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Award Number: W81XWH-12-2-0129

TITLE: Regional Anesthesia and Valproate Sodium for the Prevention of Chronic Post-Amputation Pain

PRINCIPAL INVESTIGATOR: Thomas E Buchheit MD

CONTRACTING ORGANIZATION: Duke University

Durham NC 27705-4677

REPORT DATE: October 2013

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

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose of this research is to determine if an FDA approved medication(valproic acid), commonly used for migraine headache prophylaxis, will also be effective in the prevention of chronic neuropathic pain following amputation, stump revision or surgery for mangled limb. Additionally, this research will define the alterations in DNA methylation and gene expression that occur after injury, and the extent that valproic acid, a known modulator of DNA methylation, will prevent the epigenetic effects that lead to the development of chronic post-surgical pain.

The scope of this research involves injured military service members and veterans undergoing amputation, stump revision surgery or surgery for mangled limbs with neurologic damage.

Progress to date includes protocol and SOP development, database creation in REDCap (Research Electronic Data Capture), IRB submission and approval at the Durham VAMC and Duke University, as well as submission and secondary approval from HRPO. Additionally, the protocol has been uploaded and approved by Clinicaltrials.gov. CRADA submission has been performed to the VAMC. Study enrollment will begin at the Durham VAMC as soon as CRADA approval is obtained.

15. SUBJECT TERMS
Amputation, Postamputation pain, Post-surgical pain, Neuralgia, Epigenetics, Valproic Acid, DNA Methylation, Neuropathic pain

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U	U	U	UU	53	USAMRMC

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INTRODUCTION:

Chronic pain is a significant problem in patients undergoing surgery following military trauma and chronic vascular disease. Symptoms are typically treated with medications such as narcotics, anti-inflammatory drugs, and local anesthetics. Despite these therapies, however, more than 50% of patients who have an amputation or significant limb injury experience long-term chronic pain. Chronic pain in military personnel and veterans may impair their ability to ambulate or wear a prosthetic device, and may ultimately require the use of chronic narcotic medications. Although sometimes effective for pain, chronic narcotic medications also carry risks of sedation, confusion, and possibly addiction. Identifying preventive mechanisms that can be employed at the time of surgery is of utmost importance for military and veteran health systems. Valproates such as valproic acid have a unique advantage over other classes of medicines used for neuropathic pain, as this drug actually modifies the epigenetic mechanisms (such as DNA methylation) and therefore may demonstrate efficacy in preventing the transition from acute to chronic pain. In this study, we will additionally define the gene expression changes that occur in the transition from acute to chronic pain, and any effect that valproic acid may have on these genes.

In summary, this research will investigate the effectiveness of valproic acid vs placebo when added to regional anesthesia in the prevention chronic pain after amputation, stump revision, or surgery for mangled limb with neurologic damage. It will also define the gene expression changes that occur after surgery and the ability of valproic acid to prevent the epigenetic changes that lead to the development of chronic pain.

BODY:

Below is a detailed list of events and accomplishments during Year 1 of this project. Attachment 1 is additionally a visual timeline of key events in Year 1.

The research team is ready to begin enrollment in this protocol with completed IRB approvals from the Durham VAMC, Duke University and HRPO, preparation of all associated documents and meeting with the involved surgical teams.

We have been recently informed that patient enrollment cannot begin until the CRADA is approved by the VA. The Duke Office of Research Administration sent the redline CRADA to the Institute for Medical Research (IMR) for acceptance on September 23. (The IMR is a non-profit, tax-exempt institute whose mission is to support research and education at the Durham Veterans Affairs Medical Center). The CRADA was reviewed by the VA attorney and unfortunately, the language in article 12.3, regarding 'Costs of Subject Injury' was rejected on October 10 with comments made by the attorney. Duke has responded to the rejection on October 10. As of today, October 23, the VA attorney is seeking approval from the director of the VA legal team in Washington D.C. Enrollment cannot begin until the CRADA is approved and fully executed.

2012 December

Protocol submitted to VA IRB

2013 January

Clinical Research Coordinator start

VA IRB Initial Review received

April

Received full protocol approval from VA IRB

May

VA approved protocol documents and notice sent to HRPO for secondary approval

June

New Clinical Research Coordinator, replacing previous

Cooperative Research & Development Agreement (CRADA) draft sent by Institute for Medical Research (IMR) to Duke

July

Initial review by HRPO received; request for revised documents

August

Non-perishable Supplies ordered & received

DUKE IRB approved study via expedited review

September

Submitted all revisions requested by HRPO

Protocol amendment submitted to VA IRB after HRPO request for changes/approval (v27)

REDCap electronic data capture 'Go Live' status

HRPO secondary approval granted

Vascular Surgery In-Service

Study approved on Clinicaltrials.gov

Coordination with VA Pharmacy for drug/placebo handling/flow completed

Perishable supplies ordered and received

VA study team training completed

VA ICU and floor nurse training completed

Attorney for VA rejected the CRADA,

October

Travel to WRNMMC to train, review protocol & other study documents, Standard Operating Procedure, REDCap (eCRF) with their study team

Pending full approval of CRADA between Duke and IMR

Task 1, below, is contained in our Statement of Work (SOW). The human subject approvals for Durham VAMC and Duke met our anticipated timeline. HRPO secondary approval was not obtained until month 11, delaying potential enrollment at the Durham VA an additional two months. WRNMMC protocol was reviewed in the IRB meeting held October 10, pending decision notice. The Durham VA site is currently ready to enroll, pending CRADA agreement and approval by both Duke and IMR.

Task 1 (pre-study) – Human subjects approval (including HRPO)	**Months 1-12**	***Actual***
a. Duration (Durham VAMC), months 1-9		*Month 8*
b. Duration (WRNMMC), months 1-12		*pending*
c. Exempt from Review (Duke), months 9-10		*Month 11*
Milestone Pre-Study Task 1a – IRB and HRPO approval in Durham	Month 9	*Month 12/HRPO*
Milestone Pre-Study Task 1b – IRB approval at WRNMMC	Month 12	*pending*
Milestone Task 2a – First patient enrolled in Durham	Months 9-10	*pending*
Milestone Task 2b – First patient enrolled at WRNMMC	Months 12-13	*pending*

Though we are approximately three months behind schedule, we are confident that both sites will reach Milestone Task 2d which is the enrollment of 140 subjects in months 24-26.

KEY RESEARCH ACCOMPLISHMENTS:

- We ended Year 1 with a 19% unobligated balance, which includes the equipment purchase of the *Multi-channel SOLO Robotic Pipettor* (included in our budget). The robot is scheduled for delivery 10/29/13.
- The Standard Operating Procedure has been finalized and attached (Attachment 2).
- We anticipate meeting future enrollment milestones as soon as we are able to obtain CRADA approval.

REPORTABLE OUTCOMES:

Reportable outcomes from this research study are pending enrollment and data collection. In the preparation process, we have developed the informatics database (REDCap), and continued to develop expertise in the scientific assays through work on a parallel DoD research grant, DM102142, Molecular Signatures of Chronic Pain Subtypes. Since this research involves the same research team and many of the same laboratory analyses, we are assuring reliable and reproducible work for analysis of samples in this research.

CONCLUSION:

In summary, our research team has met all milestones for protocol development and document submission to begin this important study that will investigate the efficacy of a non-narcotic analgesic and additionally elucidate the underlying epigenetic mechanisms that lead to the development of chronic pain. Study enrollment is to begin as soon as CRADA approval is obtained.

REFERENCES:

T. Buchheit, T. Van de Ven, A. Shaw, Epigenetics and the transition from acute to chronic pain, *Pain medicine (Malden, Mass.)* **13**, 1474–1490 (2012).

APPENDICES:

Attachment 1 – Year 1 Timeline
Attachment 2 – Quad Chart
Attachment 3 – Standard Operating Procedure (28 pages)
Attachment 4 – "Epigenetics and the Transition from Acute to Chronic Pain" (17 pages)

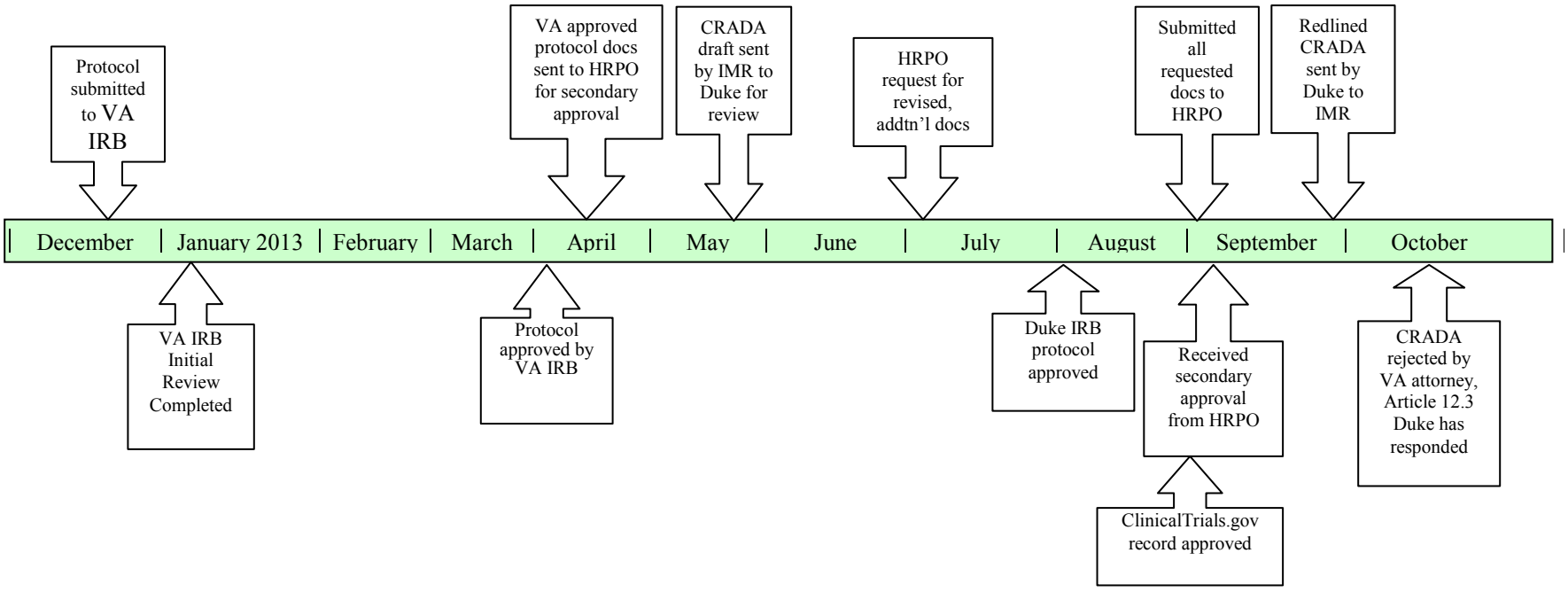
SUPPORTING DATA:

Not applicable at this time.

Year 1 Timeline

Durham VAMC & Duke

Regional Anesthesia and Valproate Sodium for the Prevention of Chronic Post-Amputation Pain



Regional Anesthesia & Valproate Sodium for the Prevention of Chronic Post-Amputation Pain

Log #PT110575 Award Number W81XWH-12-2-0129

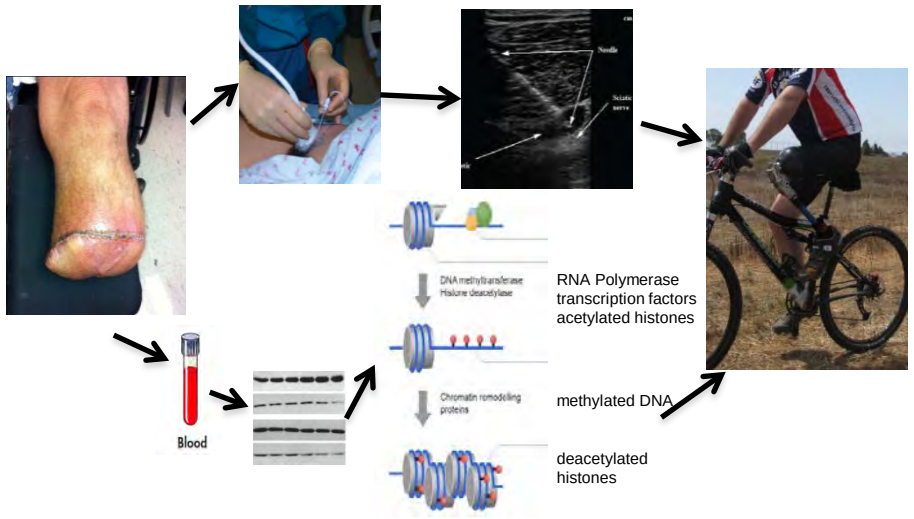
PI: Thomas Buchheit MD **Org:** Duke University **Award Amount:** $2,237,228

Study/Product Aim(s)

- Aim 1: Determine the efficacy of valproic acid combined with regional anesthesia in reducing the incidence of chronic post-amputation pain.
- Aim 2: Determine role of epigenetic DNA methylation in post-amputation pain and effects of valproic acid treatment

Approach

- In a randomized clinical trial, we will determine if the combination of valproic acid combined with regional anesthesia reduces the incidence of chronic post-amputation when compared with regional anesthesia alone.
- We will analyze DNA methylation patterns of patients with post-amputation pain and determine the way they are modified by valproic acid. We will confirm the functional relevance of these modifications using gene expression signatures.



Timeline and Cost

Activities / CY	13	14	15	16
VA/Duke/HRPO approvals; pending CRADA approval to start enrollment				
Enrollment and data collection at VA, IRB and enrollment at WRNMMC				
Enrollment and data collection, initial analysis				
Clinical study closure, outcomes analysis, final adjudication				
Estimated Budget ($K)	**$391K**	**$658K**	**$692K**	**$497K**

Updated: October 24, 2013

Goals/Milestones

CY13 Goal – Protocol planning, data use agreements, IRB & HRPO approvals, lab supply purchasing, and enrollment

☑ Fully planned, IRB approval at all institutions, lab supplies purchased, and lab analyses developed. Enrollment pending CRADA approval between VA & Duke

CY14 Goals – Patient enrollment, data and sample collection

☐Patient enrollment and data collection at Durham VAMC

☐IRB approval and enrollment at WRNMMC

CY15 Goal – Patient enrollment, data collection, epigenetic analysis

☐ Enrollment, initial epigenetic analysis and endpoint adjudication

CY16 Goal – Clinical study closure and outcomes analysis

☐ Final epigenetic analysis and endpoint adjudication

☐ Clinical outcomes analysis

Comments/Challenges/Issues/Concerns

- Enrollment pending approval of CRADA (Durham VA & Duke)

Budget Expenditure to Date

Projected Expenditure: $391K

Actual Expenditure: $317K

Attachment 3

Regional Anesthesia and Valproate Sodium for the Prevention of Chronic Post-Amputation Pain

Standard Operating Procedure

Site: Durham VAMC

October 23, 2013

CDMRP Log Number: PT110575
Principal Investigator: Thomas Buchheit, MD

VAMC IRB Protocol# 01709
DUKE IRB Protocol# Pro00047194

Valproate
Project Support

Please feel free to call if you have any questions or if we can be of assistance in any way.

Thomas Buchheit, MD, Principal Investigator
Phone: 919.286.6938

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Mary Kirkley, Research Project Manager
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INTRODUCTION

This is a randomized, double-blinded, placebo-controlled trial to test the efficacy of valproic acid (VPA) in reducing the incidence of chronic neuropathic and post-amputation pain. Additionally, it is a nested, observational study of the epigenetic modifications that occur in the transition from acute to chronic pain.

This is a collaborative study between investigators at Duke University in Durham, NC and Walter Reed National Medical Military Center (WRNMMC) in Bethesda, Maryland. Up to 420 patients from the two centers will be enrolled to determine whether the combination of perineural catheter infusion and oral valproate reduces the incidence of post-amputation pain when compared with local anesthetic infusion alone. Patients in the "control arm" of the study will receive regional anesthesia catheters prior to surgery, and have catheter infusions of local anesthetic as per current standards of care. "Intervention arm" patients will receive valproate 250mg preoperatively, and then every 8 hours for 6 days post-operatively or until the time of discharge from the hospital.

Longitudinal follow-up will occur in the Durham VAMC Post-Amputation Pain Clinic. Outcomes for patients in the intervention arm will be compared with those managed with the current institutional standards of care including regional anesthesia catheter infusions. Research blood samples will be collected preoperatively, postoperatively (at the completion of study drug intervention), and during the Pain Clinic follow-up visit, approximately 3 months post-surgery for analysis of metabolic changes, epigenetic modifications and gene expression alterations. All samples will be de-identified and subsequently studied in our laboratory in the Snyderman Genome Sciences Research Building and several core facilities at Duke University. We will also use a 3rd party metabolomics facility, Metabolon, Inc. in Raleigh, to measure plasma metabolomic differences between case and control subjects. Metabolon will receive completely de-identified plasma samples for these assays.

ABBREVIATIONS AND ACRONYMS

ALT	Alanine Aminotransferase
AST	Aspartate aminotransferase
BMI	Body Mass Index
BPI	Brief Pain Inventory
CBC	Complete Blood Count
CRF	Case Report Form
CRPS	Complex Regional Pain Syndrome
DVPRS	Defense and Veterans Pain Rating Scale
eCRF	Electronic Case Report Form
EOS	End of Study Drug
ICF	Informed Consent Form
MMSE	Mini Mental Status Exam
PHQ-2	Patient Health Questionnaire-2
PTSD	Post Traumatic Stress Disorder
RASS	Richmond Agitation Scale
S-LANSS	Self-Administered Leeds Assessment of Neuropathic Symptoms and Signs
SSN	Social Security Number
TCA	Tricyclic Anti-depressant
VAMC	Veterans Administration Medical Center
VPA	Valproate Acid
WRNMMC	Walter Reed National Military Medical Center

SCREENING & PRE-ENROLLMENT

Patients scheduled to undergo surgery for amputation, stump revision or surgery for limb injury with neurologic damage will be screened for the study. Patients who provide informed consent to participate in the study will be screened within 30 days from the scheduled surgical procedure. Screening procedures will include the following:

- medical and surgical history (including prior amputation surgeries, medications, Body Mass Index, history of depression and PTSD)
- pain scales
- clinic visits, lab and clinical test results
- demographic information
- Mini Mental Status Exam (MMSE) – Patients must answer 4 out of 6 questions correctly to be eligible for the study.

All inclusion and exclusion criteria will be verified by a second clinically trained individual within our group. Patients who meet all study entrance criteria at screening will be eligible for participation in the study.

STUDY FOLDER CHECKLIST

The study folder checklist (appendix A) will be added to the patient folder at time of enrollment and will be utilized to ensure VA IRB compliance during the study.

SUBJECT ID NUMBERING SYSTEM

Patient identification, using numerical barcode labels, will be established and available in REDCap prior to patient enrollment. Each kit to be used for the first blood collection will contain a cryo box labeled with a barcode which is the patient ID number. This same barcode number will be affixed to the patient's sample collection barcode form found in each kit as 'Patient #'. The VPA Randomization Assignment Log (appendix B) will be used to identify the order in which patients will be enrolled and the ID number (kit labeled with same number) to be used, in sequential order. The first blood collection kit to be used for each patient in this study will be labeled as 'Day of Surgery'.

SUBJECT IDENTIFICATION LOG

The Subject Identification Log (appendix C) will be updated at the time of screening and informed consent process and will be kept as an electronic file on the S: drive as is required by the Durham VAMC IRB for annual IRB review and will be maintained and housed, on the VA server.

RANDOMIZATION

During the screening process, patients will be categorized into one of three types of surgeries: amputation, stump revision or surgery for limb injury with neurologic damage. After consent and preferable one day prior to the scheduled surgery, the Research Coordinator will obtain the next study ID number and corresponding REDCap ID number (patient ID) from the randomization log for the

type of surgery to be performed. A prescription template (appendix D) will be completed and signed by the principal investigator and taken to the VA pharmacy with a copy of the signed Informed Consent Form (ICF). The pharmacist will sign to confirm receipt of the order and will follow the assignment log and dispense the study drug or placebo (250 mg) into unit dosage cups which will be labeled and barcoded for that patient for the day of surgery through the hospitalization period. The research coordinator will obtain a copy of the signed form and place in the patient folder. A bag with the unit dosages will be placed in Room C3011A, clearly labeled with the REDCap ID# and staged for the first dose prior to induction on the day of surgery.

STORAGE & ADMINISTRATION OF STUDY DRUG

The study drug and placebo will be stored in the DVAMC pharmacy at room temperature (15°C - 30°C) prior to dispensing.

The first dose will be administered by a pre-operative nurse or attending anesthesiologist in the preoperative area. Afterwards, the bag with the unit dosage cups will be placed in the appropriate Omnicell of the ward to which the patient is assigned. Subsequent doses will be administered at bedside by the ICU or floor nurse depending on patient location. A clinical warning (patient enrolled in a clinical trial) will be placed in the patient's chart. All doses of the study drug will be administered by DVAMC medical or nursing staff and will be documented in the patient's electronic Medical Record.

PATIENT RECORD

A study folder will be maintained at Durham VAMC for every patient enrolled and will be labeled with the corresponding REDCap (patient) ID#. These will contain patient data and personal health information and will be kept in locked filing cabinets in Room C3011A. The research team will comply with DVAMC regulations for future identification of subjects enrolled in this study. All information (which will be de-identified) collected in REDCap will be printed and stored at Duke, by patient ID# and used for the purpose of adjudication.

The Patient Record will contain:

a) The completed Abbreviated Mini-Mental Status Exam
b) Inclusion/Exclusion checklist with patient ID#, signed and dated by Research Coordinator
c) Original signed ICF
d) Original signed HIPAA waiver
e) Marked body diagram (appendix E) (original to Duke when samples are shipped, copy kept in patient record at Durham VAMC)
f) Blood Collection Barcode form (appendix F) (original to Duke when samples are shipped, copy kept at Durham VAMC)
g) Hard copy of REDCap data forms given to patient for his/her responses.
h) All patient's personal health information gathered for this study

Copies of all data collection forms in REDCap will be printed for each patient and kept on file at Duke, GSRB1, Room 1002A filed by patient ID#, for both sites.

STUDY DATA

The following information will be collected by reviewing the patient's medical chart in combination with information obtained during the screening/interview process:

- Demographics (name, address, telephone number, SSN, gender, ethnicity/race, age, Body Mass Index; dates to be collected are date of surgery, birth, discharge, 3 and 6 month follow-up appointments)
- Significant medical history
- Previous surgical interventions (vascular, diabetic, infection-related procedure or amputation for other cause)
- Narcotic medication (total daily morphine equivalent dose)
- Current non-narcotic medications (anticonvulsant, tricyclic antidepressant medications [daily dose of TCA must be less than 50 mg], beta blockers, NSAIDs, fish oil supplements, and Steroids)
- History of PTSD
- History of depression
- History of heterotrophic ossification

QUESTIONNAIRES/ASSESSMENTS

The following questionnaires will be printed from REDCap and given to the patient to complete:

DEPRESSION QUESTIONNAIRE

To be completed at enrollment:

- Patient Health Questionnaire-2 (PHQ-2)

DAILY ASSESSMENT QUESTIONNAIRE - DVPRS

To be completed at time of study enrollment, daily during study drug administration, and subsequently at 1, 3 and 6 months:

- Defense and Veterans Pain Rating Scale (DVPRS)

SEDATION ASSESSMENT QUESTIONNAIRE

To be completed at time of study enrollment pre-operatively and during the study drug/placebo administration, by the Research Coordinator, Nurse or assigned study personnel:

- Richmond Agitation Sedation Scale (RASS)

SYMPTOM QUESTIONNAIRES

To be completed at time of study enrollment, 1, 3 and 6 months:

- Brief Pain Inventory (BPI)
- Self-Reported Leeds Assessment of Neuropathic Symptoms and Signs pain scale (S-LANSS)

To be completed at time of study enrollment, 3 and 6 months:

- Complex Regional Pain Syndrome and Neuroma Questions

AMPUTATION QUESTIONNAIRES

To be completed at study enrollment, if patient has had a prior amputation on the study limb; and at the 3 and 6 month follow-up visits for patients that have amputation:

- Phantom and Residual Limb Pain Questions

EXAM & VISUAL DOCUMENTATION AT ENROLLMENT, 3 & 6 MONTHS

Subjects will be presenting for amputation, stump revision, or surgery for a limb injury with neurologic damage. This exam of the affected limb will take place at the time of enrollment if the patient has had a prior amputation on the limb pending surgery. It will also be conducted at the 3 and 6 month follow-up visits for all patients who have undergone amputation on the affected limb.

The investigators will perform an exam of the affected limb by removing the prosthesis and/or dressings. This will not apply to dressings for open wounds or infected areas.

1. The exam will take place in a well-lit room.
2. Prosthesis will be removed. If dressings are applied to affected part, they will not be removed and the examination will be performed as close as possible to the affected part.
3. Visual inspection of the limb will then be carried out, noting asymmetry of sweating, color, skin changes, hair growth, and tremor.
4. The research coordinator will then assess sensation of the injured limb. The subject should have eyes closed or head turned during this process.
    a. **Allodynia:** Clean cotton wool will be gently brushed against the skin in 2 areas when a painful limb is present: inside the previously marked painful area, and subsequently 5 cm proximal to the proximal edge of the marking. If the bandage cannot be removed, the skin will be tested proximal to the bandaged area. The skin will be brushed in a straight line of approximately 2 cm and this process will be repeated 3 times. Allodynia will be noted if any of the 3 brushes are uncomfortable or unpleasant to the touch. If there is no stump/residual limb pain, testing will be performed at only one position, 5cm proximal to the surgical scar on the anterior aspect of the limb. Note: if dressing exists, do not remove it; rather, test as close as possible to the painful area. Record in REDCap as unavailable due to area covered by dressing. The cotton wool will be discarded after use.

    b. **Tinel's Sign:** The research coordinator will then gently tap on the painful area of skin (if present). If a focal area of "pins and needles" or nerve sensitivity is noted, it will be considered to be Tinel's sign POSITIVE. (Note) if dressing has been applied, do not remove it and skip the test this time; record in REDCap as "unavailable due to the area covered by dressing".

    c. **Sensory and Motor Deficit:** For exam of an injured, non-amputated limb, evidence of sensory deficit outside of the area tissue injury or motor deficit will be noted.

ANALYTIC TESTS

BLOOD SAMPLE COLLECTION

The information below outlines the timeframes for blood collection and lab supplies to be used. Clinical blood samples will be collected and analyzed in the Durham VAMC Clinical Laboratory. Research blood samples will be collected and analyzed using the below noted protocols.

A clinical blood sample will be collected to test the level of valproate acid at the completion of the trial medication administration (day 6 or on the day of discharge from the hospital); this sample will be sent to Quest Diagnostics for analysis and results will be returned in a sealed envelope.

If liver function tests and complete blood count have been performed within 24 hours of anticipated study labs, these will not be duplicated.

DAY OF SURGERY

A *research* blood sample of 22ml (less than 1.5 tablespoons) will be collected from subjects for plasma, RNA and DNA.

COMPLETION OF STUDY DRUG

The second blood draw of 34ml (approximately 2 tablespoons) will be performed for *research analysis* (plasma, RNA and DNA), and *clinical analysis* (liver function tests: Aspartate aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase, bilirubin, complete blood count (CBC) and VPA level. It will be obtained at the completion of the trial medication administration. If liver function tests and complete blood count have been performed within 24 hours of anticipated study labs, these will not be duplicated. Blood collection for VPA level will be turned into the VA lab for pick up by Quest Diagnostics.

TCA DRUG LEVEL TESTING

If the patient is on a tricyclic antidepressant (TCA) medication and the daily dose is increased to exceed 50mg during the week of study drug administration, a blood sample will be collected for TCA drug level and sent to Quest Diagnostics for analysis. If the drug level is greater than 400ng/ml or the patient appears to be experiencing drug-related side-effects, the TCA will be reduced to the original dose or discontinued.

THREE-MONTH FOLLOW-UP

The third and final blood draw of 30ml (approximately 2 tablespoons) will be performed for *research analysis* (plasma, RNA and DNA), and *clinical analysis* (complete blood count (CBC) and liver function tests: Aspartate aminotransferase [AST], Alanine Aminotransferase [ALT], Alkaline Phosphatase, and bilirubin. These will be taken during the pain clinic follow-up (approximately 3 months following surgery). The time of day and duration of pre-sample fasting will be collected to control for any potential interaction with circadian rhythms and fasting status.

At the three-month follow-up, any additional surgeries will also be documented. A description of the type and location of the surgery will be noted. If the surgery involves the injured "study" limb, this will also be recorded.

BLOOD COLLECTION SUMMARY

Analysis	Tube / ml	Day of Surgery	Post-Op Day 6	3 Month Follow-up
Plasma	BD™ P100 / 8.5	X	X	X
RNA	2 x PAXGene RNA / 2.5	X	X	X
DNA	PAXGene DNA / 8.5	X	X	X
Liver Function	BD™ Vacutainer SST / 5.0		X	X
CBC	BD™ Vacutainer K2 EDTA / 3.0		X	X
VPA level	BD™ Vacutainer Serum / 4.0		X	
TCA level	BD™ Vacutainer Serum / 4.0		X	

BLOOD SAMPLE KITS

Duke University will supply lab kits to the Durham VAMC research team packaged per collection time point: 1) Pre-op/Day of Surgery; 2) Post-Op Day 6; 3) Three-month follow up.

PRE-OP / DAY OF SURGERY KIT

Contents:

a) One (1) cryo box (6"x6"x5") labeled with patient's barcode ID number (box bottom will be labeled by hand)
b) One (1) BD Vacutainer Safety-Lok Blood Collection Set
c) One (1) Blood Collection Barcode Form labeled with patient ID number (*appendix F*) and seven barcodes labels, in duplicate.
d) One (1) body chart labeled with patient ID number (*appendix E*)
e) One bag labeled 'Pre-op Kit' containing 1xP100 tube, 3 blue-top cryovials, 2xPAXgene RNA tubes, 1xPAXgene DNA tube.

POST-OP DAY 6 KIT (OR END OF STUDY DRUG ADMINISTRATION)

Contents:

a) Samples collected at this time point will be added to the patient's cryo box provided with the 'Pre-op Kit'.
b) One (1) BD Vacutainer Safety-Lok Blood Collection Set
c) Seven (7) barcode labels in duplicate.
d) One bag labeled 'Post-Op Day 6 Kit' containing 1xP100 tube, 3 blue-top cryovials, 2xPAXgene RNA tubes, 1xPAXgene DNA tube
e) Provided by Quest: pouch, requisition, BD™ Vacutainer Serum tube, and blood collection tube for VPA and TCA levels.

THREE-MONTH FOLLOW-UP KIT

Contents:

a) Samples collected at this time point will be added to the patient's cryo box provided with the 'Pre-op Kit'.
b) One (1) BD Vacutainer Safety-Lok Blood Collection Set
c) One (1) body chart labeled with patient ID number
d) Seven (7) barcode labels in duplicate.
d) One bag labeled 'Post-Op Three months Follow Up Kit' containing 1xP100 tube, 3 blue-top cryovials, 2xPAXgene RNA tubes, 1xPAXgene DNA tube

Each kit contains a strip of barcode labels in duplicate to be used for labeling of tubes to be sent to Duke; the duplicate barcode labels are to be placed on the Blood Collection Barcode Form in the applicable box. Tubes and vials must not be interchanged with contents from another kit.

BLOOD SAMPLE COLLECTION

All human body fluids should be handled as potentially infectious. Wearing gloves and other personal protective equipment during sample collection and preparation is required by the OSHA Bloodborne Pathogens Standard. All materials contaminated with blood or body fluids should be disposed of in accordance with OSHA guidelines.

Please follow this recommended order of draw, when collecting samples using multiple tubes, to avoid cross contamination caused by tube contents:

	Collection Tube	Mix by Inversion
1	BD Vacutainer Serum (VPA level) (day 7/end of study drug only)	No mixing
2	BD P100	8-10 times
3	PAXgene RNA x 2	8-10 times
4	PAXgene DNA	8-10 times

When collecting blood in these tubes, place a barcode label on each and affix the duplicate barcode label in the appropriate box on the Sample Collection Barcode Form.

Venous blood samples may be obtained via direct venipuncture or via other available venous access (e.g., an existing peripheral intravenous line or hep-lock) – as long as the hospital staff follows their protocol for first withdrawing blood to flush the line.

BD™ P100 BLOOD COLLECTION FOR PLASMA

BD™ P100 tubes contain proprietary stabilizers that immediately solubilize during blood collection, enhancing recovery and preservation of plasma analytes such as proteins and polypeptides.

Using the Vacutainer Safety-Lok System, center the P100 tube in the single patient use holder and push tube in one swift movement. The non-patient needle must penetrate the tube stopper and the mechanical separator in the center.

Position the tube vertically below the patient's arm during collection. Allow vacuum to be exhausted (approximately 10 seconds) prior to removing the tube from the non-patient needle.

Slowly invert tube 8-10 times immediately after blood collection to mix the blood and additives then place upright in the 49-count cryo box.

PAXGENE BLOOD RNA (2.5ML)

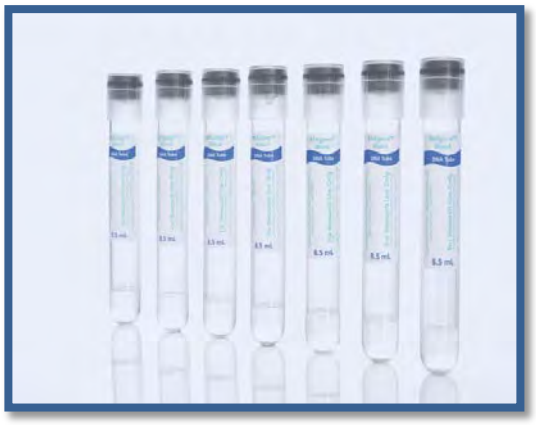
The PAXgene RNA tube time two will be used for blood collection, RNA stabilization, specimen transport and storage. It is prefilled with an RNA stabilization reagent to provide immediate RNA stabilization. The blood cell lysis in the tube simplifies subsequent RNA purification. It also allows for consistent blood draw volume and blood-to-additive ratio.

Collect blood into the two (2) RNA tubes using the Vacutainer Safety-Lok system.
Hold the tube vertically, below the subject's arm, during collection.

Allow at least 10 seconds for a complete blood draw to take place.
Ensure that the blood has stopped flowing into the tube before removing the tube from the holder.

Gently invert the PAXgene Blood RNA tube 8 to 10 times and then place upright in the 49-count cryo box. Repeat process for second RNA tube.

PAXGENE BLOOD DNA (8.5ML)



Blood is collected in PAXgene Blood DNA Tube (blue top), which contains a proprietary blend of reagents that both prevents blood coagulation and stabilizes white blood cells.

The tube must be at room temperature (18°C to 25°C) prior to use.

The PAXgene Blood DNA Tube should be the last tube drawn.

Collect blood into the tube using the same technique as before.

Ensure blood has stopped flowing into tube before withdrawing tube from holder.

VPA LEVEL BD VACUTAINER SERUM (4.0ML)

All supplies necessary will be provided by Quest Diagnostics.

USE OF BD VACUTAINER SAFETY-LOK™ BLOOD COLLECTION SET AND HOLDER

1) It is important to use a 12 inch blood collection set when collecting blood using this device.
2) Wear gloves during venipuncture and when handling blood collection tubes to minimize exposure hazard.
3) Select tube or tubes for required specimen.
4) Assemble a blood collection set with 12 inch tubing into a BD Vacutainer® One Use Holder. Be sure that blood collection set is firmly attached to holder and does not unthread during use.
5) Place tube into holder. Note: Do not puncture stopper.
6) Select site for venipuncture.
7) Place patient's arm in a downward position.
8) Apply tourniquet. Prepare venipuncture site with appropriate antiseptic technique. Do not palpate venipuncture site after cleansing.
9) Remove needle shield. Perform venipuncture with arm downward and tube stopper up (refer to the Prevention of Backflow section).
10) Center tube in the holder to prevent sidewall penetration and resultant premature vacuum loss.
11) Push tube onto non-patient-end (NP-end) of needle in one **swift action** in order to minimize premature separation of the mechanical separator from the stopper. Hold tube on NP-end during drawing.
12) Do not allow contents of the tube to contact the stopper or end of the needle during procedure.
13) Allow vacuum to be exhausted prior to removing the tube from the NP (non-patient) end of the needle.
14) Slowly invert each tube 8 to 10 times immediately after blood collection.

PREVENTION OF BACKFLOW

Since PAXgene Blood RNA and DNA tubes contain a chemical additive, it is important to avoid possible backflow from the tube. To guard against backflow, observe the following precautions:

1) Place donor's arm in a downward position.
2) Hold tube with the stopper uppermost.
3) Make sure tube additives do not touch stopper or end of the needle during venipuncture.

BLOOD COLLECTION TIME TABLE – VALPROATE STUDY

P100 (1) 1) Day of surgery 2) End of study drug 3) 3 month follow-up	Centrifuge at 2500g for 20 min * Please refer to attached P100 instructions for use	Label each blue 1.8ml cryovial with barcode label; place duplicate label on patient's Sample Collection Barcode Form.	Aliquot 1ml plasma into 2-3 cryo vials	Place in patient's cryo box and put in 20^{0}C freezer for 24-36 hours. with all other patient's samples; then move box with all the samples to the -80^{0}C freezer until shipped.
PAXgene RNA 1) Day of surgery 2) End of study drug 3) 3 month follow-up	2 tubes per draw. Place upright in rack at room temperature for 2-4 hours	Label both tubes with barcode label and place duplicate labels for each on patient's Sample Collection Barcode Form.	Place tubes upright in the -20^{0}C freezer for 24- 36 hours	Add the tubes to patient's cryo box, upright, in the -80^{0}C freezer until shipped.
PAXgene DNA 1) Day of surgery 2) End of study drug 3) 3 month follow-up	1 Tube per draw. Place upright in rack at room temperature for 2-4 hours	Label tube with barcode label and place duplicate label on patient's Sample Collection Barcode Form.	Place tubes upright in the -20^{0}C freezer for 24- 36 hours	Add the tube to patient's cryo box, upright, in the -80^{0}C freezer until shipped.
VPA level BD Vacutainer serum 4.0ml 1) End of study drug	To be drawn and processed by the designated study team member.			Sample will be sent to Quest Diagnostics for VPA level analysis
Result for **Liver function test** at end of the study drug and at 3 month follow-up visit will be obtained from the LOCAL hospital lab.				
Result for **CBC** test at end of the study drug and at 3 month follow-up visit will be obtained from the LOCAL hospital lab.				
Result for **TCA** level if needed during treatment period will be obtained and sent to Quest for analysis.				

*__NOTE:__ P100 – spin at 2500*g* for 20 minutes in a swing bucket or 45 degree fixed angle rotor. Alternate Centrifugation for P100 conditions if 2500*g* cannot be met: 1100*g* for 30 minutes or 1600*g* for 30 minutes

BLOOD COLLECTION SUMMARY

Samples will be collected and processed as outlined in Blood Sample Collection and Blood Sample Processing sections.

The patient's cryo box will contain:

1) Three blue top cryo vials (processed from P100) for each of the following time points: day of surgery, end of study drug administration and three months follow up visit.
2) Two (2) PAXgene Blood RNA tubes for each of the following time points: day of surgery, end of study drug administration and three months follow up visit.
3) One (1) PAXgene Blood DNA tube for each of the following time points: day of surgery, end of study drug administration and three months follow up visit.

When transported to Duke, the patient's box will contain the patient's 12 cryo vials, 6 PAXgene RNA tubes and 3 PAXgene DNA tube, all in the patient's labeled cryo box (one cryo box per patient), along with a the patient's Sample Collection Barcode forms. Barcode labels on the box and each tube will correspond with all barcode labels on the patient's Sample Collection Barcode forms. The original Barcode forms will be sent to Duke with the frozen samples and copies will be kept at the VA in the subject's folder.

Kits will be provided to both sites by Duke; they will be hand-carried to, and stored at, Durham VAMC for future use and replenished as needed.

REDCAP DATABASE

This study will utilize the REDCap database to capture study data via electronic Case Report Form (eCRF). REDCap is a web-based electronic data capture application designed to facilitate the collection and cleaning of clinical trial data. The site staff will need to have access to a computer capable of accessing the REDCap website.

SOURCE DOCUMENTATION

- Data should be collected from source documents which must be filed in the patient folder, then entered into the electronic CRF.
- Information will be collected on the appropriate printed REDCap data collection forms for each time point. These forms should be printed in advance and readily available when a patient is enrolled.

COMPLETION OF ECRFS

- The eCRFs are not to be completed until it has been determined whether or not the subject is a Screen Failure or if the subject will be randomized. Only subjects who are randomized will have eCRFs completed. Data for Screen Failures are not to be entered in the REDCap database.
- Once subject eligibility has been determined, the data collected from each subject must be entered into the REDCap database after the subject has completed or withdrawn from the study.
- Completion of the electronic Case Report Form (eCRF) for all subjects at the site must be the responsibility of one or more study staff member(s) listed on the Delegation of Authority form.

DATA ENTRY – EVENT GRID

The Event Grid displays the form-by-form progress of data entered into the project for one particular Study ID for all defined events. You may click on the colored buttons to access the form for a specific event.

Valproate ID **100**

Data Collection Instrument	Events											
	Screening (1)	Day of surgery (2)	Post-Op Day 1 (3)	Post-Op Day 2 (4)	Post-Op Day 3 (5)	Post-Op Day 4 (6)	Post-Op Day 5 (7)	Post-Op Day 6 (8)	End of Sudy (9)	Follow-up 1 month ± 2 weeks (10)	Follow-up 3 month ± 2 weeks (11)	Follow-up 6 month ± 2 weeks (12)
Inclusion / Exclusion Criteria	●											
Demographics	●											
Narcotic Medications Within Last 24 Hours	●	●	●	●	●	●	●	●	●	●	●	●
Current Non-Narcotic Medications	●	●	●	●	●	●	●	●	●			
Past Medical History	●											
Previous Surgical Interventions	●											
PHQ-2	●											
Defense And Veterans Pain Rating Scale Dvprs	●	●	●	●	●	●	●	●	●	●	●	●
Richmond Agitation Sedation Scale Rass	●	●	●	●	●	●	●	●	●			
Brief Pain Inventory Short Form (BPI)	●									●	●	●
S-LANSS Pain Score	●									●	●	●
Type of surgery	●											
Amputation Injury at enrollment	●											
Amputation injury at follow up visit											●	●
Neuroma focal Neuralgia Pain Questions	●									●	●	●
Complex Regional Pain Syndrome Questions	●									●	●	●
Phantom Limb Pain	●										●	●
Residual Limb Questions	●										●	●
Prosthesis	●										●	●
Exam And Visual Documentation	●										●	●
Study Drug Administration		●	●	●	●	●	●	●	●			
Blood Samples and Results		●							●		●	
Valproate Level Blood Test									●			
Tricyclic antidepressant level (TCA)		●	●	●	●	●	●	●	●			
AE or SAE		●	●	●	●	●	●	●	●	●	●	●
Completion Data												●

DATA ENTRY PAGE

Go to https://redcap.dtmi.duke.edu/redcap/ and log on with your user name and password. Click on 'Valproate Study', then 'Data Entry'. Choose the 'existing Valproate ID' from the dropdown list for the patient's ID you are ready to enter. Most pages include detailed instructions. To create a new record/response, type a new value in the text box below and hit Tab or Enter. You may view an existing record/response by selecting it from the drop-down

lists on the Data Entry Page. To quickly find a record without using the drop-downs, the text box will auto-populate with existing record names as you begin to type in it, allowing you to select it.
Eligibility: The first data collection instrument is 'Inclusion/Exclusion Criteria'. Click on the red bullet and answer all questions, then 'save record'. Once saved, the bullet will turn green.

Add the completed and signed Inclusion/Exclusion form, ICF and HIPAA Authorization form to the patient folder. Pull the 'Body Diagram' form (*appendix E*) from the sample kit (will be marked with patient number). Answer the first 2 questions on the Demographics form by confirming that a signed ICF and HIPAA Authorization have been added to the Patient Record.

Gather the applicable REDCap data collection forms and questionnaires (printed on paper) to be answered by the patient and the Body Diagram for the 'Pre-op / Day of Surgery time point. If the patient does not currently have an amputation, several of the forms relating to a current amputation will not be given to the patient.

NOTE: Listed below is information which can be recorded **after** interviewing the subject:

- Past Medical History – point total
- Amputation and Injury form (if needed for this visit)
- Narcotic Meds (before or after interview from medical record)
- Current non-narcotic medications
- PHQ-2 – point total and history of depression
- Demographics
- Past Medical History. This is an entry form where you can ask the patient about their medical history. Return to this form later to calculate total points.
- Previous Surgical Interventions

The DVPRS Questionnaire (actual colored forms for patient to fill in) is to be completed at the time of enrollment, daily during study drug administration, as well as the 1, 3 and 6 month time points.

The RASS questionnaire is to be completed by the Research Coordinator, Nurse or delegated study personnel at the time of study enrollment and during study drug administration.

The BPI questionnaire is to be completed at time of enrollment, as well as the 1, 3 and 6 month time points. Give the 'body diagram' to the subject and begin reading the section on how to fill in the diagram. (X=area of most pain; shaded=all areas of pain) **The subject must mark the body diagram with the pen provided.**

- All patients will be assessed with the following forms at the time of enrollment, as well as the , 3 and 6 month time points:
  - Neuroma/Focal Neuralgia Pain
  - Complex Regional Pain Syndrome

- S-LANSS Pain Score is to be completed at time of enrollment, 1, 3 and 6 months. Continue to allow the subject to refer to the 'body diagram' and answer questions in Redcap. After #7, put the 'body diagram' aside. Mark 'incomplete' (total score later) and then 'Save and go to next form'.

- Amputation and Injury – If patient does not have a current amputation pre-surgery, mark 'no' and none of the following questionnaires will be applicable. This form will be completed at the 3 and 6-month follow-up visits.

- Patients with a current amputation will be given the following questionnaire forms to complete at time of enrollment, 3 and 6 months:

    1) Phantom Limb Pain
    2) Residual Limb Pain
    3) Prosthesis

DATES AND TIMES

Visit dates will be chosen from drop box.
The 24-hour clock time designation should be used (00:00 – 23:59) and entered as hh:mm.

CONCOMITANT MEDICATIONS

Information regarding narcotic dosage can be taken from the patient's medical record.
Drugs are to be recorded using the medication classes.

ADVERSE EVENT COMPLETION GUIDELINES

- Use acceptable medical terms
- Do not capture a procedure; the reason for the procedure will be the event name
- Do not capture the event term as hospitalization or death; capture the cause if known
- Do not capture symptoms of a disease; the diagnosis will be the event name
- Documentation on the AE and SAE CRF page must be supported by the source documents

APPENDICES

VAMC IRB Protocol# 01709

Checklist for VA Research Study Compliance

PRE-ENROLLMENT	WHO (initials)	Date	Notes
Screening in Vista (if approaching before surgery)			
Inclusion/Exclusion criteria (double-checked by **2nd person**)			
Abbrev. Mini Mental State Exam (MMSE) completed			
ENROLLMENT			
Current ICF signed before **ANY** facet of research study begun			
Patient & CRC signature on ICF & date			
Pt. initials, Genetic Testing, ICF, pg. 4			
CRC adds full SS# to 1st pg. of ICF & HIPAA			
Patient signature on HIPAA			
Copy of ICF & HIPAA to patient; research brochure to patient.			
Original ICF & HIPAA to VA folder			
Put signed copy of script in pt. folder			
Add patient data to VA tracking log			
CPRS NOTES & SCANNING within 24 hrs. of enrollment			
Patient consent note in CPRS by whoever witnessed consent			
Patient study participation note in CPRS by whoever witnessed consent			
Note in CPRS that brochure was given to patient			
Scan ICF, HIPAA Auth. & Form 10-9012 into CPRS			
DURING STUDY			
CPRS follow-up during patient hospital stay for AE/SAE's			Circle Day when complete: 1 2 3 4 5 6 7
Read D/C note for potential AE/SAE's			
Check CPRS notes for AE/SAE - 2 wks. post discharge			
Mail 1-month questionnaires to patient			
Check CPRS notes for AE/SAE- 4 wks. post discharge			
END OF STUDY			
Remove study participation note from CPRS.			

Valproate Study Randomization Assignments: **Durham VA Site**

SSN	Redcap	Primary Amputation		
	ID #	Study ID	Assignment	Date Assigned
	127501	1001	Val or Pla	
	127502	1002	Val or Pla	
	127503	1003	Val or Pla	
	127504	1004	Val or Pla	
	127505	1005	Val or Pla	
	127506	1006	Val or Pla	
	127507	1007	Val or Pla	
	127508	1008	Val or Pla	
	127509	1009	Val or Pla	
	127510	1010	Val or Pla	
	127511	1011	Val or Pla	
	127512	1012	Val or Pla	
	127513	1013	Val or Pla	
	127514	1014	Val or Pla	
	127515	1015	Val or Pla	
	127516	1016	Val or Pla	
	127517	1017	Val or Pla	
	127518	1018	Val or Pla	
	127519	1019	Val or Pla	

SSN	Redcap	Revision		
	ID #	Study ID	Assignment	Date Assigned
	127643	1201	Val or Pla	
	127644	1202	Val or Pla	
	127645	1203	Val or Pla	
	127646	1204	Val or Pla	
	127647	1205	Val or Pla	
	127648	1206	Val or Pla	
	127649	1207	Val or Pla	
	127650	1208	Val or Pla	
	127651	1209	Val or Pla	
	127652	1210	Val or Pla	
	127653	1211	Val or Pla	
	127654	1212	Val or Pla	
	127655	1213	Val or Pla	
	127656	1214	Val or Pla	
	127657	1215	Val or Pla	
	127658	1216	Val or Pla	
	127659	1217	Val or Pla	
	127660	1218	Val or Pla	
	127661	1219	Val or Pla	

SSN	Redcap	Surgery on Limb w/ Neurologic Damage		
	ID #	Study ID	Assignment	Date Assigned
	127743	1401	Val or Pla	
	127744	1402	Val or Pla	
	127745	1403	Val or Pla	
	127746	1404	Val or Pla	
	127747	1405	Val or Pla	
	127748	1406	Val or Pla	
	127749	1407	Val or Pla	
	127750	1408	Val or Pla	
	127751	1409	Val or Pla	
	127752	1410	Val or Pla	
	127753	1411	Val or Pla	
	127754	1412	Val or Pla	
	127755	1413	Val or Pla	
	127756	1414	Val or Pla	
	127757	1415	Val or Pla	
	127758	1416	Val or Pla	
	127759	1417	Val or Pla	
	127760	1418	Val or Pla	
	127761	1419	Val or Pla	

Subject Identification Log (Durham VAMC) page 1

From_____ Present Date_____

Project Title: "Regional Anesthesia and Valproate Sodium for the Prevention of Chronic Post-Amputation Pain" MIRB#01709

Subject Study Number	Subject Name	Last 4 SSN (1234)	Date of Informed Consent	Informed Consent obtained by:	Notes

Subject Identification Log (Durham VAMC) page 2

From_____ Present Date_____

Project Title: "Regional Anesthesia and Valproate Sodium for the Prevention of Chronic Post-Amputation Pain" (MIRB#01709)

Subject Study Number	Subject Name	Last 4 SSN (1234)	Date of Informed Consent	Informed Consent obtained by:	Notes

INVESTIGATIONAL STUDY SCRIPT

Title: Regional Anesthesia and Valproate Sodium for the Prevention of Chronic Post-Amputation Pain
PI: Thomas Buchheit, MD
VAMC IRB Protocol# 01709

INV-VA VALPROATE Study VPA/PBO 250 mg/5mL

REDCap ID # 127501 **Study ID#** [ ] **Date/Time: 10/24/2013 2:18 PM**

DISP: 4 oz.

Take 1 teaspoon (5ml) every 8hours for 7 days or until discharged from hospital

Patient Name: John Doe

SSN: 123-45-6789

Physician Signature: _____ Print name: _____

Received by: _____PharmD Date: _____ Time: _____

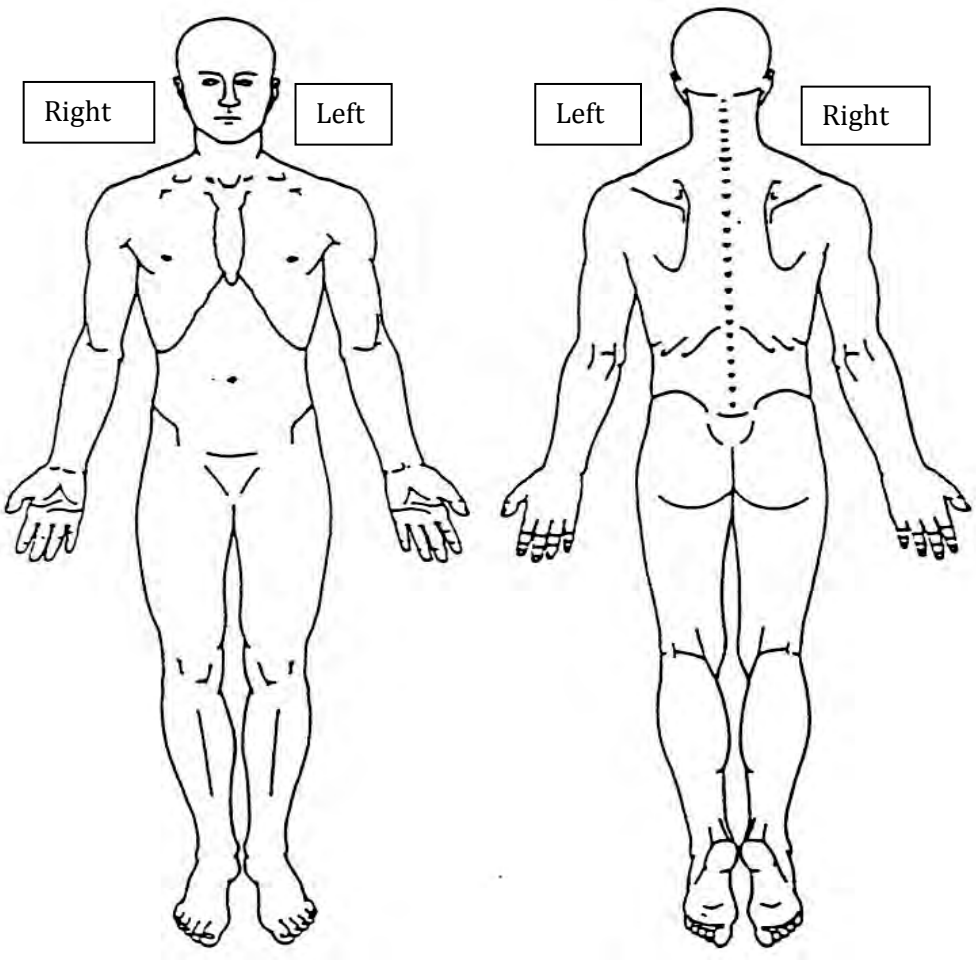
APPENDIX E – BODY DIAGRAM (VPA)

Patient ID#: _____ **VISIT: (circle one)** **Date: _____**

1-Pre-op/enrollment
2- One month follow up
3-Three month follow up
4-Six month follow up

Instructions:

On the body diagram below, please shade in the areas where you feel pain. Put an X on the area that hurts the most.



Indicate on the line below how you would describe your present pain by placing a mark on the line between the two extremes of experiencing no pain at all and experiencing the worst pain you have ever felt.

No Pain — Worst Pain Possible

VISIT: (circle one) 1-Day of Surgery **Date of Blood Draw: _____** **Time: _____**
2-End of Study Drug
3-Three Month Follow up

Patient No.		_____	**COLLECTION TUBES** 	_____	_____
Aliquots **P100-1**		_____	**P100**		_____
P100-2		_____	**DNA - 1**		_____
P100-3		_____	**RNA - 1**		_____
		_____	**RNA - 2**		_____
_____	_____	_____	**BD Vacutainer Serum 4.0ml** VPA level (sent to Quest)		_____

When collecting draws 2 and 3, please write in patient ID# by hand on form.

Red – requires a barcode number (label), will be processed, frozen and shipped.

P100 tube will be aliquoted then discarded.

Pain Medicine 2012; 13: 1474–1490
Wiley Periodicals, Inc.

Attachment 4

ACUTE & PERIOPERATIVE PAIN SECTION

Original Research Articles

Epigenetics and the Transition from Acute to Chronic Pain

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Conflict of interest/disclosure: The authors report no conflicts of interest.

Abstract

Objective. The objective of this study was to review the epigenetic modifications involved in the transition from acute to chronic pain and to identify potential targets for the development of novel, individualized pain therapeutics.

Background. Epigenetics is the study of heritable modifications in gene expression and phenotype that do not require a change in genetic sequence to manifest their effects. Environmental toxins, medications, diet, and psychological stresses can alter epigenetic processes such as DNA methylation, histone acetylation, and RNA interference. As epigenetic modifications potentially play an important role in inflammatory cytokine metabolism, steroid responsiveness, and opioid sensitivity, they are likely key factors in the development of chronic pain. Although our knowledge of the human genetic code and disease-associated polymorphisms has grown significantly in the past decade, we have not yet been able to elucidate the mechanisms that lead to the development of persistent pain after nerve injury or surgery.

Design. This is a focused literature review of epigenetic science and its relationship to chronic pain.

Results. Significant laboratory and clinical data support the notion that epigenetic modifications are affected by the environment and lead to differential gene expression. Similar to mechanisms involved in the development of cancer, neurodegenerative disease, and inflammatory disorders, the literature endorses an important potential role for epigenetics in chronic pain.

Conclusions. Epigenetic analysis may identify mechanisms critical to the development of chronic pain after injury, and may provide new pathways and target mechanisms for future drug development and individualized medicine.

Key Words. Epigenetics; Pain; DNA Methylation; Histone Deacetylase Inhibitors; RNA Interference

Introduction

In recent years, we have developed a better understanding of the cellular mechanisms that link inflammation, peripheral sensitization, and pain [1]. In addition, we have learned more about the human genetic code [2] and mutations (particularly single nucleotide polymorphisms [SNPs] and copy number variations) that are associated with specific chronic pain syndromes [3,4]. These physiologic and genetic advances, however, do not fully explain why one patient develops chronic pain following an injury, and another patient does not. Despite recent improvements in techniques for acute pain management, 30–50% of patients still develop chronic pain following surgeries such as amputation, thoracotomy, hernia repair, and mastectomy [5].

It is also notable that monozygotic twins may exhibit significantly different inflammatory and chronic pain phenotypes [6–8], indicating that the etiological basis of these

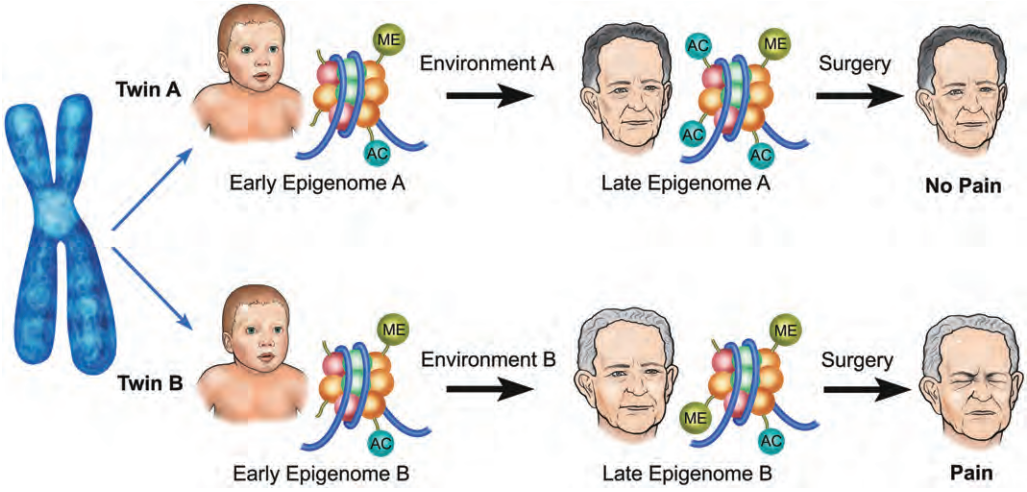

Figure 1 Epigenome and chronic pain. Twin A and Twin B demonstrate similar "epigenomes" at birth with few (if any) differences in methylation and acetylation patterns. Environmental factors throughout development affect histone acetylation patterns and cytosine methylation patterns, resulting in phenotypic differences by adulthood. With surgery or nerve injury, these epigenetic differences may result in differing risks of chronic pain.

disorders is not due simply to differences in genetic sequence. We now appreciate that response to injury is determined by complex interactions between the genome and the environment. These alterations might well be epigenetic in nature, i.e., heritable modifications that are not intrinsic to the genetic code, but that affect gene expression in a tissue-specific manner, resulting in an observable phenotype (Figure 1) [9].

Epigenetic processes are responsible for cellular differentiation during embryogenesis and are critical for normal development [10]. These processes also play an important role in memory formation, as correlations between hippocampal activity, DNA methylation, and histone phosphorylation in the brain have been found [11,12]. The spinal cord sensitization seen in painful conditions shares common mechanisms with the neural plasticity of memory formation [13], and it is likely that similar epigenetic mechanisms regulate both of these neural processes.

Multiple examples of the importance of epigenetic influences in development are found throughout nature. One of the best-described cases of environmental influence on gene expression involves the control of bee development by ingesting royal jelly. This nutritive substance induces changes in juvenile bee DNA methylation patterns and leads to development of the bee's phenotype to become a queen rather than a worker [14]. The concepts of epigenetic heritability and stability have also been described in plants [15] and mammals [16]. For instance, high-fat diets fed to paternal rats induce functional changes in β-islet cells of female offspring [16]. Similar modifications in DNA methylation were noted in the fathers and offspring, suggesting the nongenetic heritability of this metabolic disorder.

Nondevelopmental epigenetic modifications are also triggered by environment, nutrition, and stress [17–19], and may play a role in the onset of chronic pain following nerve injury [20,21]. We have long appreciated the importance of the psychosocial environment to the incidence and severity of chronic pain [22–27], and mounting evidence suggests that epigenetic mechanisms supply the link between disease expression and environment [18,28]. Nongenetic factors are important in the development of cancer [29,30], neurologic disorders [31], and painful disorders such as bladder pain syndromes [7], myofascial pain [32], and temporomandibular joint pain [8]. Twin disease models of neurodegenerative conditions [33], inflammatory periodontal disease [34], and autoimmune disease [35] demonstrate variable disease expression depending on the DNA methylation pattern [6].

Environmental factors alter gene expression and phenotype for painful disorders by inducing epigenetic modifications such as histone acetylation, DNA methylation, and RNA interference (RNAi) [36–38]. Following injury, expression of transcription factors such as nuclear factor-kappa B (NF-κB) is increased [39], sodium channels in the injured axon are upregulated [40], μ-opioid receptors in the dorsal root ganglion are downregulated [41,42], substance P expression is altered [43], and the dorsal horn of the spinal cord is structurally reorganized through axonal sprouting [44]. As with DNA variation, epigenetic modifications may be inherited and may be propagated over multiple cell divisions; however, they are flexible enough to respond to

modifying influences. This concept may in part explain how we interact with our environment at the (epi)genomic level, and is potentially of great importance in understanding the relationship between gene expression and complex diseases such as chronic pain.

Genetics, Epigenetics, and Pain

Over the past several decades, much has been written about the association of genetic polymorphisms and the development of chronic pain [45,46]. It was believed that, through knowledge of genetic variation, we could develop the foundation for individualized medicine that optimizes therapy for each patient based on one's specific genetic sequence [47]. Expectations for personalized medicine were high after completion of the human genome project [2], but thus far, our ability to use the genetic code to prevent or improve chronic pain has been somewhat limited [48]. It is the heretofore unquantifiable environmental effect that has been one of the limitations of genetic studies [45].

Multiple candidate gene association studies have been used for the investigation of pain, but have been limited by their focus on genomic regions where the pathophysiology is thought to be reasonably well understood. They are not designed to analyze painful conditions that result from interactions of multiple genes [49]. A few candidate gene polymorphisms have been linked to pain susceptibility, including catechol-O-methyltranferase (COMT). This gene modulates nociceptive and inflammatory pain and has been linked to temporomandibular joint pain syndromes [50]. Even studies of COMT, however, have demonstrated inconsistencies. Some investigators have found an association between a COMT SNP val158met [4,50] with increasing pain responses, while others failed to replicate these findings [51,52].

The SCN9A gene has also been studied as a marker for pain sensitivity. Mutations in this gene, which codes for the alpha-subunit of a voltage-gated sodium channel ($Na_v1.7$), are known to result in alterations of pain perception [53], and have been noted in rare pain disorders such as erythromelalgia and paroxysmal extreme pain disorder [54,55]. SCN9A polymorphisms have also been described in individuals who are insensitive to pain [3,56]. Although the implications of the SCN9A gene polymorphism are clear, clinical applications of this knowledge remain limited [47].

Genome-wide association studies (GWAS) have been used in an attempt to overcome some of the limitations of candidate gene analysis. These studies tell us where the genetic variation exists, but do not always fully explain the underlying biology. Furthermore, although GWAS have identified thousands of genetic variations in complex diseases, most of the variants confer only a modest risk with an odds ratio for disease of <1.5. These genetic variants, therefore, account for only a small fraction of the population attributable risk for heritable complex traits [57,58], implying a strong nongenetic predisposition to disease. GWAS directed toward painful conditions remain limited in number [45].

Specific Epigenetic Modifications

Histone Modifications

Histones octamers and their surrounding DNA form a nucleosome, the fundamental building block of chromatin (Figure 2A). The N-terminal histone tails may be modified by more than 100 different posttranslational processes including acetylation, phosphorylation, and methylation (Figure 2B). Most of the histone complex is inaccessible, but the N-terminal tail protrudes from the nucleosome and is therefore subject to additions that change the three-dimensional chromatin structure and subsequent gene expression [59,60]. One of the more common modifications involves acetylation. Histone acetyl transferases add acetyl groups, altering the histone protein structure. This change prevents the chromatin from becoming more compact, allowing transcription factors to bind more easily. This state of increased acetylation and "permissive chromatin" generally increases transcription activity and RNA production from that genetic sequence, especially when located in gene promoter regions [61,62]. Conversely, histone deacetylases (HDACs) remove acetyl groups from histones, generally suppressing gene expression. In concert, these activities serve important regulatory functions.

DNA Methylation

Another ubiquitous epigenetic modification involves methylation of DNA cytosine nucleotides. In this process, DNA methyltransferase enzymes (DNMT1, DNMT3A, and DNMT3B) add a methyl group to the 5-carbon of the cytosine pyrimidine ring, converting it to 5-methylcytosine. This methylation generally silences gene expression either by preventing the binding of transcription factors [63,64], or by attracting methylated DNA-binding proteins such as MeCP2 that themselves repress transcription (Figure 2C) [65,66]. The methylation process is vital for normal embryonic development and growth [67], and these methylation patterns are propagated during cell division.

The degree of cytosine methylation tends to mirror the degree of tissue specialization. For instance, DNA in neurologic tissue is highly methylated, while sperm DNA is relatively unmethylated [68]. More recent research has focused on the regulatory importance of cytosine methylation in promoter regions where methylation may silence a previously active gene sequence in the process of tissue specialization [69]. In addition to the cytosine nucleotides dispersed throughout the genome, there are regions particularly rich in cytosine-phosphate-guanine (CpG) linear sequences, described as "CpG islands" [70]. These "CpG islands" are found in promoter regions or first exons of approximately 60% of human genes, and are often unmethylated during development, allowing a transcriptionally active state [71]. Although promoter site methylation may

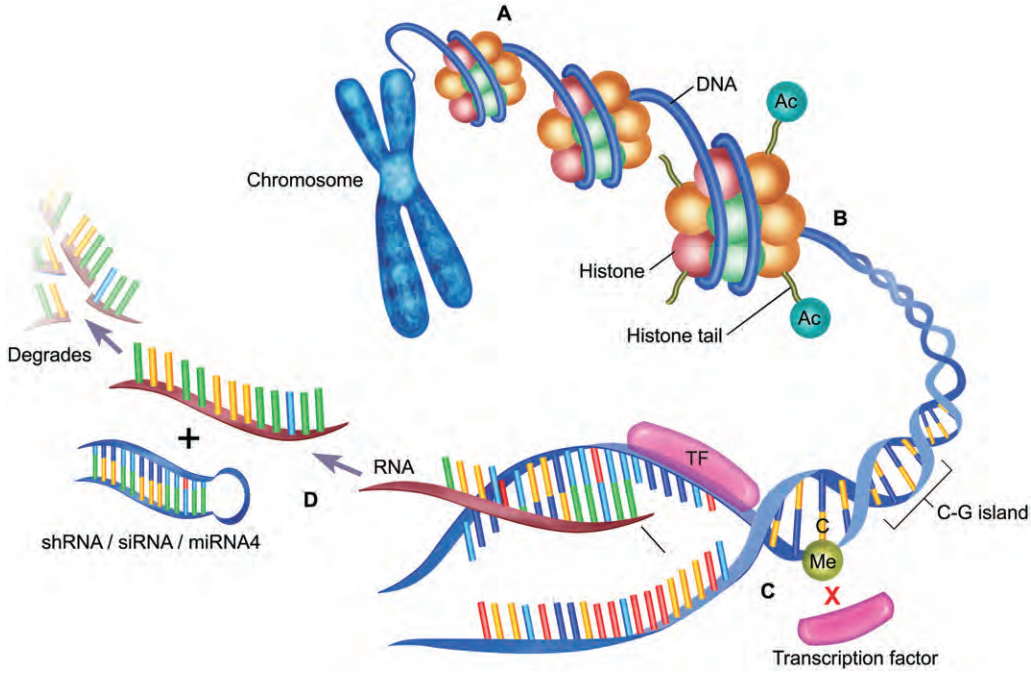

Figure 2 Epigenetic mechanisms. (A) DNA wraps around histone octamers to form a nucleosome, the fundamental building block of chromatin. (B) Histone proteins may be modified through several processes, including acetylation. The addition of an acetyl group to histone tails generally opens the chromatin structure and facilitates transcription factor binding, enhancing gene expression. (C) Methylation of cytosine nucleotides in C-G rich sequences ("CG islands") prevents the binding of transcription factors and generally silences gene expression. These CG islands are often found near promoter regions and serve a significant role in gene regulation. (D) Posttranscriptional regulatory mechanisms include short hairpin RNA (shRNA), small interfering RNA (siRNA), and micro RNA (miRNA) that bind RNA and induce their degradation.

silence gene expression during development, genes may still be reactivated even in specialized neurologic tissues [72,73]. This potentially modifiable plasticity of neural tissue methylation may hold promise for reversing the neurologic molecular remodeling that occurs during the transition from acute to chronic pain.

Several disease states, including cancer, schizophrenia, and opioid addiction, are associated with DNA methylation abnormalities [30,74–76]. In cancer, these altered methylation patterns may lead to tumor growth by downregulating tumor suppressor genes [30]. Methylated gene domains demonstrate not only stability, but also heritability [70]. The epigenetic influence across generations is demonstrated in rodent studies in which spermatogenesis is suppressed, and methylation patterns are altered for several generations after using the antiandrogenic compound vinclozolin during embryonic development [77].

Noncoding RNA

Gene expression can also be controlled by RNAi that involves endogenous molecules such as small interfering RNA (siRNA), microRNA (miRNA), and short hairpin RNA (shRNA). These small noncoding RNA molecules can silence gene expression by binding to mRNA and inducing subsequent degradation of the direct gene product (Figure 2D) [78]. These molecules can self-propagate through cell division and epigenetically transmit regulatory information across generations [79]. Interfering RNAs carry great therapeutic promise and have been used in animal trials for chronic neuropathic pain [80] and neurodegenerative disease [81], as well as in human clinical trials for cancer [82].

Our understanding of epigenetic processes has increased dramatically over the past decade. Efforts are currently underway, through such groups as the International Human Epigenome Consortium, to sequence and create maps of cell-specific DNA methylation and histone modifications [83].

Techniques of Epigenetic Analysis

There are many challenges in defining the specific epigenetic changes that lead to a particular disease state. Many earlier epigenomic studies have been limited by either inadequate genome survey or small sample size, and the

relationship in many diseases between phenotypic expression and epigenomic variation remains unclear [84]. It is unlikely that single gene epigenetic modification will explain the complex pain phenotypes seen after injury or surgery. Epigenome-wide association studies have been proposed as a possible solution to improve our understanding of the links between disease state and epigenetic modifications. Comprehensive epigenomic maps are currently being developed with promising future applications [84].

Another challenge with epigenetic studies and disease variation is the need for enhanced comprehension of the distinction between cause and consequence [84]. To fully understand if a particular biomarker represents the cause *of* a disease or the effect *from* a disease, we will need to perform analyses at multiple time points before and after the development of a disease. This initiative has already begun with the establishment of the U.S. National Institutes of Health Roadmap Epigenomics Mapping Consortium [85].

Regardless of the relationship between biomarkers and causation, however, epigenetic modifications throughout the course of a chronic disease can be used as biomarkers. In particular, DNA methylation is well suited as a potential predictive biomarker secondary to its relative chemical stability. Reliable biomarkers are critical if we are to develop personalized epigenetic interventions. Candidate markers would need to be found in an accessible space (blood), but still reflect the neurobiological process occurring at the proximal tissue (spinal cord/brain). Whether the circulating leukocyte epigenome can report on more inaccessible tissues (such as central nervous system [CNS]) is uncertain, but there is growing evidence that methylation patterns tend to be similar between proximal tissue and more easily accessible circulating blood cells. For example, it was recently shown that the pattern of CpG island methylation in the promoter region of the prodynorphin gene in both human brain tissue collected postmortem and matched peripheral blood mononuclear cells is virtually identical [86].

The burgeoning field of epigenetics is using novel technologies to measure these heritable, yet modifiable, patterns of transcriptional regulation. DNA methylation is analyzed through bisulfite sequencing that allows the epigenetic information present in the form of cytosine methylation to be retained during amplification (Figure 3B). Traditional molecular analysis of specific gene loci relies on the ability to amplify the DNA of interest using cloning and polymerase chain reaction (PCR) techniques. If this amplification is done, however, without somehow immortalizing the methylation status of a particular cytosine, that information will be lost after the first PCR cycle. To solve this problem, unmethylated cytosines can be modified through the bisulfite reaction, deaminating them to uracil. Methylated cytosines, however, are not deaminated by bisulfite, remaining unchanged during subsequent amplification. Probes can then be designed to determine whether a specific promoter region has retained a particular cytosine (previously methylated) or whether this cytosine has been converted to uracil (previously unmethylated). The methylation status of the promoter can then be determined using the cytosine/uracil ratio.

Histone protein modifications have also been studied since 1988 through a process of chromatin immunoprecipitation (ChIP) (Figure 3A) [87]. This process involves fragmentation of the chromatin and immunoprecipitation using an antibody to the protein or modification of interest. For example, an antibody to a specific acetylation site on histone H3 is used to precipitate all DNA associated with that particular acetylated histone. Following immunoprecipitation, the DNA fragments are then typically identified through microarray hybridization. More recently, "next generation sequencing" (NGS) technologies have been combined with ChIP, providing a high resolution, genome-wide analysis of histone modification. Whereas microarray techniques analyze regions of the genome previously identified, NGS carries the possibility of capturing all the DNA fragments isolated by immunoprecipitation [71]. These NGS technologies will continue to expand our understanding of epigenetic changes and the chromatin regulatory state throughout the genome.

The Role of Epigenetic Modification in the Transition from Acute to Chronic Pain

Prevention of chronic pain after injury has been the focus of numerous previous trials involving interventions such as multimodal analgesics and catheter-based local anesthetic infusions [88–90]. Although these techniques are successful in reducing the burden of acute pain [91], they have not succeeded in dramatically reducing the incidence of chronic post-injury or post-surgical pain [92–94]. The shortcomings of our preventive strategies are most pronounced following surgeries that have a higher risk for developing chronic pain such as amputation, thoracotomy, hernia repair, coronary artery bypass, and mastectomy [5,95,96].

Our therapeutic limitations may be partially due to our inability to prevent the epigenetic changes that occur following injury and surgery. A patient's gene expression profile changes rapidly in the post-injury period [97], with over 1,000 genes activated in the dorsal root ganglion alone after nerve injury [98]. There is significant evidence for epigenetic control of this gene activation in the transition from acute to chronic pain. First, immunologic response and inflammatory cytokine expression are under epigenetic control [99,100]. Second, glucocorticoid receptor (GR) function, which affects pain sensitivity, inflammation, and the development of autoimmune disease, is modulated both through posttranslational mechanisms and DNA methylation [101–103]. Third, genes such as glutamic acid decarboxylase 65 that code for pain regulatory enzymes in the CNS are known to be hypoacetylated and downregulated in inflammatory and nerve injury pain states [104]. Finally, epigenetic modifications are involved in opioid receptor regulation and function, with implications for endogenous pain modulation systems and pain severity [63,76].

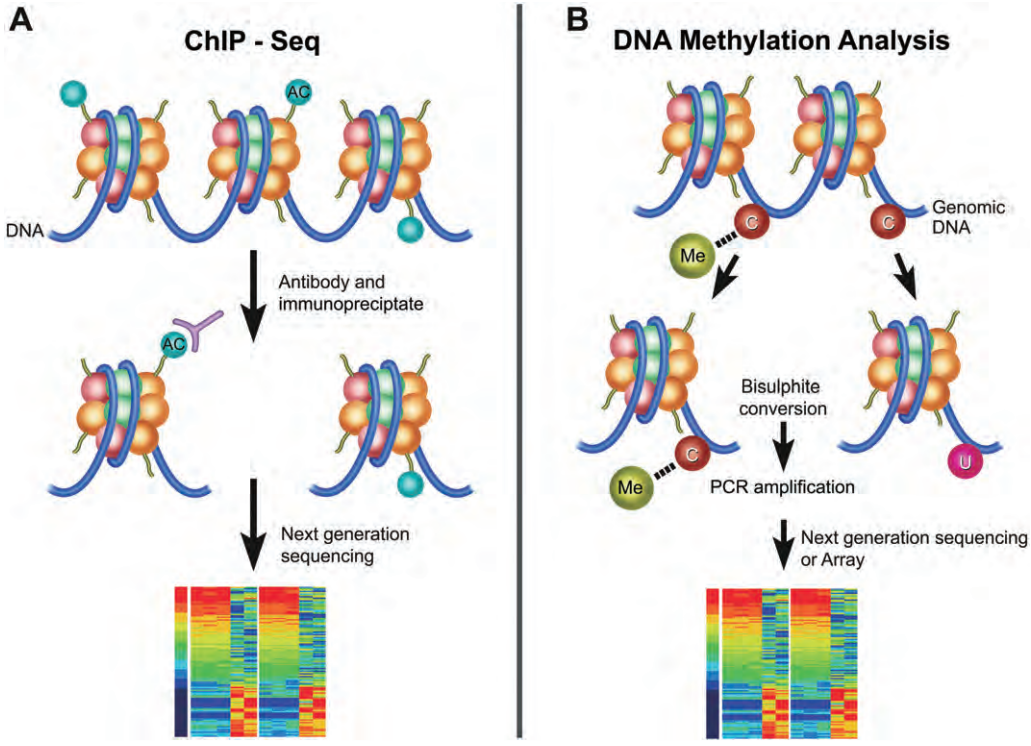

Figure 3 Laboratory techniques in epigenetics. (A) In ChIP-seq analysis, an antibody is used on chromatin to immunoprecipitate and select for acetylation and other histone modifications. The results may then be analyzed through several techniques including genome-wide next generation sequencing. In this manner, the histone acetylation patterns of a particular tissue may be determined. (B) The analysis of DNA methylation employs bisulfite sequencing to convert unmethylated cytosines to uracil. This process does not affect the methylated cytosines. The methylation patterns can be calculated by comparing the ratio of cytosine to uracil.

The important link between epigenetic regulation and pain is also supported by studies involving intervertebral disc degeneration and chronic low back pain. Tajerian et al. found that DNA methylation of an extracellular matrix protein, secreted protein, acidic, rich in cysteine, is linked to accelerated disc degeneration both in humans and in animal models of this disease [38]. The correlation between pain and epigenetics is additionally observed in a study of DNA methylation in human cancer where endothelin receptor type B (EDNRB) is heavily methylated and downregulated in painful squamous cell carcinoma (SCC) lesions [105]. The investigators noted similar findings in their mouse model of SCC, and were able to improve mechanical allodynia when EDNRB transcription was virally augmented [105]. These human and animal studies strongly support a role for gene methylation in regulating the pain experience.

Cytokines

Injury and autoimmune disease are characterized by excessive cytokine production, and anti-cytokine therapies have been successfully used to treat painful conditions such as ankylosing spondylitis [106,107] and neuropathy [108,109]. The link between cytokine expression and pain is supported by the demonstration of T-cell infiltration and inflammatory interleukin (IL) release in animal models of neuropathic pain [110]. Furthermore, interventions that modify the immune response to injury also reduce pain. Such modifications include depletion of mast cells [111], reduction of peripheral macrophages using clodronate [112], and impairment of complement activation and neutrophil chemotaxis [113].

One of the inflammatory master switches, nuclear factor-κB (NF-κB), induces multiple cytokines [114] and cyclooxygenase [115]. NF-κB is epigenetically regulated by acetylation and remodeling of chromatin [114,116,117]. When activated, this transcription factor demethylates and induces cytokines such as Tumor necrosis factor-alpha (TNF-α), IL-1, IL-2, and IL-6 [118,119]. Activation of NF-κB is associated with autoimmune and neurodegenerative disease [120]. Conversely, inhibition of NF-κB reduces pain behavior after peripheral nerve injury [121].

The link between epigenetically induced cytokine production and pain intensity has been noted in multiple disease models such as migraine headache [122], diabetes [114], and osteoarthritis [99]. In osteoarthritis, DNA demethylation at specific CpG sites in human chrondrocytes produces aberrant expression of inflammatory cytokines (IL-1β) and metalloproteinases [99]. Thus, cytokine-induced painful joint damage appears to be epigenetically modulated.

GRs

Glucocorticoids are important endogenous regulators that appear to protect against excessive inflammatory response following injury. Stress-induced glucocorticoid production suppresses immune cell release of IL-6, TNF-α, and other inflammatory cytokines [123]. Exogenous glucocorticoids also have potent anti-inflammatory actions and are used extensively in the treatment of autoimmune disease and painful conditions. However, not all patients respond equally to their clinical effects, and it is believed that glucocorticoid resistance is a likely mechanism in the development of autoimmune disease and chronic pain [124].

The GR is controlled by a system of complex regulatory mechanisms, and clinical response to glucocorticoids correlates with the number of intracellular GRs [125]. Normally, individuals demonstrate variable GR promoter methylation [103] and variable response to glucocorticoid therapy [126]. Diverse methylation patterns are believed to lead to the use of alternative promoter sites and subsequent alteration in GR sensitivity [103].

GR expression is also modified by maternal care, grooming, diet [127,128], and early-life stresses [129,130]. Human studies have demonstrated epigenetic alterations in GRs of patients who previously suffered abuse [131]. The style of maternal care appears to specifically affect methylation patterns of exon 1_7 of the GR promoter, epigenetically linking receptor function and early-life experience [132]. Abnormalities in GR-mediated immune cell function may lead to the development of inflammatory adult phenotypes [133] and autoimmune disorders such as rheumatoid arthritis [101,134]. GR dysfunction may also play a role in fatigue, chronic pain states, and fibromyalgia [102,135]. These maternally influenced expression patterns, however, are not necessarily permanent and have been reversed in cross-fostering parent studies [136]. The GR appears to provide a potential link between injury, environmental stresses, and the severity of chronic pain.

Opioid Receptors

Both demethylating agents and HDAC inhibitors increase expression of the μ-opioid receptor [137], indicating that the endogenous opioid system is under significant epigenetic control. Consistent with these laboratory findings, increased CpG methylation has been noted in the promoter regions of the μ-opioid receptors of heroin users, consistent with receptor downregulation [76], Likewise, DNA methylation of the proenkephalin gene promoter inhibits transcription and gene expression of this opioid peptide [63].

Beyond the direct role of methylation in the regulation of opioid peptide expression, spinal opioid receptor activity also appears to be partially modulated by central GRs [138]. This association is of particular importance given the synergy between the increased central expression of GR following peripheral nerve injury [139] and direct epigenetic manipulation of the endogenous opioid system [63,137]. The interaction between modifications of the GR and the opioid receptor demonstrates the complex role that epigenetic alterations play in controlling the inflammatory and pain-modulating pathways.

"Epigenetic Intervention" to Prevent Chronic Pain

Genetic studies have taught us that variability in pain sensitivity results from multiple genetic and environmental factors. Environmental influences upon pain severity have been previously described and linked to early-life stress [47,140–143]. Although precise mechanisms have yet to be elucidated, epigenetic modifications are increasingly appreciated as a likely factor in this linkage [36,104,122].

Our need for targeted therapies has never been greater. Multiple analgesic drugs are now in use; however, most of these share a common function with opioids or anti-inflammatory medications. These medications have improved symptoms in some patients, but have created the additional morbidities of systemic toxicity, opioid tolerance, and addiction. Our options for safe and effective treatments for chronic pain remain limited with few recent "breakthroughs."

Since the sequencing of the human genome, there have been increasing calls for "personalized medicine" that tailors drug therapy to a patient's pain phenotype [47,144]. Although such therapies have demonstrated some efficacy as cancer treatments [145–147], we have not yet had great success with targeted pain therapies. We will now review some of the potential targets for "personalized epigenetic intervention" (Table 1).

Intervention: HDAC Inhibition

Given the association between histone deacetylation and cancer, neurodegenerative disease, and pain, histone deacetylase inhibitors (HDACis) have been evaluated as therapeutic agents for these diseases [30,36,148]. Thus far, HDACis are primarily used in cancer therapy. In these patients, HDACis alter the balance of acetylation/deacetylation and activate genes that suppress tumor growth and invasion [30,149–152]. In neurodegenerative disease, HDACis have been evaluated secondary to their ability to induce neural growth and to improve memory [153]. HDACis have also demonstrated evidence for

Table 1 Epigenetically active drugs and their mechanisms

Epigenetics Mechanism	Drug	Action	Clinical Use	Comments
Histone deacetylase inhibitor	Valproic acid	Inhibits classes I and II HDAC	Seizures, pain	Effective for migraine prophylaxis
	Givinostat	Inhibits classes I and II HDAC	Juvenile idiopathic arthritis	Effective in human arthritis trial
	Tricostatin A (TSA)	Inhibits classes I and II HDAC	Laboratory only	Produces analgesia in animal models. Enhances μ-opioid receptor transcription
	Suberoylanilide hydroxamic acid (SAHA)	Inhibits classes I HDAC	Laboratory only	Produces analgesia in animal models
DNA methylation	Glucosamine	Prevents demethylation of IL-1β gene promoter	Arthritis pain	Common clinical use; effect on IL-1β reduces inflammatory cytokine production
	Valproic acid	Induces demethylation of reelin promoter	Seizures, pain	Reelin modulates NMDA function and pain processing
	L-methionine	Induces methylation at glucocorticoid receptor promoter gene	Dietary supplement	Alters experimental stress response; used as dietary supplement for arthritis
RNA interference	siRNA targeted to NMDA receptor subunits	Gene silencing of NR1 and NR2 subunits of NMDA	Experimental	Produces analgesia in animal models
	siRNA to P2X3	Gene silencing of P2X3	Experimental	Produces analgesia in animal models; no observed neurotoxicity with intrathecal use
	siRNA to TNF-α	Gene silencing of TNF-α	Experimental	Produces analgesia in animal models

analgesia in both inflammatory and neuropathic pain [151,154,155]. The clinical effect of many of these drugs is thought to be partially attributed to reduced production of inflammatory cytokines such as TNF-α and IL-1β [156].

HDACis are organized into several different structural groups. Trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) are hydroxamate-based HDACis. TSA inhibits both class 1 (ubiquitously expressed) and class 2 (selectively expressed) HDACs, whereas SAHA exhibits greater selectivity for class 1 HDAC. TSA produces analgesia in animal models with an associated decrease in expression of transient receptor potential type-1 cation channel (TRPV1) and protein kinase Cε [157]. SAHA reduces the nociceptive response of animals during the second phase of the formalin test [154]. These drugs increase acetylation of the transcription factor p65/Re1A, which enhances gene expression of the metabotrobic glutamate receptors (mGlu2) in dorsal root ganglia neurons. Activation of these mGlu2 receptors inhibits primary afferent neurotransmitter release in the dorsal horn of the spinal cord and provides analgesia in animal models of neuropathic pain [158]. TSA also enhances μ-opioid receptor transcription [159], indicating partial HDAC modulation of the endogenous opioid system.

Another HDACi, Givinostat, has not only demonstrated evidence of analgesia in animal models, but also efficacy in a human trial for juvenile idiopathic arthritis. Although randomized studies have not yet been performed, its use for this autoimmune inflammatory disease is especially encouraging given its relative lack of systemic toxicity [160].

The most commonly used HDACi, valproic acid (VPA), is part of the aliphatic-based drug class that inhibits classes I and II HDACs [151,161], and is effective following systemic or intrathecal administration [162,163]. VPA is of particular interest because it has been successful with long-term clinical use [164]. Although it is now used predominantly to treat chronic painful conditions [163–165], its inhibition of HDAC and potential to prevent specific epigenetic alterations may lead to preemptive use in the acute setting. It is not yet clear whether VPA-induced analgesia results from HDAC inhibition or its ability to potentiate gamma amino butyric acid (GABA) in the CNS.

Although therapies based on HDAC inhibition have been effective in treating pain and oncologic disease, nonspecific HDACis such as TSA affect the regulation of multiple

genes, which increases the possibility of side effects with this therapy [166,167]. The success of future drug development will likely depend upon our ability to target specific subclasses of HDACs that selectively alter pain processing without the toxicities of nonselective agents. The importance of this selectivity concept has been demonstrated in a mouse model in which a full knockout of the HDAC4 gene (a class IIa HDAC) is lethal, whereas a conditional knockout of this gene provides analgesia [168]. Further investigations of HDAC subclass function are needed in order to identify novel drug targets.

Intervention: DNA Methylation

DNA methylation is another key epigenetic mechanism. Methylation patterns, although generally stable throughout the genome, are responsive to pharmacologic intervention. One common medication that appears to act through epigenetic mechanisms is glucosamine [169]. In arthritis models, it has been demonstrated that glucosamine prevents demethylation of the IL-1β gene promoter, thereby decreasing expression of this cytokine. Decreased IL-1β subsequently reduces NF-κB expression and downstream inflammatory cytokine production [119,170].

In addition to its function as an HDAC inhibitor, VPA induces demethylation of multiple genes [171]. One of these important genes encodes for reelin, a glycoprotein synthesized by GABAergic neurons of the CNS [172,173]. Reelin modulates N-methyl-D-aspartate (NMDA) receptor function [174], and is important for sensory processing [175]. Mutations of this gene cause alterations in mechanical and thermal hypersensitivity [173], which indicates the potential significance of VPA regulation of reelin in the development of chronic pain.

L-methionine administration has also been tested as a potential drug for epigenetic intervention. This amino acid appears to increase methylation patterns of the GR gene, thereby altering the hypothalamic-pituitary-adrenal response to stress [176]. In addition, dietary methyl supplementation in an animal model improves the health and longevity of offspring [177]. Both of these findings suggest that nutritional status partially controls the activity of the GR and its role in inflammatory disease.

The combined action of pharmacologic DNA demethylation and HDAC inhibition increases activity at the proximal promoter site of the μ-opioid receptor gene, increasing μ-opioid receptor expression [137]. Carried out in concert, these processes may represent an important balance that allows less stable histone modifications to lead to more stable changes in DNA methylation, thus facilitating longer-term modifications in the endogenous opioid receptor system.

Intervention: RNAi

Epigenetic therapies based on RNAi also hold promise for preventing and treating chronic pain. These methods target specific disease pathways.

RNAi is an endogenous mechanism for gene silencing in plants [178] and mammals [179], and involves subgroups such as siRNA, miRNA, and shRNA. Given their ability to silence undesirable gene products in malignancy, these small RNA molecules have been used for cancer therapy [82]. They have also been shown to improve chronic neuropathic pain [80].

siRNA targeted for the NR2 subunit of NMDA receptors abolishes formalin-induced pain behavior in rats [180]. Likewise, injection of siRNA aimed at the NR1 subunit of the NMDA receptor alleviates experimentally induced allodynia in mice [181]. Successful RNAi studies have targeted TRPV1 channels [182], brain-derived neurotrophic factor [183], cytokines such as TNF-α [184], and pain-related cation channels ($P2X_3$) [80]. Importantly, direct intrathecal administration of siRNA targeting $P2X_3$ in animals has not demonstrated significant toxicity [80], indicating that this intervention may be applicable to humans in coming years.

Conclusions

The transition from acute to chronic pain is a complex process involving local inflammation and nociceptor activation that may resolve in some patients and may lead to the development of chronic pain in others. As we learn more about the various ways that injury and environment change gene expression, we can begin to elucidate disease mechanisms and gain insight into potential therapies. Epigenetic alterations such as DNA methylation, histone acetylation, and RNAi are necessary for normal tissue specialization and neurologic development. However, these same modifications play a significant role in the induction of the chronic pain phenotype following neurologic injury.

In contrast to the genetic determinism inherent in genomic studies, the field of epigenetics strives to understand the environmental control over gene expression. Such knowledge will open up opportunities for developing novel analgesics. Future personalized therapies will likely be based on epigenetic interventions that alter the transcriptional expression that occurs in chronic pain states. Given the strong mechanistic implications of epigenetic modifications in the development of chronic pain, and our current treatment limitations, we possess both the promise of epigenetic tools and the imperative to prevent the transition from acute to chronic pain.

Authors' Contribution

TB, TV, and AS conceived, wrote, and performed the final editing of this manuscript. Medical illustrations were created in collaboration with Stan Coffman from Medmedia Solutions, Durham, NC. We also wish to thank Kathy Gage, BS, Duke University Department of Anesthesiology, for her editorial assistance in the preparation of this work.

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