budgets.

While we have not investigated the issue systematically, a variety of experiments indicated that the presence of Ca^{2+} in buffers used to dissociate primary neurons is deleterious to cell survival and that the presence of Ca^{2+} during electroporation is especially harmful. We make every effort to prevent the introduction of Ca^{2+} into the various buffers. We now use Ca^{2+} -free Hibernate media to dissociate our neurons.

Electroporation in Neurons

Several laboratories have reported successful transfection and protein expression in neurons after electroporation. Mertz and colleagues (8) used a Bio-Rad Laboratories system to transfect cerebellar neurons in basal media. They used 5 million cells/well and had a transfection efficiency of 10.4% with a viability of 44%. This technique most closely resembles ours but requires the use of a much larger number of cells and is not high-throughput. Teruel et al. (9) used another method in which hippocampal neurons were electroporated after plating. They called this method microporation, and they reported between 1% and 30% transfection efficiency. This system could potentially be modified to use multiple microporation heads and thereby be made compatible with high-throughput applications. Organotypic culture systems are also commonly used as *in situ* ways to study neurons. Murphy and Messer (10) used BTX paddle electrodes to electroporate slice cultures and reported 26% transfection efficiency.

Amaya Biosystems (Cologne, Germany) has helped to lead neuroscientists to more reliable and effective transfections with the introduction of their Nucleofector™ technology (2). Recently, Leclerc and colleagues (11) used the Nucleofector technology successfully in transfection of retinal ganglion cells and dorsal root ganglia. Using Amaya system's proprietary solutions and transfection conditions, Leclerc et al. achieved 28% and 20% transfection efficiency, respectively, with these two neuronal populations.

The electroporation methods described above have enabled a variety of studies on neuronal differentiation, synapse formation, and neuronal disease. However, they are not appropriate for projects in which hundreds or thousands of targets need to be screened. A significant problem with earlier methods concerns the numbers of cells needed per transfection. We have optimized the BTX transfection system to work with as few as 25,000 cells per transfection. This is significantly less than the 1 million or more required by most of the other methods listed above. Given that 6 million neurons can be derived from one postnatal mouse cerebellum, there are enough cells to test 240 different cDNAs using the present method. The alternatives mentioned above would require 40 mice for an experiment of similar scale.

ACKNOWLEDGMENTS

We would like to thank Cristina Vila, Robin Smith, Anthony Oliva, Alexis Tapanes-Castillo, and the rest of the Lemmon/Bixby laboratory, as well as Darcie Moore, Tony DeFazio, Ella Bossy-Wetzel, and Akos Gerencser for their assistance and advice on this project. This work was supported by the Miami Project to Cure Paralysis, the Buoniconti Fund, DOD W81XWH-05-1-0061, and grant no. 2396 from the Paralyzed Veterans of America Research Foundation. W.J.B. is a recipient of a Lois Pope LIFE Scholar award. V.P.L. holds the Walter G. Ross Chair in Developmental Neuroscience at the University of Miami.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES

1. Washbourne, P. and A.K. McAllister. 2002. Techniques for gene transfer into neurons. *Curr. Opin. Neurobiol.* 12:566-573.
2. Hamm, A., N. Krott, I. Breibach, R. Blindt, and A.K. Bosserhoff. 2002. Efficient transfection method for primary cells. *Tissue Eng.* 8:235-245.

3. **Cheng, L. and V. Lemmon.** 2004. Pathological missense mutations of neural cell adhesion molecule L1 affect neurite outgrowth and branching on an L1 substrate. *Mol. Cell. Neurosci.* 27:522-530.
4. **Dityateva, G., M. Hammond, C. Thiel, M.O. Ruonala, M. Delling, G. Siebenkotten, M. Nix, and A. Dityatev.** 2003. Rapid and efficient electroporation-based gene transfer into primary dissociated neurons. *J. Neurosci. Methods* 130:65-73.
5. **Beattie, C.E. and R.E. Siegel.** 1993. Developmental cues modulate GABAA receptor subunit mRNA expression in cultured cerebellar granule neurons. *J. Neurosci.* 13:1784-1792.
6. **Keilhauer, G., A. Faissner, and M. Schachner.** 1985. Differential inhibition of neurone-neurone, neurone-astrocyte and astrocyte-astrocyte adhesion by L1, L2 and N-CAM antibodies. *Nature* 316:728-730.
7. **Majoul, I., M. Straub, S.W. Hell, R. Duden, and H.D. Soling.** 2001. KDEL-cargo regulates interactions between proteins involved in COPI vesicle traffic: measurements in living cells using FRET. *Dev. Cell* 1:139-153.
8. **Mertz, K.D., G. Weisheit, K. Schilling, and G.H. Luers.** 2002. Electroporation of primary neural cultures: a simple method for directed gene transfer in vitro. *Histochem. Cell Biol.* 118:501-506.
9. **Teruel, M.N., T.A. Blanpied, K. Shen, G.J. Augustine, and T. Meyer.** 1999. A versatile microporation technique for the transfection of cultured CNS neurons. *J. Neurosci. Methods* 93:37-48.
10. **Murphy, R.C. and A. Messer.** 2001. Gene transfer methods for CNS organotypic cultures: a comparison of three nonviral methods. *Mol. Ther.* 3:113-121.
11. **Leclerc, P.G., A. Panjwani, R. Docherty, M. Berry, J. Pizzey, and D.A. Tonge.** 2005. Effective gene delivery to adult neurons by a modified form of electroporation. *J. Neurosci. Methods* 142:137-143.

Received 2 May 2006; accepted 10 August 2006.

Address correspondence to Vance P. Lemmon, University of Miami Miller School of Medicine, Lois Pope LIFE Center, 1095 NW 14th Terrace, Miami, FL 33136, USA. e-mail: vlemmon@miami.edu

To purchase reprints of this article, contact: Reprints@BioTechniques.com