# STATISTICAL METHODS

* 1. Sample Size Estimation

In a previous series of investigations in chronic neuropathic pain we assessed resting state connectivity and infra-slow oscillations. On average we found that regions displaying significant differences such as the thalamus, had an effect size of at least 0.83 (e.g., thalamus ISO power Cohen’s D = 0.83). We expect that these thalamic changes may relate to altered activation or density of neuroglia – since altered astrocytic engagement induce changes in infra-slow oscillations (Hughes et al. 2011). A power analysis suggests that we will need approximately 30 subjects in each group to detect similar effect sizes for the brain imaging part of the proposal. Given this, we are confident that 40 trigeminal neuropathic pain and 40 control participants will provide more than enough power to detect significant differences in the MRI/PET measures in this proposal. The additional participants will ensure that we have enough power to detect more subtle changes in indices such as genetic and immune markers, as well as counteract any dropout between sessions 1 and 2.

* 1. Population to be analysed

Our dataset will form two groups which we will draw direct comparisons between – 40 healthy controls age and gender matched with 40 chronic orofacial neuropathic pain sufferers.

* 1. Statistical Analysis Plan

Data from this investigation forms three streams: behavioural, neuroimaging-based, and genetic/endocrine markers derived from blood.

Behavioural:

Psychological questionnaires listed in the above sections will be scored and compared between our two groups for statistical differences (two-sample t-tests, p<0.05 Bonferroni corrected). Additionally, within our TNP sample, characteristics such as symptom severity and length of time since diagnosis will be compared with individual scores on these same questionnaires for linear or non-linear relationships between these variables (linear regression R-squared values / Principal component regression and multiple linear regression rho values).

fMRI Imaging:

The fMRI images will be processed using statistical parametric mapping (SPM) and custom software. Image analysis including brainstem specific analysis procedures, will include removal of cardiac and respiratory frequencies, motion correction, global signal and movement correction, spatial normalization and smoothing procedures. For the resting state fMRI, two analysis procedures will be used to determine significant differences between trigeminal neuropathic pain and control groups: i) resting connectivity within the descending pain modulation system and higher cortical regions, ii) pain- and task-specific connectivity of the descending pain modulatory circuit. For the pain alone, offset and conditioned pain modulation (CPM) scans, significant changes in signal intensity during each pain period will be determined using a repeated box-car model. To assess the brain circuitry underlying offset and CPM analgesia, significant differences between the first pain-only scan and the offset and CPM scans will be determined for each individual. The resultant brain maps will then be placed into a second level, between group analysis to determine differences in offset and CPM analgesia in PTN compared with controls (random effects, p<0.05, corrected).

The ASL images will be used to create cerebral blood flow maps. These maps will spatially normalized to a standard template, smoothed using a Gaussian filter and significant differences between PTN and control groups determined using second level, between group analysis (random effects, p<0.05, corrected). DWI images will be motion corrected and mean diffusivity and fractional anisotropy maps calculated. These diffusion maps will spatially normalized to a standard template, smoothed using a Gaussian filter and significant differences between PTN and control groups determined using second level, between group analysis (random effects, p<0.05, corrected).

PET data analysis:

For each participant, using the PET data analysis software PMOD (Zurich, Switzerland; pmod.com), semi-quantitative analysis will be performed by calculating regional Standard Uptake Values (SUVs) and normalised SUV (SUVratios) and a voxel-by-voxel level. Furthermore, kinetic modelling, using appropriate tissue compartment models will be performed using PMOD to provide more accurate, quantitative voxel values. Using SPM12, these brain maps will spatially normalized to a standard template, smoothed using a Gaussian filter and significant differences between PTN and control groups determined using second level, between group analysis (random effects, p<0.05, corrected).

EEG data analysis:

Using SPM, EEG data will be filtered, artefacts removed and full-band (0.1–25Hz) FFT transformed, power values calculated, logged, plotted and differences between groups over the entire frequency band and within infra-slow, theta, alpha, and beta ranges determined (p<0.05, one-way ANOVA). Differences in power across each EEG channel will be determined (p<0.05, p t-tests). Changes in power during noxious stimuli will also be determined and relationships between pain intensity, EEG power, resting state thalamocortical connectivity, resting infra-slow oscillations as well as markers of astrocyte activation determined.

Blood transport and analysis:

Immediately following blood collection, all tubes will be labelled with a subject ID and will be therefore immediately de-identified. The following blood fractions will be prepared and frozen in a -80℃ freezer located securely at the MBIU. Three different processing procedures will be used:

1. For immune cell analysis, a 5 ml sample will be transferred to 10 x 1.5ml tubes, where 0.5ml of blood will be transferred into each tube containing 0.7ml of proteomic stabliser buffer (Smart Tube Inc, CA, USA), and mixed by inverting 2-3 times. Samples will then be incubated in proteomic stabliser buffer at room temperature for 10 minutes, before being transferred to a -80℃ freezer.
2. For endocrine measures using commercially available ELISA or RIA kits, 5 ml of the blood sample will be left at room temperature for 15 minutes and allowed to clot, before being centrifuged at 2,000 × g for 10 minutes. Serum will then be transferred to 10 x 0.5ml tubes before being transferred to a -80℃ freezer. An additional 5 mls of the blood sample will be collected in anticoagulant, before being centrifuged and plasma collected which will then be transferred to 10 x 0.5ml tubes before being transferred to a -80℃ freezer.
3. For genotyping, a final 5 ml of the blood sample will be collected in anticoagulant, before being centrifuged and the buffy coat and plasma collected which will then be transferred to 10 x 0.5ml tubes before being transferred to a -80℃ freezer. These samples will be used to process COMT, ORPM1, ABCB1, MC1R, CYP2D6 and FAAH and other pain-related genotyping. Also, tested for the second generation TSPO binding affinity, via rs6971 polymorphism. Time of blood collection will be noted.
   1. Interim Analyses

Given this is a cross-sectional cohort proposal we will not be performing interim analyses apart from data quality assessments.

# DATA MANAGEMENT

* 1. Data Collection

Psychological questionnaires will be collected via personal links to a secured REDCAP server being sent to participants elected email address. This REDCAP project is only accessible by investigators listed on this project and participant codes are used for de-identification. Neural imaging data will be collected on site at the Melbourne Brain Centre Imaging Unit (99 Commercial Road Parkville) on their in house 7-Tesla MRI (Siemens MAGNETOM) and their PET/CT scanner (Siemens Biograph Vision 68 PET/CT)

* 1. Data Storage

All MRI and PET images and EEG recordings acquired at the Melbourne Brain Centre Imaging Unit are immediately deidentified and acquired on the database DARIS server. They are then transferred securely to the University of Sydney’s RDS server. The deidentified data is accessed and processed only on computers in Professor Henderson’s Neural Imaging Laboratory at the University of Sydney Brain and Mind Centre. Participants shall be coded by number and each participant’s name and contact details will be kept in a locked filing cabinet in the Neural Imaging Laboratory at the Brain and Mind Centre (BMC), 94 Mallett Street Camperdown, building M02A-04 room A4.20, The University of Sydney and accessed only by the Chief Investigators. Individual participants will not be identified in any publications. The questionnaires will be recorded on REDCAP and electronically stored. All data files and questionnaires will be kept at the University for at least 20 years, after which they will be deleted when the primary data is no longer required. The blood samples will be immediately de-identified and transported securely to the University of Sydney where they will be stored and processed in Professor Keay’s secure laboratory.

* 1. Data Confidentiality

All data will be kept in re-identifiable formatting. Participants will be coded by number on initial recruitment by Professor Henderson who will keep a record of these codes matched to participants in a locked filing cabinet to which he holds the only key.

* 1. Study Record Retention

We are collecting MRI and PET images, EEG recordings, pain rating data and psychological questionnaires and have chosen the 20-year storage period as required. After this period all electronic records will be deleted, and any hard copy records shredded.