Development of a Novel Method to Assess the Effects of Predictability and Chronic Stress on Neuronal Morphology and Decision-Making in Rats

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Declaration

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Signature

Mustafa Kassem

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Abstract

The Golgi-Cox stain remains the gold standard for studying changes in neuronal morphology offering the greatest detail and clearest spine visualisation. Nevertheless, the method has limitations particularly in thick sections where laser penetration, inadequate 3D cell reconstructions, background staining and inadequate cell visualisation within fixed or otherwise non-fresh tissue offer challenges to the microscopist. Here I describe the development of a more efficient, cost effective and more broadly applicable stain together with my attempt to apply this new stain to modern tissue clearing techniques. Not only did the new methodology improve the staining of cells, it enhanced CLARITY and CUBIC clearing techniques allowing the clearing of brain tissue within a fraction of the usual time. Having developed this new methodology, I then applied this new approach to study the changes in neuronal morphology induced by chronic stress in rats. Changes in morphology in a large number of brain regions were analysed and relate to the functional effects of chronic stress. Chronic stress has been repeatedly shown to change morphology in profound ways, and I observed both increases and decreases in dendritic lengths and spine densities in different brain regions. In addition to the morphology analysis, I investigated the concomitant effects of chronic stress on choice and decision-making in the instrumental conditioning situation. Chronically stressed rats presented with decreased sensitivity to changes in the value of the instrumental outcome and in the action-outcome contingency. Furthermore, chronically stressed rats expressed a facilitation of outcome specific Pavlovian-instrumental-transfer (sPIT). The ability to control or to predict the application of stress has been reported to ameliorate its effects and, in a subsequent experiment, I compared the effects of random chronic stress and predicted chronic stress. Consistent with the previous literature, when the rats could predict the application of stress, its detrimental effects were reduced. Although the effects of random chronic stress were similar to those observed in the previous experiment, rats exposed to predictable stress showed sensitivity to changes in outcome value and in the action-outcome contingency comparable to unstressed controls. Nevertheless, in other tests of decision-making predictably stressed rats showed a further facilitation in the sPIT effect together with a deficit

in delayed discounting. These changes in decision-making were correlated to changes in neuronal morphology caused by chronic stress. Again, randomly stressed rats presented with degenerated dendritic length and synaptic spine density across many subregions of the prefrontal cortex, and proliferated morphology within the nucleus accumbens core and basolateral amygdala (BLA). Consistent with the idea that predicted stress would protect the animal from the detrimental effects of chronic stress, predicted stress rats expressed as controls within the dorsomedial striatum (DMS), hippocampus and medio-orbitofrontal cortex (MO), along with other morphology changes not consistent with random stress rats, such as a proliferation of the BLA more so than controls but not as much as random stress rats. Of the many arguments made, principally we propose that the protection of the DMS and MO and their crucial involvement in goal direct action, explains why, unlike random stress rats, predicted stress rats maintain sensitivity to outcome devaluation and contingency degradation. These morphological changes were analysed with the newly developed ultra-rapid Golgi (URG) stain. Which with further development and use of two-photon microscopy has been designed to excite stained neurons to auto-fluoresce. The two-photon laser was manipulated to excite the mercury compounds impregnated into the cells, energising the mercury to the point of electron displacement, this is a completely novel visualisation of Golgi stained neurons, offering even greater visual clarity and analysis.

NOTE:

Hyper-links will be present within text, they will be highlighted in blue as such, e.g. this will take you to the title page, or this to the abbreviations. These links will help the reader navigate the document with ease and to the desire of the writer. A helpful hint, if you intend to return to where you are currently reading, press spacebar near where you are reading, after taking the hyperlink, if you press ctrl+z you will return to your original reading point.

Contents

Abbreviations

NaC – Nucleus Accumbens Core

NaS – Nucleus Accumbens Shell

PLC – Prelimbic Cortex

IFC – Infralimbic Cortex

DLS – Dorsolateral Striatum

DMS – Dorsomedial Striatum

BLA – Basolateral Amygdala

CN – Central Nucleus of the Amygdala

OFC – Orbitofrontal Cortex

LO – Lateral OFC

VO – Ventral OFC

MO – Medial OFC

ACC – Anterior Cingulate Cortex

HIP – Hippocampus

InC – Insula Cortex

ES – Escapable Shock

IS – Inescapable Shock

CRS – Chronic Restraint Stress

CMS – Chronic Mild Stress

CaMKII – Calcium and Calmodulin Dependent Kinase 2

ERK – Extracellular Signal Regulating Kinase

ATP – Adenosine 5'-Triphosphate

CeM – Medial CN

CeL – Lateral CN

PFC – Prefrontal Cortex

sPIT – Specific Pavlovian Instrumental Transfer

gPIT – General Pavlovian Instrumental Transfer

GC – Glucocorticoids

CRH – Corticotrophin Releasing Hormone

DASS – Depression Anxiety and Stress Scale

ROI – Region of Interest

ETC – Electrophoretic Tissue Clearing

SDS – Sodium Dodecyl Sulphate

ITI – Inter trial interval

LH – Learned Helplessness

DRN – Dorsal Raphe Nucleus

VTA – Ventral Tegmentum Area

Figure and Table List

Preface

This thesis describes four experiments, which demonstrate the detrimental effects chronic stress has on neuronal morphology and goal directed action, and how the influence of predictive stressors within this chronic stress paradigm can protect against these effects. A large battery of ROIs (NaC, NaS, PLC, IFC, DMS, DLS, BLA, CN, LO, VO, MO, HIP, ACC and InC) are analysed through the experiments within to justify changes in behavioural maladaptation via morphological change. The novel methodologies used within this thesis were developed out of a desire for better tools to use when analysing morphology. I found that the speed, depth and other factors of the traditional Golgi-Cox stain, as it currently stands, to be limiting. A clearer representation of the data to further develop our understanding demanded a better stain. I knew that the Golgi stain offered, and continues to offer, the best neuronal detail and clearest synaptic spine delineation, so I worked to improve it. I doubted the idea that the stain could not be modified to rectify the restricitons it still had. Through intuitive modification of temperature, extraction and tissue care, I was able to develop the URG stain. Further, the prominence of tissue clearing offered the opportunity to combine the detail of the URG stain with the transparency of CUBIC and CLARITY. A difficult task, Experiment 1 took several months to adequately modify the new URG stain with that of the CLARITY and CUBIC techniques, ironically resulting in only slight changes to offer the best results. Lastly, in my exhaustive search to optimize and improve, I had an ephiphany, that the Golgi stain itself may be fluorescent. During Experiment 3, via delicate modification of two photon power levels, I discovered that the mercury compounds within the URG stain could become energised and fluoresce. My desire to improve on this most revered of methods was a labour of love, it saw me braching out into the quantum and spectral sciences, however, I most certainly believe this labour was fruitful, that these new methods will allow greater visualisation of neurons and their feartures, with an ease not comparable to any other method. Using these newly found techniques I believed we could approach the next level of experimentaiton with the appropriate tools. By using these methods, I got a comprehensive catalouge of morpholgical data, generating information about regions not previosuly researched

under chronic stress nor decision making. I hope that these together make a contribution to the reservoir of neuroscientific knowledge.

Through the research and study of this thesis I personally believe that two things are lacking within the neuroscience and psychological fields, one, morphological study is surprisingly sparse within the psychological literature. If we are to believe Franz Nissl's philosophy that all irregular behavioural phenotypes may be expressed as an irregular change within neuronal morphology, then further morphological study is needed, particularly in prevalent disorders, such as depression, which are known to have changes in morphology and decision making, and of which we use chronic stress as a model. The second thing lacking, although purely anecdotal, is the regard and reference to history within the fields. Rarely is there mention of the pioneer or seeding work in modern literature. How are we as scientists able to fully recognise and understand our acheivments and direction without conetxt from history? Both Fontenelle and Volaire, at the birth of the Age of Englightenment, argued, reference to history was required for progress (Morley, 1901, Steven, 1971, Laborde-Milaà, 1905), Diderot's Encyclopédie is a prime example of how the collection and study of a history of knowledge can allow for such progress. We have at our fingertips over a century of modern science, and although much has been disproven or no longer canon, the further we progress, the more we have to look back on, and the more we have to learn. It was from the study of the original Golgi and Cajal work, that I was able to develop the novel methodolgy presented here, and I hope to have ensured reference to history within the following introduction, and that it places the rest of the body in a context that, we the scientific community, can see our progress and direction.

Chapter 1: Aims and background

1.1. Introduction

The study of cellular morphology within the nervous system has contributed crucial information to neuroscientific knowledge. In the early 1900s we see Franz Nissl's Cresyl Violet stain (although originally Dahlia Violet) used to study neurons and their fibres within grey matter (Nissl, 1903), earning him a Nobel Prize nomination for medicine in 1912. Two other pioneers, Camilo Golgi and Ramon Cajal, together shared the 1906 Nobel Prize in medicine in recognition of their work on the structure of the nervous system, which was studied via the Golgi stain developed by Golgi and used by Cajal (originally called the Black or Silver stain) (Golgi, 1873b, Cajal, 1899-1904). Cajal's work is often seen as the standard by which neuronal morphology is analysed. From these stains and the many others that followed, the study of neuroscience expanded to aid in understanding the nature and purpose of the axon, the dendrite, the soma and the synaptic spine as well as revealing that these can change in both healthy and unhealthy conditions and that these changes can manifest themselves in both physical and psychological ways. Nissl (1896) articulated this best saying that: "As soon as we agree to see in all mental derangements the clinical expression of definite disease processes in the cortex, we remove the obstacles that make impossible agreement among alienists." All irregular behavioural phenotypes may be expressed as an irregular change in neuronal morphology. Nevertheless, we have, I believe, reached a plateau in the methodology used to analyse morphology and restrictions in the use of the Golgi stain, although improved over the last century, still remain. The Golgi stain is used to measure and visualise whole neurons, however the stain only allows sections of up to a max of 200-300um in depth to be visualised (Glaser and Van der Loos, 1981), and so struggles in the context of 3D cell reconstruction. In addition, the stain is prone to difficulties induced by background staining and cannot be applied to non-fresh tissue without a loss in image quality. These restrictions have caused researchers to look elsewhere to study cellular

morphology, or to ignore changes in morphology entirely but the alternatives so far developed are heavily restricted in their application and may not be viable in vivo or for the study of large cell populations. A reinvention of the Golgi stain for use with modern techniques would have the benefit of allowing researchers to study changes in cellular morphology in their current experimental preparations with little additional effort. This thesis describes the development of one approach to reinventing the Golgi stain, and its application in the context of modern histological techniques to study changes in neuronal morphology.

Nissl's argument that psychological phenomena reflect the expression of changes within the brain has been demonstrated repeatedly. From early studies into dementia, Nissl worked with his good friend Aloysius Alzheimer, to study massive cellular degeneration within his patients. Besides gross volume changes, Nissl stains revealed that neuronal morphology was deteriorated (Alzheimer, 1904, Perusini, 1909, Hippius and Neundörfer, 2003). These physical changes caused the patients to express the psychological symptoms associated with what is now called Alzheimer's Disease. However, psychiatric phenotypes can express themselves from less overt deterioration. Golgi staining has revealed for decades, and still do, that individuals suffering from schizophrenia, depression and PTSD, just to name a few, express neuronal morphology significantly different from healthy patients (Adamec et al., 2012, Belichenko, 1989, Faherty et al., 2003, Garrett and Wellman, 2009, Lazcano et al., 2015, Mitra et al., 2009, Nashed et al., 2015, Senitz and Winkelmann, 1981, Sierakowiak et al., 2014, Totterdell and Smith, 1986) . These morphological changes are argued to be the cause of the symptoms of these disorders. The study of neuronal morphology, however, should not be restricted to disease; any maladaptive psychological change can be analysed from a morphological perspective. For example, the literature contains many examples of changes in an animal's decision-making ability induced by lesions to specific regions of the brain. Lesions induce, of course, marked cell loss that may be more or less regionally specific; however, given Nissl's contention, subtler environmental treatments that induce changes in cellular morphology within these regions should also induce concomitant changes in decision making. Nevertheless, as

discussed later, the current literature has only very few studies in which the changes in decision-making associated with changes in neuronal morphology have been assessed.

The best example of an environmental treatment that changes cellular morphology is chronic stress. Chronic stress is often used as a model of depression; the changes that occur mirror the changes in morphology that are presented in depression patients (Bennett, 2008, Koolschijn et al., 2009, Kroes et al., 2011, Thompson et al., 2007, Zhu et al., 2011). Typically, stress alters morphology by decreasing dendritic length and synaptic spine density to the point where grey matter volume changes become evident (Cook and Wellman, 2004, Kassem et al., 2013, Murmu et al., 2006, Radley et al., 2006, Radley et al., 2004, Shansky et al., 2009a, Vyas et al., 2002), however it does uniquely increase dendritic length and synaptic spine density in a few regions (Mitra et al., 2005, Padival et al., 2013, Vyas et al., 2006, Bessa et al., 2013), an effect not induced by other treatments. Importantly, given the arguments above, were we to apply chronic stress to animals, we might expect there to be changes in their decision-making capacity compared to non-stressed animals; studies have shown that chronic stress causes changes to neuronal morphology in regions which have also been associated with the decision-making process (Cook and Wellman, 2004, Martinez-Tellez et al., 2009, Muhammad et al., 2012, Radley et al., 2004, Vyas et al., 2003, Vyas et al., 2006, Vyas et al., 2002).

The chronic stress treatment can be titrated to potentially reveal further unique changes within the animal, and those changes associated with changes within decision-making. The stress literature has revealed that single instances of intense stress (acute stress), can produce discrete deficits with a different severity and symptomology to chronic stress (McEwen, 2004, Moisan and Le Moal, 2012) albeit via some of the same mechanisms as chronic stress; e,g, the engagement of the hypothalamus–pituitary-adrenal (HPA) axis (Del Rey et al., 2008, Hargreaves, 1990, Meserve and Leathem, 1981), the complexities of which are elaborated later. Previous studies have demonstrated that if an animal has control over a stressor and can remove itself from an acutely stressful situation, then many of the negative effects of acute stress are ameliorated (Maier, 1990, Baratta et al., 2007, Amat et a., 2014). If we were to apply the same logic to the chronically stressful situation it might be

anticipated that at least some of the negative effects associated with chronic stress will also be ameliorated. The difficulty however, is giving control over the stressor to an animal in a situation in which applying the treatment necessitates that the animal lacks at least some elements of control; indeed, chronic stress is usually defined as the repeated exposure to a stressful situation that a subject cannot escape (Lazarus, 1966, McEwen, 2007). In this thesis a treatment is described in which we provide the animal, not with the ability to directly control or escape from the stressor, but with the ability to predict its onset. It has been argued that the predictability of stressful events can to some degree replicate the effects of stressor controllability and, as such, by giving the animal at least this degree of amelioration, we hope to protect it from many of the harmful effects of chronic stress.

To summarise the broad goals of this thesis, we aim (i) to develop an improved method using which researchers can study and analyse changes in neuronal morphology (ii) to use this new method to study changes in cellular morphology induced by chronic stress and collect a large catalogue of morphological information about changes within regions either only briefly or never studied before in chronic stress literature (iii) to develop a 'treatment' for chronic stress to provide some degree of amelioration by making chronic stress predictable and, finally, (iv) to apply these new methods and treatments to study the effects of chronic stress on decision making, changes in which we hope to show are at least partly explained by region-specific changes in neuronal morphology. The large amount of morphological information gathered will add data on a number of previously unmeasured regions to the reservoir of scientific knowledge, potentially revealing a new avenue of study within the stress and learning fields.

In the following subchapters the topics introduced above will be elaborated to cover (i) the grounds for the development of the improved Golgi methodologies, (ii) an explanation of how stress effects various regions of Interest (ROI) in the brain which will be investigated in the later parts of this thesis, (iii) background to the various behavioural aspects of this thesis that will be used to investigate decision making, their neural bases and what is known about how stress affects these brain areas and (iv) the influence that stressor prediction and control have been found to have on

decision-making. Finally, as guided through the content, the four experiments performed will be put into a context to be elaborated in the methods.

1.2. The Ultra Rapid Golgi-Cox Method and its application to Brain Clearing Techniques

Camillo Golgi developed the first histological method to fully delineate entire neurons on clear backgrounds, with a resolution that allowed synaptic spines to be visualised (Golgi, 1873a, Mancuso et al., 2013) The stain was quickly improved by Cox (1881) to more reliably stain neurons (Scheibel and Tomiyasu, 1978). The Golgi-Cox stain has since played an enormous role in neuroscience, the low cost, ease of use and great detail of the Golgi-Cox method has seen the prevalence of its use maintained within neuroscience to the present day. Typically used to investigate neuronal morphological features (Sholl, 1953, Rutledge et al., 1969, Sousa et al., 2000, Brown et al., 2005, Mychasiuk et al., 2013) and dendritic spine density (Levine et al., 2013, Mancuso et al., 2013, Mitra et al., 2005, Radley et al., 2006, Vetere et al., 2011), it is an attractive tool to use in the lab; however the Golgi-Cox method is restricted by tissue transparency, as imaging is typically restricted to tissue samples sectioned to 200-300 ums in depth (Glaser and Van der Loos, 1981). With advances in tissue clearing techniques, the Golgi-Cox method's use is likely to continue to develop. Removing the thickness restriction will allow for low cost and fast visualisation of extremely accurate entire neuron 3D reconstructions via reflective confocal microscopy.

Modernisations

The modern Golgi-Cox stain still works via "la reazione nera" (the black reaction) (Golgi, 1873a). The resultant combination of potassium di/chromate ($K_2Cr_2O_7$/K_2CrO_4) and mercuric chloride ($HgCl_2$) to form mercurous chloride (Hg_2Cl_2) which, when alkalised, transforms into black mercuric sulphide (HgS) crystals within the cell membrane (Stean, 1974, Fregerslev et al., 1971a, Fregerslev et al., 1971b, Das et al., 2013). When observed under brightfield the neurons are stained black against a

clear background. These metallic impregnations within the neuron can reflect laser light and can be visualised under reflective confocal imaging, allowing for clear visualisation with a black background and 3D reconstructions (Spiga et al., 2005, Spiga et al., 2003, Richardson and Lichtman, 2015).

The Golgi-Cox method has also been modified over the last century to stain in reduced time. Originally taking months to process (Golgi, 1873), modifications to concentrations and care of the samples brought the time to 8 weeks (Sholl, 1953), to the current standard of 2 weeks (Levine et al., 2013). However, recent studies have shown that clear Golgi-Cox stains can be achieved in under 24hrs (Ranjan and Mallick, 2010, Ranjan and Mallick, 2012). Ranjan and Mallick (2010) describe how an increase in temperature (to 37^{o}C) of the staining solution would increase the random motion of the metallic compounds facilitating their impregnation into neurons and reducing the time required. The rapid method however cannot stain adequately if tissue is fixed. A modification to the staining procedure to allow the Golgi stain to work rapidly within both fresh and fixed tissue would be very desirable.

Tissue clearing

Tissue clearing has been practiced since the beginning of the 20th century. The original method involves a series of dehydration and bleaching steps (Spalteholz, 1914) which, although effective for the time, damages fixed tissue and prohibits further histological investigation (Steinke and Wolff, 2001). Modern clearing techniques either continue to involve dehydration such as BABB (Dodt et al., 2007) and iDISCO (Renier et al., 2014) or involve aqueous-based clearing such as CUBIC (Tainaka et al., 2014, Susaki et al., 2014) and CLARITY (Chung et al., 2013).

Tissue transparency can be greatly improved via removal of lipids; lipids increase light scatter as photons attempt to pass through the tissue. By either removing the lipids or increasing the tissue's refractive index, greater transparency is achieved (Richardson and Lichtman, 2015). CUBIC, which depends on the removal of lipids, is an emersion-based technique that achieves transparency via replacing the refractive index of the liquid in and around tissue with that of a higher refractive index

solution, aiming to match the average refractive index of the tissue itself. CUBIC uses high levels of Triton-X to maximise lipid removal. While this results in a faster clearing technique, higher levels of protein loss are experienced making immuno-staining difficult (Susaki et al., 2014, Tainaka et al., 2014). CLARITY however, uses a hydrogel embedding technique that maintains the integrity of the proteins. Further, the process can be hastened with the uses of electrophoresis to remove the lipids from within the tissue (Chung et al., 2013). The application of a Golgi-Cox stain to cleared brain tissue would allow complete neurons to be visualized and reconstructed, offering the most accurate data on neuron morphology. The Golgi-Cox stain is not restricted by protein loss and modification to the procedure could see it work on fixed tissue within 48hrs.

Imaging

As clearing techniques advance even further microscope imagining will be one of the few limitations remaining (Richardson and Lichtman, 2015). Although, traditionally, confocal microscopes are limited to approximately 200 um of depth in non-cleared tissue (Graeden and Sive, 2009, Brakenhoff et al., 1988), cleared tissue does not have this restriction. Manufacturers now produce objectives that are built for cleared tissue with working distances greater than 5mm while still maintaining high resolution; making confocal microscopy an appropriate choice for the potential of reflective imaging of Golgi-Cox stained neurons within cleared tissue. Another technique which could be applied is Light-sheet. Light-sheet imagining is a technique that allows larger visual fields to be imaged at once (Dodt et al., 2007). Through the use of planar excitation as opposed to laser excitation (Eberle et al., 2015, Richardson and Lichtman, 2015), light-sheet imaging illuminates an entire plane at once, making imaging of large tissue samples exceedingly fast compared to confocal and other imaging techniques (Becker et al., 2008, Becker et al., 2013). Light-sheet imaging allows for structural and whole tissue 3D imaging, illustrating regional changes in morphology (Dodt et al., 2007, Becker et al., 2008, Becker et al., 2013, Eberle et al., 2015). With the application of a Golgi stain, light sheet imaging of neurons will be able to visualise entire tissue samples and analyse

regional information in a 3D environment, offering even greater analysis of neuron populations, while maintaining the information gathered using a Golgi stain.

Development and Improvement

Fixed Tissue

The development of an improved Golgi-Cox stain is aided by the work done by Ranjan and Mallick (2010, 2012), however they were still restricted by the limitations of the stain. To improve stain efficacy within fixed tissue, further manipulation of handling and temperature may be required. Following traditional PFA tissue fixation via perfusion, residual fixative is left within the tissue. A small pilot study performed by myself revealed that the reduced image quality using the traditional Golgi-Cox stain with PFA fixed tissue was dependent on residual PFA left within the brain following perfusion. That is, if a larger amount of PFA were left within the tissue, it impacted Golgi staining. Washing the tissue in PB or PBS, as per traditional protocol, was not helpful in reducing the impact of residual PFA on image quality. To overcome this factor, a slower perfusion was used following which a perfusion of PBS was applied to remove residual PFA left within the tissue. The tissue was found to be adequately fixed, displaying many of the trademark characteristics of PFA fixed tissue. This first step allowed a traditional Golgi-cox stain to work slightly more effectively within fixed tissue. However, this improvement was not sufficient; images were still plagued by large amounts of artefact (amorphous blotches of mercuric sulphide). I imagined that, comparatively, PFA fixed tissue is more difficult to penetrate than fresh tissue and, as Ranjan and Mallick (2010, 2012) demonstrated, an increase in temperature promotes an increase in particle movement, allowing increased penetration. PFA fixed tissue has a higher resistance to temperature (Fox et al., 1985, Mayers, 1970, Puchtler and Meloan, 1985, Srinivasan et al., 2002) and could, therefore, accommodate increased temperature before damage presents itself. With this in mind, a higher temperature was applied, which resulted in further improved image quality in PFA fixed tissue. After multiple runs, a temperature of 42^{o}C was revealed to be the most optimal. Interestingly, however,

within fresh tissue, increasing the temperature higher than 37^{o}C did not improve image quality. Only by combining these two changes (perfusion and temperature) was Golgi staining found to work within PFA fixed tissue. Furthermore, staining was best when incubation was extended to 36 hours instead of 24, as described in Ranjan and Mallick (2010), for both fresh and fixed tissue. With this issue resolved, the application of the Golgi stain to modern tissue clearing techniques can be addressed and, if successful, could resolve issues with background staining and improve the imaging of entire neurons at a greater depth within the tissue.

Clearing techniques

The visualisation of Golgi stained neurons within cleared tissue would elevate the Golgi-Cox stain to the level of complete detail offered by neuron injection staining but with the utility that it can be applied to the study of whole populations of neurons, and can be applied relatively easily to whole tissue without using any more apparatus than an incubator. The application of the Golgi-Cox stain to cleared tissue has not yet been reported in the literature. With the work I have done to modify the application of the stain to non-fresh tissue, it should be predicted that neurons within CUBIC and CLARITY cleared tissue will now stain appropriately. With the tissue being cleared, imaging via confocal and lightsheet would allow the best laser penetration and hence visualisation.

Design of studies aimed at developing the use of Golgi-Cox stain with cleared tissue

To properly investigate, develop and optimise a new method, the design will be constantly updated to accommodate any improvements or alternative avenues found to be of value in the process. Initially, I plan to take the modified for non-fresh tissue appropriate modifications to the Golgi-Cox stain and to apply those modifications at crucial steps in the tissue clearing process. Tissue clearing via the CLARITY technique, involves first embedding the tissue into a hydrogel solution, and then polymerising the tissue and clearing it. In addition, the CLARITY technique has both an active and passive clearing protocol (see Chung et al., 2013). By applying the Golgi stain before and after

the embedding step and in both active and passive clearing protocols will provide the full battery of possible periods for best staining. The CUBIC technique is simpler, only involving submersion of the tissue within a clearing solution that clears the tissue over many days. By applying the stain before and after clearing, we can determine the optimal period. Following clearing and stain development, imaging via confocal and lightsheet microscopy will be used to reveal the results of these studies and the acuity of any neuronal visualisation. Jump to Experiment 1 methods here.

1.3. Novel Photoluminescence of Golgi Stained Neurons Using Multi-Photon Excitation

During the course of visualising cleared tissue impregnated with the Golgi-Cox stain, I developed an alternative means of visualising the effects of this stain using 2-photon excitation. While it is an attractive tool to use in the lab, the Golgi-Cox method is restricted by tissue transparency where imaging is typically restricted to tissue samples sectioned to 200-300 ums in depth and background staining (Glaser and Van der Loos, 1981). By using deeper imaging microscope techniques with greater specificity and the ability to reduce background staining the use of the Golgi stain will continue to offer low cost and fast 3D reconstructions with morphological delineation unmatched by many other modern histological techniques.

Two-photon excitation was first theorized in (1931) by the physicist Maria Goeppert-Mayer although evidence for such excitation was not established until the early 1960s (Kaiser and Garrett, 1961, Abella, 1962), and not applied to fluorescent imaging until decades later in 1990 (Denk et al., 1990a). Denk and colleagues have repeatedly demonstrated the use of two-photon absorption with modifications to a laser microscope to excite fluorophores (Denk et al., 1994, Denk and Svoboda, 1997, Helmchen and Denk, 2005). Now eight decades after the original theory, two-photon excitation microscopy is reasonably prevalent within the scientific community. Two-photon imaging has the great advantage of deep laser penetration, penetrating up to 2 mm into tissue (Theer and Denk, 2006, Dufour et al., 2006). Comparatively, conventional confocal imaging is typically limited to

approximately 200um (Graeden and Sive, 2009, Brakenhoff et al., 1988). Two-photon imaging allows the user to delineate neuronal morphology very deep into the tissue, allowing for the most representative 3D reconstructions and dendritic measurements.

Two-photon microscopy, works via exciting cells using two photon beams, which can combine their wavelengths (Denk et al., 1990a, 1994). This means that, unlike traditional confocal microscopy where the excitation photon-beam is usually of a shorter wavelength, i.e. higher energy, than the emission wavelength, two-photon microscopy instead, allows the combination of two longer wavelengths, i.e. lower energy, exciting the cells simultaneously, so still reaching the required energy level needed for excitation and light emission. The main advantage of this method is the change in the point-spread function (PSF) of the images (Kaminer et al., 2013). The PSF is the degree of blurriness surrounding a point within an imaging system. This is most easily represented by the quality of an image rendered in 3D but captured from the x-y plane. For example, consider an image stack captured from the x-y plane and sampled at points in the z-direction depth; i.e. a conventional z-stack. Once that image stack is compiled and viewed from the z-direction or rendered in 3D, the PSF determines the resolution of the image. Two-photon microscopy has a better PSF compared to confocal microscopy, allowing for better resolution of image stacks and 3D rendering.

Development

Election Displacement

As mentioned, two-photon microscopy works via the excitation of cells through the absorption of two simultaneous photons, which can allow for greatly increased energy levels to be applied to cells. Two-photon lasers cause emission of light via excitation due to the displacement of electrons within the atoms of the cell or stain (Haar, 1967, Arons and Peppard, 1965) See Fig. 1 for an illustration. This process is often used to excite photoluminescence from fluorophores to allow visualisation (Xu and Webb, 1996, Denk et al., 1990b, Berland and So, 2000, So et al., 2000). However, fundamentally this property can be used to excite any photosensitive material (usually

metals) to emit light. The Golgi-Cox stain works via impregnating mercury compounds into the neuron membrane, where neurons are stained completely and in black, allowing for visualisation under brightfield imaging (Stean, 1974, Fregerslev et al., 1971a, Fregerslev et al., 1971b, Das et al., 2013). Mercury compounds have been shown repeatedly to be sensitive to photoexcitation and to exhibit photoluminescence, with a rather prevalent example being the common mercury lamp which uses electrical excitation of vaporous mercury to the point where it is energised enough to emit a light bright enough to illuminate (Kunkely and Vogler, 1989, Zeng et al., 2010, Gunning, 1958, Esmaeili-Zare and Salavati-Niasari, 2015, Waymouth, 1971). If instead we excite the metallic mercury compounds within the Golgi stained tissue, the neurons should photoluminate, allowing the researcher to take advantage of an improved PSF, reducing background staining and allowing synaptic spines that are often hidden due to perspective to now be observable via luminance intensity.

Design of a study to develop 2-photon imaging of Golgi-Cox stained tissue,

Although this experiment was developed halfway through the thesis, it is presented here because it relates directly to the previous experiment. No previous literature has looked at the spectrum of solid compound based HgS crystals, hence there is nothing on which to indicate what power levels are needed to excite these mercury atoms. However, after consultation of the chemistry/physics databases (NIST, Nave, 2010), we would expect normal mercury atoms to be excited and have emission between 184nm to 578nm. By designing a protocol in which the two-photon laser intensity is applied at different points between 200nm and 1100nm, and emissions are prepared to be caught within this range, we expect to see photoluminescence. The range is extended because excitation and emission for HgS may fall outside that of simple Hg, and the mechanics of a two-photon beam, as described, dictate that that longer wavelengths be used to reach the combined higher power, i.e. lower wavelength, within the tissue. Adjustment to various laser intensities will

also be applied throughout this range as the exact intensity required is still to be revealed. See Fig. 1 for an illustration. Jump to Experiment 3 methods here.

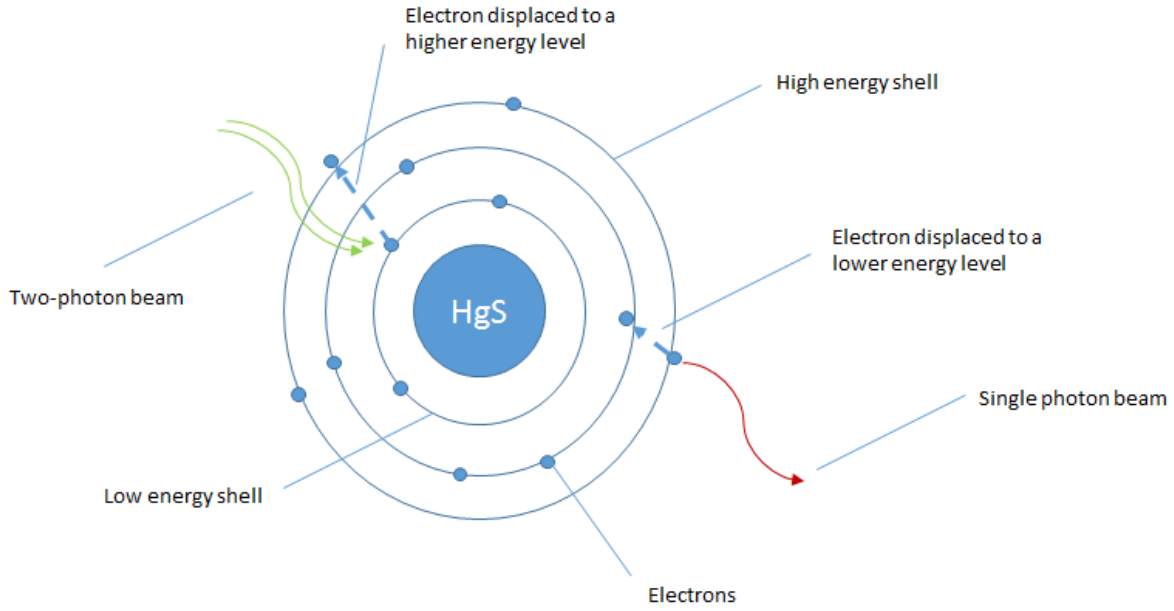

Figure 1: An illustration, depicting the application of a two-photon beam displacing electrons within a compound to a higher energy level. The HgS is the metallic mercury compound used in a Golgi stain, what can be seen from this illustration is that we expect to use the two-photon beam to displace an electron to a higher energy level, causing a resultant shift in energy of the HgS compound, and resulting in a photon beam emitted, which we will capture as fluorescence.

1.4. Stress and the Brain

The use of these two novel methods is best applied to the study of cellular morphology. The two methods will offer improved visualisation and analysis of structural changes within the neuron. As already discussed in subchapter 1.1, the treatment that offers the best opportunity to study morphological changes is chronic stress. Chronic stress affects neurons by changing dendritic length and synaptic spine densities. The application of the methods described in subchapters 1.2 and 1.3 would allow novel investigation of various regions of the brain and generate a catalogue of morphological data, for regions briefly or never investigated for the effects of chronic stress. The

current section will detail how stress affects neurons, which systems it engages, and distinguish between acute and chronic stress. Acute stress refers to exposure to a single instance of intense stress, whereas chronic stress refers to being exposure to repeated stressors without the ability to escape from the situation. The latter is the focus of this section, as it produces the most reliable changes in morphology and, as will be discussed in subchapter 1.5, decision-making. This review of chronic stress will describe evidence relating to the battery of the 14 ROIs to be investigated in Experiments 2 and 4.

Stress is a physiological and psychological trait that motivates an animal to remove itself from a harmful situation and return to a state of homeostasis; in essence, events are perceived and experienced as stressful to the extent they cause deviation from a homeostatic state (Korte et al., 2005). This defensive mechanism involves the HPA axis, which engages the release of corticotropin-releasing-hormone (CRH) to promote release of glucocorticoids (GC) (Makino et al., 1995, Smith et al., 1995, De Souza et al., 1985, Kling et al., 2009, Makino et al., 1994b, Watanabe et al., 1992a). An important distinction that must be made between acute and chronic stress is that stress responses are designed to help in acute situations. The release of GCs promotes increased attention and a flight or fight response (Korte et al., 2005, Lemos et al., 2012, Melo et al., 2011). Additionally, GC provides a negative feedback to the HPA axis to stop production of CRH as the stressful event passes (Bennett, 2008, Thompson et al., 2007). In contrast, chronic stress, as a stressful situation that cannot be escaped and is recurring causes the continued production of CRH. Excessive levels of CRH, as observed in chronic stress, have been shown to cause the deterioration of neuronal integrity and to alter behaviour. (Watanabe et al., 1992b, Wellman, 2001, Radley et al., 2004, Cerqueira et al., 2007b, Perez-Cruz et al., 2007).

It is true that acute stress has also been shown to affect neuronal integrity. Chen et al. (2008) showed that after a single 5-hour restraint session, rats showed decreased hippocampal dendritic spines, a finding that was demonstrated again in 2010 (Chen et al.). Additionally, acute stress has been shown in humans to impair self-control in decision-making (Maier et al., 2015). Nevertheless,

the effects of chronic stress are far greater and more consistent. Considerable evidence over the decades has shown that chronic stress causes neurodegeneration in the hippocampus (Alfarez et al., 2003, Alfarez et al., 2008, Brunson et al., 2001, Conrad et al., 2004, Conrad et al., 1999, Donohue et al., 2006) and prefrontal cortices (PFC) (Bennett, 2011a, Bennett, 2011b, Brown et al., 2005, Cerqueira et al., 2007a, Cook and Wellman, 2004, Radley et al., 2005, Radley et al., 2006, Radley et al., 2008, Radley et al., 2004, Shansky et al., 2009a), as well as sub-cortical regions (Blix et al., 2013, Makino et al., 1994b, Makino et al., 1999, Martinez-Tellez et al., 2009, Mitra et al., 2005, Muhammad et al., 2012, Padival et al., 2013, Taylor et al., 2014, Vyas et al., 2006, Vyas et al., 2002, Wang et al., 2012). Based on this evidence, the selection of the ROIs for this thesis includes the NaC, NaS, PLC, IFC, BLA, CN, DMS, DLS, LO, VO, MO, ACC, HIP and InC (please refer here for full names). Stress related changes to these regions should be predicted to cause concomitant changes in decision making (see subchapter 1.5). I will now present briefly the evidence relating to the effects of chronic stress on each of these regions starting with the HIP and ACC, which although involved in decision-making, are consistently and heavily influenced by chronic stress and so will be used as key indicators of successful chronic stress treatment.

Hippocampus and Anterior Cingulate Cortex

The hippocampus was the first region of the brain shown to be morphologically affected by chronic stress. Although shown since the 1960s to have changes in GC concentrations (Mandell et al., 1963, Kawakami et al., 1969, Slusher, 1964), it wasn't until the 1990s that reliable replications demonstrated dendritic and synaptic spine decay (Fuchs et al., 1995, Sapolsky et al., 1990, Uno et al., 1989, Watanabe et al., 1992b). Hippocampal changes continue to be reported, and as will be seen through the other ROIs to be discussed, the HIP has long been used as a standard against which to assess whether a putative stress treatment caused dendritic change. The ACC, although not as often used as such a standard, has repeatedly demonstrated to be degenerated in grey matter volume and dendritic arborisation after chronic stress treatments (Cerqueira et al., 2005, Gos et al., 2008, Kasai

et al., 2008, Treadway et al., 2009). Further, as will be expanded upon in subchapter 1.6, using an extra ROI to act as a standard ensures that any protective effects associated with prediction (see Experiment 4), do not entirely obscure the effectiveness of the stress treatment itself, i.e. If only one region is used as a standard, any treatment induced reduction in morphological changes could reflect the treatment or the failure of the stress treatment. Using two standards doesn't completely protect against this issue but is a more conservative practise. Generally, however, the HIP and ACC are both detrimentally affected by chronic stress and changes to these regions are expected following any chronic stress treatment.

Pre- and Infra-limbic Cortices

As discussed, the PFC has consistently demonstrated deterioration in neuronal morphology following chronic stress. However, delineation of the PLC and IFC within the PFC is important as they have distinct functions and previous research into chronic stress has failed to delineate these regions amongst many others. Radley et al. (2004) demonstrated that rats exposed to chronic restraint stress (CRS) for 6 hours a day for 21 days, had significantly decreased dendritic length and arborisation within pyramidal neurons of the PLC. A study using the same paradigm observed not only dendritic lengths and arborisation decrease following CRS, but also a decrease in synaptic spine density (Radley et al., 2006), with further studies showing that neurons within the PLC showed reduced synaptic transmission (Negron-Oyarzo et al., 2014). Shansky et al. (2009b) showed, using a less intensive stress paradigm of 2 hours a day for 10 days, that IFC neurons exhibited reduced dendritic length and arborisation, although failed to demonstrate decreases in spine density. This failure to see decrease in spines was likely due to the weaker stress paradigm used. Cerqueira et al. (2007b) demonstrated that both PLC and IFC dendritic decreases were also present following treatment with subcutaneous injections of GCs. After 4 weeks of daily corticosterone injections, PLC and IFC neurons had dendrites reduced by up to 33%. However, congruent to Shansky et al. (2009b), this study also

failed to see spine density changes. Clearly it can be seen that across both the PLC and IFC that chronic stress degenerates dendrites and synaptic spines.

Orbitofrontal Cortex

The Orbitofrontal Cortex (OFC) has been investigated thoroughly, and the many varied models of chronic stress that have been used consistently generate decreases in dendritic length, arborisation and synaptic spine density (Cerqueira et al., 2007a, Goldwater et al., 2009, Perez-Cruz et al., 2007, Radley et al., 2006, Radley et al., 2008, Radley et al., 2004, Shansky et al., 2009a). However, few studies have clearly delineated the OFC from other PFCs, and on closer review of the literature there are conflicting changes in morphology reported. Liston et al. (2006) demonstrated that 21 days of 6 hour CRS led to an increase in OFC dendritic length. Conversely, in prenatally stressed rats, offspring exhibited reduced OFC dendritic lengths and spines (Murmu et al., 2006). In a somewhat congruent result, prenatal stress was reported to decrease dendritic lengths but increase synaptic spines (Mychasiuk et al., 2012, Mychasiuk et al., 2011, Muhammad et al., 2012). And lastly, Muhammad and Kolb (2011), demonstrated that prenatal stress had no effect on OFC spine density. The conflicting results between the studies are most likely due to the stress paradigm used and, although prenatal stress and chronic stress may be similar, the difference here is likely due to the more intense paradigm used by Liston et al (2006), which has been argued to be the most effective and consistent stress paradigm (McLaughlin et al., 2007). It is also important to recognise that no chronic stress study has delineated the OFC into its distinct regions for morphological analysis. It can be interpreted from the Liston et al (2006) paper that the region of the OFC that they sampled from was in fact the LO (Paxinos and Watson, 2006). Regional analysis of the LO, VO and MO may reveal differing morphology, as is discussed in subchapter 1.5, the specificity of analysis is important and pertinent to understanding the effects of chronic stress on decision-making. Summarising, unlike the PLC and IFC, it is less clear the direction if any, of morphological changes within the OFC. Further

research is, therefore, imperative to establish clear evidence of the morphological changes induced by CRS in this region.

Dorsal Striatum

There are very few studies that have investigated morphological changes within the dorsal striatum following chronic stress. Taylor et al. (2014), demonstrated that rats which underwent chronic stress for 14 days, consisting of either daily 1 hour restraint or 30 minutes shaking ground, exhibited increased DMS dendrites. Blix et al. (2013) showed with MRI that humans patients suffering from chronic work-related stress had reduced grey matter volumes within the caudate and putamen. The caudate and putamen are homologous to the DMS and DLS in rodents (Balleine and O'Doherty, 2010). We should expect, therefore, to see similar decreases within the DMS and DLS to those observed by Blix et al. (2013) following chronic stress in rodents. One other study has investigated changes within the DMS and DLS following chronic stress, Dias-Ferreira et al. (2009) reported a decrease in dendritic length in the DMS and an increase in the DLS, however these results will be discussed in detail later (see subchapter 1.6).

Basolateral and Central Amygdala

The BLA responds interestingly to chronic stress. It has previously been reported that chronic stress causes an increase in dendritic arborisation within the BLA, one of only two regions with morphological evidence indicating an increase in dendritic length and spine density following chronic stress. Vyas (2002) showed that rats which underwent chronic immobility stress, where animals were restrained for 2 hours a day for 10 days, had typically decreased hippocampal dendritic lengths, however saw increased dendritic length and branch points in the BLA. This was seen again by Vyas (2006), using the same chronic immobility paradigm, spine densities were reported to increase on BLA neurons, this effect again reported across all dendritic orders greater than 2 on which the authors reported a progressively larger increase in synaptic spines (Mitra et al., 2005). Using a 20

minute restraint stress design across 7 days, Padival et al (2013) found that both dendrites and synaptic spines increased on BLA neurons, specifically showing that it is the intermediate and distal dendrites that saw an increase in synaptic spine density. The unique effect that chronic stress has on the BLA is well documented, and if chronic stress is applied as a treatment, it has a unique ability to proliferate neuronal morphology. In contrast, the CN does not appear to respond similarly to CRS although there has been no systematic investigation of morphological changes in the chronic stress literature, the CN literature is mostly restricted to GC or CRH measurements. Makino et al. (1994a, 1994b) showed that rats given chronic systemic injections of GC over 14 days in the form of corticosterone saw significantly increased CRH concentrations within the CN. Additionally, a slow release corticosterone pellet over 60 days caused similar increases in CRH within the CN. The evidence for this has been consistent, (Makino et al., 1999, Hatalski et al., 1998, Iwasaki-Sekino et al., 2009). There is a lack of evidence, however, of dendritic measures within the CN following increases in CRH and GC which, based on other studies might be predicted to cause decreases in dendritic arborisation according to the model of dendritic regression argued by Bennett (2008). Research into the morphological effects of chronic stress within the CN is clearly required.

Nucleus Accumbens

Morphological research into the effect chronic stress has on the nucleus accumbens is also limited. Matinez-Tellez et al (2009), showed that a mild CRS treatment, i.e., 2 hours of chronic restraint per day for 11 days on pregnant rats until birth, produced a decrease in nucleus accumbens spine density in the birthed pups, a deterioration that was still present up to week 9. Congruent decreases in dendritic length have been reported when pups were exposed to chronic stress post weaning (Wang et al., 2012, Monroy et al., 2010). Conversely, chronic maternal separation stress for 3 hours a day from birth has been shown to induce an increase in both dendritic length and spine density in the nucleus accumbens (Muhammad et al., 2012), with similar results reported in a chronic stress model (Taylor et al., 2014). Resolution to these conflicting results is further impeded by the

lack of discrimination in these studies between the NaC and NaS. There are only two studies currently that distinguish between the NaC and NaS. Morales-Medina et al. (2010) demonstrated that chronic stress during pregnancy saw a decrease in dendritic length in the NaS whereas Bessa et al. (2013) investigated the effect that chronic mild stress (CMS) on rats within the nucleus accumbens and its relations to anhedonia. The CMS design lasted for 6 weeks, with daily unpredictable stressors consisting of: restraint stress for 1 hour, tilted cage for 3 hours, damp bedding for 8 hours, 18 hours food or water deprivation followed by inaccessible but viable food or water for 1 hour and reversing the dark/light cycles affecting sleep. Following stress, morphological analyses revealed increased dendritic length within the NaC and NaS, however, only the NaC showed concomitant spine density increases. The conflicting results within the NaS requires further investigation, however Morales-Medina et al. (2010), reported that some pockets of NaS neurons showed increases, which may indicate that sampling was imprecise or that there is a heterogenous effect on NaS neurons under different chronic stress treatments. Conversely, as Bessa (2013) did not report synaptic spine density increases on NaS neurons, the proliferation of these cells may be transient or irregular. Further research into the effect stress has within the nucleus accumbens is required to establish a stronger understanding.

Insula Cortex

The effects of stress on InC have been understudied, and data from a rodent model are completely lacking. For the purposes of this review inferences from human studies will be made. Chen et al. (2006) showed, via MRI, that post-traumatic stress disorder (PTSD) patients had decreased grey matter volumes of the InC. This result was replicated to show that veterans with PTSD had reduced InC volume (Herringa et al., 2012). The InC has also been reported to have reduced volume and irregular activity within sufferers of major depression (Horn et al., 2010, Wiebking et al., 2010, Wiebking et al., 2015), for which we use chronic stress as a model (Liu et al., 2011, Bennett, 2008, Bennett, 2007, Bennett, 2011a, Bennett, 2011b). We can assume that the grey

matter volume decreases reported are explained by dendritic and synaptic spine changes within the InC (Kassem et al., 2013), and so following chronic stress it would be expected that similar degenerative morphology changes present themselves.

Summary

Together this section illustrates the range at which chronic stress effects the brain. Across the many regions introduced and discussed, chronic stress has been reported to consistently effect some, i.e. degeneration of the HIP, ACC, PLC IFC and proliferation of the BLA. Other regions however, have more conflicting results with reports of degeneration and proliferation or no change at all following chronic stress i.e. OFC and nucleus accumbens. Lastly across all the ROI's except the HIP, ACC and BLA, further or pioneering research is required to generate sufficient morphological information to understand the effects chronic stress has on the brain. Regardless, it is evident that chronic stress affects the brain, and drastically changes neuronal morphology. If we remember Nissl's philosophy on cellular morphology, these cellular changes within neuronal morphology can only be expected to change behaviour. And as will be discussed in subchapter 1.6, chronic stress has been reported to affect decision-making. Before that literature can be discussed, however, we must put it into context and introduce decision-making research and the tests used to measure changes in this capacity. Essentially three main tests have been used to assess the associative structure (and changes in the associative structure induced by various treatments) that underlies action selection in animal models of decision-making: outcome devaluation, instrumental contingency degradation and Pavlovian-instrumental transfer. The nature of these assessments and their neural bases will be described next.

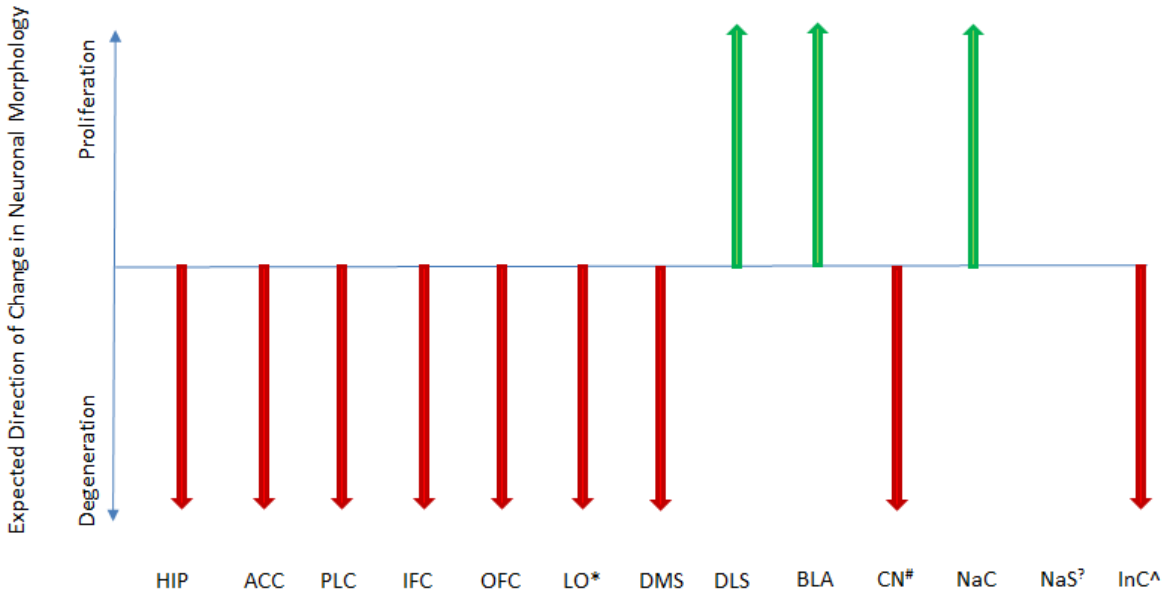

Figure 2: Illustration for reference of collected predictions of change to neuronal morphology following chronic stress treatment. *Inferred from images – compared to atlas, # Inferred from GC and CRH concentrations. ?Conflicting data. ^Inferred from human studies.

1.5. Decision-Making

Introduction

Since as early as Descartes's Principles of Philosophy (1983 [1644]), where he proposed that humans inherently exist and act only in response to the environment, theory has depended on the associations animals make between stimuli, actions and outcomes to explain learning. Animals must be able to recognise and estimate the relationship between their actions and the resultant outcomes, as well as understand the causal relationship between actions and outcomes, to best determine and select a course of action. Further, the appropriate evaluation of an outcome drives the decision making process, how rewarding an outcome is determining the action taken (Balleine et al., 2011, Balleine, 2005). The neuroanatomical correlates that dictate this decision making behaviour have been linked to frontal cortices (Goldman-Rakic, 1996, Goldman-Rakic, 1995, Fuster, 2000,

Ostlund and Balleine, 2007a, Ostlund and Balleine, 2007b, Balleine et al., 2011), and recent evidence also points towards basal ganglia and amygdala involvement as crucial for successful decision making (Shiflett and Balleine, 2010, Parkes et al., 2015, Bradfield et al., 2013a, Yin et al., 2005b, Yin et al., 2005a, Lingawi and Balleine, 2012, Izquierdo et al., 2013). The interaction of the aforementioned regions generates two distinct heterogeneous behaviours to accommodate decision-making, habits and goal directed actions (Balleine et al., 2007, Tanaka et al., 2006). Habits are actions that are relatively inflexible and are mediated more by environmental stimuli then by their consequences, generating a stream of automatic behaviours which have been well established in the animal via a process of stimulus-response (S-R) association (Yin et al., 2004, Tricomi et al., 2009). Goal directed actions are relatively flexible and mediated both by encoding the relationship between actions and their consequences or outcome, i.e., the formation of an action-outcome association (R-O), and by evaluation of the desirability of those consequences (Balleine and Dickinson, 1998, Balleine, 2011). Both forms of action control can generate adaptive behaviour in specific situation, with over dependence on either or neuroanatomical damage leading to maladaptive behaviours, as can be seen in Parkinson's (Antonini et al., 2001, Redgrave et al., 2010) and Huntington's diseases patients (Hodges et al., 2006, Joel, 2001). Outcome devaluation and contingency degradation are two well established paradigms used to test these phenomena, with insensitivity to changes in the action-outcome contingency or in outcome value indicative of an increased dependency on habits (Balleine and Dickinson, 1992, Balleine et al., 1995, Balleine and Dickinson, 1998, Balleine et al., 2003, Parkes et al., 2015, Wiltgen et al., 2007, Yin et al., 2005b).

In addition, Pavlovian-instrumental transfer (PIT) provides a measure of how well an animal uses Pavlovian cues to control the performance of actions and comes in two forms, general and specific PIT, both of which will be described in more detail later. A deficit on a PIT test indicates that the animal is unable to use Pavlovian outcome expectancies to guide decision-making.

Outcome Devaluation and Contingency Degradation

For almost 90 years we have known that animals associate a stimulus within an outcome, Pavlov's early work with dogs (1927), provided evidence that animals do encode a relationship between predictive stimuli and outcomes, but it wasn't until Adams and Dickinson (1981) demonstrated that animals encode the relationship between instrumental actions and their outcomes that the same was known to be true of instrumental conditioning. By training a rat to lever press for sucrose and then pairing sucrose with lithium chloride, which produces illness, rats learned quickly that the sucrose had decreased in value. When given the opportunity to lever press for sucrose in the absence to any reinforcer, lever press performance dropped (Adams and Dickinson, 1981). This provided evidence that the animals were able to encode a different form of learning, a response-outcome, or R-O, association, where responding was dictated by outcome value. If the value of the outcome is reduced, so is responding. Adams (1982) replicated this phenomenon using the same procedure, but comparing undertrained with overtrained lever pressing rats and observed that overtraining rendered the rats insensitive to outcome devaluation, i.e. rats did not reduce lever pressing after the sucrose outcome was paired with lithium chloride. This suggested that animals use R-O processes initially to govern their actions; however, after repeated use and/or overtraining, the animal's performance becomes habitual and dependant on an S-R process. This switch can in nature be beneficial. If an animal can learn to make a decision which then develops into a habit, it can give the animal an advantage; a rough example, imagine a pair of early primates, one cannot develop habits. When they need to crack open a coconut to eat using tools in the process, the primate that can develop a habit will, after successful tries, no longer need to evaluate the situation, the habit will engage and the primate will know to use the stone to crack the coconut open. Meanwhile, the other primate will have to figure it out every time it wishes to eat. As previously mentioned, over dependence on either goal directed or habitual actions can be maladaptive, and a healthy animal will use both. As will be revealed, chronic stress can cause such maladaptations to develop, via changing neuronal morphology within regions crucial to decision making.

Contingency degradation is a measure of how reliant the performance of an action is on the action-outcome contingency. Hammond (Hammond, 1980) was the first to develop this test. He trained rats to press a lever on a schedule in which there was a fixed probability of reinforcement given that the animal made at least one press in the immediately preceding second. He then introduced a non-contingent schedule by arranging for the delivery of a free reinforcer with the same probability following each second without a lever press. This schedule is non-contingent in the sense that the rate of reinforcement is the same if the animal presses or not. In spite of the fact that the probability of contiguous reinforcement remained unchanged by the transition from the contingent to non-contingent, the rate of lever pressing declined under the non-contingent schedule, indicating that the animal was aware that its actions were not contingent on that outcome. This effect has been replicated many times (Dickinson and Mulatero, 1989, Balleine and Dickinson, 1998, Ostlund and Balleine, 2008), and is often used in conjunction with outcome devaluation (Bradfield et al., 2013a, Corbit and Balleine, 2003, Yin et al., 2005a, Yin et al., 2005b) to illustrate a better picture of changes in decision making following a treatment. An animal that cannot detect contingency degradation and change decision making in response to that change is dependent on habits, which as discussed are insensitive to such changes. Now that we have established the methods used to measure goal directed action, we will discuss them in the context of anatomy.

Pre- and Infra-limbic Cortex

Similar to the way we have discussed the effects of chronic stress, goal directed learning and habitual actions will be elaborated neuroanatomically. Beginning with PFC, we will work our way through the same ROIs discussed in subchapter 1.4, demonstrating the involvement of each region and relating it to changes within the same regions that are affected by chronic stress. Outcome devaluation and contingency degradation has been used repeatedly to show that animals encode R-O associations and that these govern instrumental performance, and that manipulations to the animal's ability to learn R-O associations leads to a switch or dependence on S-R associations. The

PLC has been demonstrated to be of importance in goal directed actions. In a study first demonstrated by Balleine and Dickinson (1998) where rats had lesions to the PLC and were trained to lever press for pellets or sucrose. Lesioned rats acquired the R-O association slower than sham rats and, in addition, were insensitive to outcome devaluation and contingency degradation, this result has been replicated (Corbit and Balleine, 2003). Ostlund and Balleine (2005) found congruent results, PLC lesion rats showed insensitivity to outcome devaluation, however, this was present when the lesions were applied pre-training. When studied after a post-training lesion the rats responded healthily. Ostlund and Balleine argued that the PLC is crucial for goal directed action; however, it is primarily required for the acquisition of action outcome contingencies. It is important to note that the PLC projects to the dorsomedial striatum DMS (Gabbott et al., 2005), which has been reported to be involved in reward related circuitry (Dayan and Balleine, 2002, Tanaka et al., 2006).

Killcross and Coutureau (2003) demonstrated that although PLC lesion rats were insensitive to outcome devaluation, IFC lesion rats were not, but in fact were more sensitive than shams. Rats were trained to lever press for two outcomes, and were under- or over-trained. Overtrained sham rats were insensitive to outcome devaluation as expected, however IFC lesion rats remained goal directed, and during undertraining showed elevated lever press from the non-devalued outcome compared to shams. This result was replicated using a very similar paradigm, where overtrained rats that were insensitive to outcome devaluation, were able to recover sensitivity to the outcome value following lesioning of the IFC (Coutureau and Killcross, 2003). These studies indicate the importance of the PLC and IFC in decision-making. Based on Fig. 2, we predict that chronic stress would degenerate neuronal morphology within both of these regions. This leads to a conflicting change in decision making behaviour. As a decrease in PLC would lead to a biasing to habitual actions, but a decrease in the IFC would lead to a biasing to goal directed action. Research into this will reveal which maladaptive behaviour most presents itself.

Orbitofrontal Cortex

Bradfield et al. (2015) showed in their pioneering study that the MO is required to retrieve information about the value of an outcome. In rats that were trained to lever press for two different outcomes, those with MO lesions were insensitive to outcome devaluation, indicating an inability to appropriately encode action outcome contingencies. However, in an outcome devaluation task not done in extinction, but with the rewards present, the MO lesion rats performed the same as shams. Bradfield et al. (2015) argued that the MO is required to retrieve value-based information when the outcome is not observable. This indicates that the MO is crucial for goal directed actions, especially in premeditated decisions where the outcome is less salient. In a similar result, Gouley at al. (2016) demonstrated that DREADD stimulation of the MO increased sensitivity to outcome devaluation, whereas, BDNF knockout mice were insensitive. The importance of the MO in retrieval of value information during a decision is made evident here, and based on Fig. 2 we would predict a degeneration in MO morphology, and hence a decrease in the rat's performance on outcome devaluation. However, as already discussed, the OFC has never been delineated under the effects of chronic stress, although literature has consistently reported a decrease in the OFC in general, without specific delineation of the sub-regions this may not be true for the MO. Further research into sub-regional changes will reveal any differences that exist between the sub-regions, and how this may affect decision making. This is particularly true, as will be discussed in the PIT sub-chapter, LO and VO lesions do not abolish outcome devaluation, but do abolish outcome specific PIT (Ostlund and Balleine, 2007b).

Dorsal Striatum

An exemplary study into goal directed action demonstrates the crucial importance of the posterior DMS (pDMS) in an animal's ability to successfully generate and maintain R-O associations. Yin et al (2005b) lesioned either the anterior DMS (aDMS) or the pDMS either before or after training in different groups of rats. Food deprived rats were trained to press two levers, one for pellets and

one for sucrose solution at increasingly higher random ratios. Following this, outcome devaluation via specific satiety was performed, where instead of devaluing the value of an outcome via a negative attribute (i.e. sickness), rats are given free access to one of the outcomes for 1 hr, before testing in extinction. During this free 1hr feeding session, rats have free access to eat as much as desired, so as to devalue the outcome, rats should press for this outcome less compared to the non-free fed outcome. Control rats pressed less for the devalued outcome whereas rats which had lesions to the pDMS saw no reduction in lever press on the action that previously delivered the now devalued outcome, indicating the importance of the pDMS to goal directed action This insensitivity to change in outcome value was extended to contingency degradation. Rats which had pDMS lesions were insensitive to the contingency of their actions being degraded. Further Yin et al. injected muscimol as a temporary GABA agonist, inhibiting the pDMS. Rats injected with muscimol also saw impaired goal directed action, pressing the same for both the devalued, non-devalued outcomes, and contiguous and non-contiguous outcomes, again illustrating the crucial importance of the pDMS in goal-directed action both during acquisition and performance. This was demonstrated again in a similar study in which Yin et al. (2005a), showed that blockade of NMDA receptors in the pDMS produced similar insensitivity to outcome devaluation and contingency degradation. A more recent study showed that the input into the pDMS is also required for adequate evaluation of outcomes. Bradfield et al. (2013a) demonstrated that not only the DMS, but its afferents can alter decision making. Lesions to the parafascicular thalamus (PF), which interrupt the thalamostriatal pathway connecting the PF to the DMS via cholinergic interneurons (CIN), reduced sensitivity to contingency degradation, i.e. rats continued to press for an outcome no longer contingent on their actions. And following a change in action-outcome contingency, lesion rats were insensitive to outcome devaluation. This indicated that lesion rats were unable to use action-outcome information to guide performance after the initial contingencies were altered reinforcing the importance of the DMS.

There are parallel circuits within the dorsal striatum that influence goal directed and habitual actions. The DMS is crucial for goal directed actions, however, the DLS is crucial for habits. Yin et al

(2004) lesioned the DLS in rats, then overtrained them along with shams. As expected, the sham rats were insensitive to outcome devaluation induced by taste aversion due to overtraining and a shift to habitual behaviour; however, the DLS lesioned rats remained goal directed and reduced pressing on the devalued lever, indicating that the DLS is crucial for habit formation and, with its removal, decision processes were biased towards goal directed actions. This was demonstrated again with the use of muscimol to temporarily inactivate the DLS. Using a similar paradigm, rats which had the DLS inactivated became sensitive to change in outcome value and bias goal directed actions over habitual (Yin et al., 2006). The DMS and DLS are involved in goal directed and habitual actions respectively, and, in the context of chronic stress, from Fig.2 we should predict a decrease and increase in dendritic length in the DMS and DLS, respectively. This should be predicted to lead to a strong bias towards habits.

Insular Cortex

The InC has also been reported to play a crucial role in the performance of goal-directed actions through its role in encoding changes in the value of the instrumental outcome. Balleine and Dickinson (2000) demonstrated that rats with lesions to the InC trained to lever press for two outcomes were insensitive to outcome devaluation. Similar results were seen when a NMDA antagonist was infused into the InC post training (Parkes and Balleine, 2013). A further study showed that in addition to the InC, its connection to the NaC is also crucial for appropriate goal directed action (Parkes et al., 2015). Parkes et al (2015) trained rats to lever press for two outcomes and infused muscimol to temporarily inactivate the InC and NaC asymmetrically to disconnect the InC to NaC pathway prior to testing. They observed that rats that were able to evaluate outcomes became insensitive to outcome value when these regions were inactivated. This indicates the importance of the InC and NaC to goal directed actions. Parkes et al. (2015) argued that the interaction specifically mediates the retrieval of stored information regarding the incentive value of the outcome at test. It is evident that the MO and InC both play specific but crucial roles in ensuring healthy retrieval of the

outcome and outcome value, respectively, for goal-directed action. Based purely on human stress studies, it would be expected that degeneration of neuronal morphology would present itself following chronic stress within the InC, and this would lead to an insensitivity in outcome devaluation.

Nucleus Accumbens

The NaC has been shown to be necessary for the performance but not for the acquisition of goal directed actions. Corbit et al. (2001) demonstrated that rats with lesions to the NaC were insensitive to outcome devaluation. Rats that were trained on two levers for two outcomes continued to press equally for both after one had been devalued via specific satiety. Further, they showed that NaS lesions did not disrupt outcome devaluation, but removed Pavlovian cue enhancement of action selection in an outcome-specific PIT (sPIT) test (see p. 47), indicating that the NaC is required for changes in performance based on R-O associations, and the NaS is required for changes in performance based on S-O associations. These results were replicated in a study in which disconnection of the BLA from to the NaC saw rats become insensitive to outcome devaluation whereas disconnection from the NaS caused a deficit in sPIT (Shiflett and Balleine, 2010). Additionally, Laurent et al. (2012) demonstrated that when CTAP, a powerful μ-opioid receptor antagonist, is infused into the NaC; or μ-opioid receptor knockout mice are trained to lever press for two outcomes and tested via outcome devaluation, they are insensitive to changes in outcome value, demonstrating that a μ-opioid receptor process in the NaC is required for an animal to effectively update and recall value of an expected outcome. When similar infusions and knockouts were performed targeting the NaS, no change in outcome devaluation was seen. Fig.2 predicts that the NaC should proliferate following chronic stress, should we see a proliferation of the NaC it would be expected that an increase in outcome devaluation performance would be observed. Appling chronic stress treatment will help reveal what effects NaC proliferation will have on decision making.

Summary

A series of regions have been reviewed so far and found to be involved in decision-making. The PLC, DMS, MO, InC and NaC are all crucial for an animal to be able to evaluate, update, recall and use expected outcome value with manipulations that reduce the function of these regions expected to lead to insensitivity to outcome devaluation and contingency degradation. This suggests that such manipulations bias performance towards a habit. Conversely, the IFC and DLS are crucial for effective habitual action, with manipulations to these regions expected to render animals unable to utilise S-R associations to control performance, biasing towards goal directed actions, even after rigorous overtraining.

Additionally, parallel circuits appear to mediate goal directed and habitual behaviour, with the PLC → DMS and associated regions controlling goal directed performance and the sensorimotor Cortex → DLS and associated regions controlling habitual performance. These two dissociable circuits, although anatomically close, relate to two heterogeneous forms of action control, (i) flexible goal directed actions, where the decision is sensitive to outcome feedback and updated and changed outcome value and, (ii) relatively inflexible habitual actions, where the decision is sensitive to stimuli. Aside from lesions and pharmacological intervention, it could easily be argued that other modifications to these regions would cause correlated behavioural changes. If we were to decrease dendritic length and arborisation in the PLC or DMS we would see a correlated decrease in sensitivity to outcome devaluation and contingency degradation. Conversely, if we were to increase the dendritic length and arborisation in the NaC, conceivably, an increase in sensitivity may be observed. Next, we discuss the effect chronic stress has on the third test used as a measure of decision-making, PIT.

Pavlovian-Instrumental-Transfer

As discussed, we have known that animals associate a stimulus with an outcome, from Pavlov's early work with dogs (1927), where he observed anticipatory behaviour demonstrated when

exposed to a stimulus associated with food. It has also been well established for over 100 years that animals are capable of instrumental performance, which is strengthened by a desired outcome. Thorndike's (1898) early work with cats saw what he described as the "Law of Effect"; cats strengthened their behaviours and more readily performed them if they resulted in a desired outcome, in this example, escape from a puzzle box. Subsequently, Estes (1948) bought these two strands of research together showing that, when a rat is exposed to a tone which has been paired with food whilst pressing a lever paired with the same food, then the tone tends to increase lever pressing. This facilitatory effect of a Pavlovian stimulus on instrumental performance, Estes argued, was because the Pavlovian stimulus gained discriminative control over the instrumental action. This experiment constituted the inception of PIT. Subsequently, Lovibond (1983) training rabbits to raise their heads for a sucrose outcome. He paired a stimulus with this outcome, and in addition, another stimulus was presented not paired with an outcome. When tested in extinction, rabbits only increased instrumental performance when the stimulus paired with sucrose was presented, and not when the unpaired stimulus was presented. What this established more clearly was that a paired but not an unpaired stimulus will elevate instrumental performance. One issue in this literature is whether the outcome predicted by the stimulus and the action need to be identical to show transfer. In fact, transfer can be revealed when the outcomes differ – the stimulus can produce a general arousing effect on instrumental performance – so-called general PIT (gPIT) – but, in situations in which animals are given a choice between two actions, it is clear that performance is elevated only for actions delivering the same outcome as that predicted by the stimulus – called outcome specific PIT (sPIT). This distinction has been demonstrated most clearly within the Corbit-Balleine papers (Corbit and Balleine, 2005, Corbit and Balleine, 2011, Corbit et al., 2007).

Corbit and Balleine (2005) demonstrated that the effect of a conditioned stimulus (CS) on choice between instrumental actions depends on outcome identity. In their study they trained rats to press two levers each delivering a different outcome chosen from three outcomes: sucrose, pellets or polycrose (with 0.9% sodium chloride), pairing those two outcomes with left and right levers. Once

this was established rats received sessions of Pavlovian conditioning, pairing the aforementioned outcomes with tone, white noise or clicker stimuli, the result being that rats had instrumental and Pavlovian associations with two shared outcomes but only Pavlovian associations with a third, creating a $S_1/R_1 \rightarrow O_1$, $S_2/R_2 \rightarrow O_2$, $S_3 \rightarrow O_3$ set-up. This allowed them to investigate the effects of stimuli associated with a specific outcome on actions that deliver that specific outcome, and also the effect that stimuli generally associated with outcomes have on responding. During two extinction tests, rats were presented with one of each of their levers, while throughout the test all three stimuli were presented. They demonstrated that animals presented with a stimulus (S_3) associated with an outcome which was not earned by the actions (i.e., O_3) caused a general increase in instrumental performance; gPIT. However, what was also clearly established within the paper was that there was a specific enhancement in performance too; i.e., stimuli associated with outcomes that were also earned by instrumental actions (e.g., S_1/R_1-O_1) elevated the performance of those actions with which they shared an outcome but left other actions (e.g., R_2) unaffected. This effect was referred to as sPIT. Now, just as previously, we will discuss these effects within the context of anatomy.

Neural Correlates

Corbit and Balleine (2005) also establish the neural bases to these two types of PIT. In the same experiment rats were given lesions of the BLA or CN. These rats showed no discernible effect of the lesion on their training measures; they were able to adequately learn both the Pavlovian and instrumental associations. When tested for general and specific PIT, however, the rats with the BLA lesion were unable to show lever press enhancement via sPIT but could show gPIT whereas the CN lesion rats were unable to show gPIT but showed normal sPIT. Corbit and Balleine (2011) investigated further neurological correlates to general and specific PIT using a similar design to the 2005 study, i.e. they used the three outcome design and established three Pavlovian predictors, S_1, S_2, S_3 for O_1, O_2 and O_3, and two instrumental actions $A_1 \rightarrow O_1$ and $A_2 \rightarrow O_2$. In this study they inactivated the NaC or NaS. In a similar result, the NaC inactivated rats did not show an increase in responding on the levers

to S_3; i.e., they failed to show gPIT, but showed normal outcome sPIT. In contrast, NaS inactivation abolished outcome sPIT but left gPIT unaffected. These studies point to clear neural correlates that allow the facilitatory effects of specific cue-guided (sPIT) and general arousal (gPIT) increases in instrumental performance.

Other studies have found evidence supporting the argument that these structures are crucial in general and specific PIT. A disconnection study revealed that when the BLA is specifically disconnected from the NaS, a failure of sPIT is observed (Shiflett and Balleine, 2010) whereas disconnection of the BLA from other outputs did not affect sPIT, i.e. when disconnected from the NaC, there was no change compared to sham animals. Other studies have used specific pharmacological approaches. For example, Laurent et al (2012, 2014) injected a variety of dopamine and opioid antagonists into the NaS showing that the D1 antagonist SCH23390, but not the D2 antagonist raclopride, abolished sPIT when infused into the NaS (but not into the NaC). Likewise, infusing the δ-opioid receptor antagonist natrindole into the NaS abolished sPIT, but not the mu-antagonist CTAP, and neither drug had an effect on specific transfer when infused into the NaC. Finally, Laurent et al (2014) established that an interaction between D1 and δ-receptor-related processes in the NaS using an asymmetrical infusion design in which SCH was infused on one side and naltridole on the other. Although neither drug infused unilaterally had any effect alone, when infused contralaterally they abolished specific transfer (Laurent et al., 2014).

Both the amygdala and nucleus accumbens are sub-cortical, there is however, also crucial involvement in the cortex. A study by (Ostlund and Balleine, 2007b) demonstrated that rats with lesions to the OFC abolished sPIT. This involvement of the OFC was investigated further by Balleine et al. (2011) who demonstrated that specifically the LO and VO play a crucial role in sPIT performance. In a lesion study, where the LO and VO were damaged prior to training, Balleine et al. (2011) found that rats did not show any selective facilitation of lever press in the presence of Pavlovian cues (Balleine et al., 2011). These lesions did not affect goal directed learning, or pavlovian or instrumental performance. Balleine et al. (2011) argued that a BLA → OFC → NaS pathway was

disrupted via these lesions, and that this disruption damaged the circuit for which the animal needs to perform sPIT. We see now the full involvement of the neuronal correlates discussed, a circuit is present within the animal, with disruption to any of these components abolishing the sPIT effect.

Summary

What is clearly evident from these studies as a whole is that the facilitation of instrumental performance, first seen by Estes (1948), has distinct behavioural and neural correlates. Performance may be enhanced by a general state of arousal, gPIT, and by cue specific triggers, sPIT. Further, anatomically close yet functionally distinct regions modulate these effects. The BLA, as well as its connection to the NaS, are both crucial to sPIT whereas the CN is required for gPIT. This is seen again in the NaC and NaS, where the NaC crucial for gPIT and the NaS necessary for sPIT. And full circle we see the circuit complete with the OFC, with its connections to both the amygdala and nucleus accumbens as crucial for sPIT performance. It has become clear that modifications to these regions have distinct behavioural outcomes. Aside from lesion and pharmacological manipulations, it could be argued that other modifications to these regions should cause behavioural changes. If we were, for example, to increase dendritic length and arborisation in the BLA, conceivably, an increase in the facilitatory effects in sPIT could be observed. Next, we discuss examples within the literature of the application of chronic stress and its effects on decision making.

1.6. Effects of Chronic Stress on Decision Making

In this section we discuss literature that has investigated the effects of stress on decision-making. Specifically, we will look at studies which have used outcome devaluation, contingency degradation and PIT as measures of decision making. As we have discussed in subchapter 1.5, the content will be compartmentalized into literature which has studied outcome devaluation and

contingency degradation as measures of goal directed action and others which have studied PIT as a measure of stimulus control of action selection.

Stress and Goal Directed Action

There is only one study that has investigated the effect chronic stress has on goal directed action using measures of outcome devaluation and contingency degradation as indices of goal-directed control. Dias-Ferrerira et al. (2009) showed that instrumental actions of rats which underwent chronic stress for 21 days, which consisted daily of 15 minutes social defeat, 10 minutes forced swim or 30 minutes restraint stress, had significant deficits compared to controls. Stressed rats were trained to lever press for pellets and sucrose. Following training these rats showed insensitivity to outcome devaluation and contingency degradation. Chronically stressed rats continued to press a lever delivering an outcome that was also delivered non-contiguously or when the value of the outcome was reduced via specific satiety. Rats were histologically examined to reveal that chronic stress had caused significant atrophy to the dendritic lengths of the PLC, IFC and a downward trend in DMS, however, an increase in dendritic length was found within the DLS. The animal's insensitivity to outcome value was explained by the concomitant changes within these regions. The decrease in DMS and PLC dendrites indicated a decrease in goal directed action, with the increase in DLS suggestive of a potential increase in habitual action. Together these morphological changes create a clear picture to explain the behavioural shift from one decision making process to another. It is important to note, however, that synaptic spine densities saw no significant changes within any regions; all regions saw a downward trend, contradictory to the DLS dendritic increase. As proposed earlier, a more reliable and rigorous stress paradigm may have led to a more consistent trend in spine density (McLaughlin et al., 2007). Regardless, this study demonstrates through combined examination of a reliable behavioural assay and histology that chronic stress causes changes within regions crucial for goal directed behaviour and these changes result in modified decision making.

Analogous studies

Although there are no other papers that directly look at chronic stress and goal directed actions, analogues may be drawn from similar paradigms. A study demonstrated, using the same stressor paradigm as in Dias-Ferreria et al. (2009), that chronically stressed rats required to switch their learned associations between a lever and its outcome, and encode a new action-outcome association to earn a reward, took significantly longer compared to controls. i.e. Rats learn that $R_1 \rightarrow O_1$ and $R_2 \rightarrow O_2$, with only O_1 earning a reward, if the levers change to $R_1 \rightarrow O_2$ and $R_2 \rightarrow O_1$, stressed rats took significantly longer to switch and encode this new association (Iguchi et al., 2015). Furthermore, Iguchi et al., (2015) demonstrated that chronically stressed rats continued to press the un-rewarded action longer, even when presented with the new R-O association. This manipulation of contingencies is referred to as reversal learning; reversal learning is a procedure requiring an animal has to change from one strategy to another and the rate at which this shift occurs can reflect both the reliance on a prior stimulus-response habit but also the ability to learn the new action-outcome contingency. In similar fashion to Iguchi et al. (2015), Bradfield et al. (2013) demonstrated as discussed earlier (p. 43) that the pDMS and its afferents were required to adjust to reversal learning. Rats with the PF to pDMS connection interrupted via lesions, demonstrated an insensitivity to outcome devaluation after the contingencies learned had been reversed. Further, Izquierdo et al. (2013) demonstrated that following lesions of the OFC, the length of time required for a rat to adequately encode a new association in a reversal learning task increased, however, with lesions to the BLA, this amount of time decreased. As has already been discussed, OFC neuronal morphology decreases following chronic stress, and although there are few reports, if we assume the DMS also decreases, conceivably a degeneration of these regions by chronic stress would hinder reversal learning within the animal, and conversely a proliferation within the BLA would accelerate it. What these studies demonstrate collectively is that chronic stress affects decision-making specifically, it would appear, by hindering the animal's ability to encode new associations. However, what these studies also show collectively is that this field of research is lacking in systematic research and further

study is required to elucidate the effects stress has on decision-making. We must currently draw on studies using acute stress because studies specifically into chronic stress are lacking. Nevertheless, although they differ, analogies may still be drawn because acute stress still works via the HPA axis; the system suspected to mediate the deleterious effects of chronic stress.

Acute stress has quite different effects on cellular morphology and behaviour, however, Braun and Hauber (2013) reported that an acute stressor applied prior to testing (via systemic administration of yohimbine, a synthetic corticosterone), decreases a rat's sensitivity to outcome devaluation. Schwager et al. (2014) demonstrated with the same paradigm, that rats administered yohimbine prior to a delayed discounting task performed more poorly compared to controls. A delayed discounting task requires rats to understand their action outcome associations in finer detail; two trained levers are presented with one offering no delay of outcome delivery and the other an increasing or decreasing delay, but with a larger outcome delivered. Initially, the delayed lever begins with no delay and gradually increases or the reverse. When the delay is short rats quickly preference the large outcome/delay lever, as the delay increases they return to the no delay lever. Rats must be patient and inhibit certain responses to continue to gain the larger reward. The authors argued that this task provides a measure of choice flexibility; control rats change their preference to the lever with no delay when the delay is largest and change back to the delay lever when the delay is sufficiently short. Yohimbine administation caused the rats to stick to the lever they were first exposed to, i.e. to continue to press the delayed lever if it was initially presented even with ascending delays, or to press the non-delay lever even when it is presented against a changing delay lever with a short delay. The authors suggest that this type of inflexibility has been linked to alcohol (Corbit and Janak, 2007, Dickinson et al., 2000, Furlong et al., 2016), and drug abuse (Zhang et al., 2011, Becker et al., 2011, Marinelli and Piazza, 2002). These two acute stress studies demonstrate that acute stress can impact on goal directed action in a similar way to chronic stress, via insensitivity to outcome devaluation (Braun and Hauber, 2013), and effects other measures of goal directed action such as delayed discounting (Schawager et al., 2014). If these studies are analogous to the

effects one would expect to see in chronic stress animals, it increases the reliability of the prediction that chronic stress will make an animal insensitive to outcome devaluation, but also raises the suggestion that chronic stress animals may bias animals towards inflexibility in their decisions.

Lastly, a study by Soares et al. (2012) demonstrated that stress can affect decision-making in human subjects. Students who had just finished a long period of preparation for a medical residence exam were compared to students under normal studying conditions. Stress was measured by a perceived stress questionnaire and the Hamilton scales of anxiety and depression. Participants were instructed to fast for 12 hours prior to the experiment and were taught to respond to two different images, which would result in chocolate milk or tomato juice being delivered. Outcomes were measured on a pleasantness scale prior to the exam to ensure desire to press for the outcomes. Following training, participants were given an hour to consume as much of an outcome as they liked, to induce specific satiety outcome devaluation. When placed in the task again, stressed participants pressed for the devalued outcome significantly more than the controls. This method of human outcome devaluation has been replicated previously (Valentin et al., 2007). The finding that the effects of chronic stress on decision making translate to human studies further increases the reliability of the prediction that chronic stress should make an animal insensitive to changes in outcome value, but also highlights that these effects are not exclusive and appear to translate across species.

Stress and PIT

There are few investigations into the effects of chronic stress on sPIT. As a direct assessment in the literature is lacking, we will focus on analogous experiments from acute stress and human studies. Morgado et al. (2012) presented evidence that CMS, in a paradigm less intense than that used by Bessa et al. (2013, see p. 34) showed a transient decrease in the sPIT effect. Their stress paradigm lasted 28 days and only consisted of exposure to 1 hour of restraint, overcrowding, cold water, vibration or steam, and was presented prior to behavioural training. During extinction, rats

showed no sPIT effect, pressing equally on both levers regardless of the presentation of the pavlovian cue. Morgado et al. (2012) argued that this decrease in sPIT performance was the result of the effect stress has on the PFC. As discussed in subchapter 1.4 chronic stress has been reported to decrease grey matter volume, dendrite lengths, and synaptic spines within PFC and its many regions. With degeneration across the PFC, this could be a likely explanation, however, as discussed in subchapter 1.5, sPIT performance is primarily driven by the BLA and NaS. If we include the OFC as a part of the broad umbrella that is PFC, and we recognise its crucial involvement in sPIT, then the argument made by Morgado et al. (2012) would explain the decreases observed. What could equally be predicted however, is a facilitation of the sPIT effect, as the BLA is known to proliferate following chronic stress, further research is required to validate the effect chronic stress has on sPIT performance. What needs to be considered however, particularly in the Morgado et al. study, is the strength of the treatment. An important study that compared stress paradigms within rats showed that 21 days of 6 hours restraint stress (i.e. CRS) was required to see the most consistent decrease in dendritic lengths and arborisation within the hippocampus (McLaughlin et al., 2007). Other milder forms of restraint stress, such as 2 hours for 10 days, saw morphological decreases; however, these were not as prominent or consistent as those induced by 6 hours for 21 days. Regards the Morgado et al. (2012) study, their effect was only transient, with the rats returning to normal sPIT performance after 6 weeks without stress, if a more severe chronic stress paradigm were used a more resilient change may have been observed.

Pecina et al. (2006) showed using rats that consistent high dose (500ng) injections of CRH induced facilitation of gPIT. Although not technically chronic stress, the chronic administration of CRH should simulate its effects. Rats were trained to lever press for sucrose pellets using a variable interval schedule, and following this, given Pavlovian training in which one CS was paired with sucrose pellets whereas another CS was presented unpaired. To prevent the unpaired CS becoming inhibitory and so suppressing lever press performance it was only introduced towards the end of training. Cannulas were implanted bilaterally into the NaS, and, following re-training, rats received 5

sessions of microinjections of vehicle, 200ng or 500ng CRH. PIT test revealed that rats that received the 500ng microinjections showed a significant increase in gPIT, increasing lever pressing during the paired CS compared to vehicle rats. As we have discussed, stress and CRH derived from stress is aversive; besides its morphological effects, it suppresses appetitive behaviour (Macey et al., 2000) particularly if introduced into the nucleus accumbens (Lemos et al., 2012). However, this increase in CRH is also correlated with increased dendritic lengths and spine densities in the BLA, NaC and NaS, (NaC vs. NaS evidence is lacking however). Pecina et al (2006) argued that CRH receptor activation in the NaS increased incentive salience to the paired CS, explaining the increase in lever pressing during the paired CS. Given the likely increases in both the NaC and NaS following chronic stress, it could be argued that perhaps the NaC was also affected. With the NaC crucial for gPIT, increases in NaC dendritic arborisation and resultant activity would be a better explanation for the increased gPIT effect reported. Additionally, if Pecina et al. (2006) argued that the NaS was affected, could it be predicted that sPIT would also be enhanced? Further research on these questions is required.

Further Analogues

Lastly, the effects of stress on PIT in humans have also been measured. Pool et al (2015) showed that human participants that submerged their hand in freezing water for as long as they could, "wanted" the conditioned outcome more than non-stressed individuals. Participants were taught to squeeze a handle to release a pleasant chocolate odour and, following this, received pairings of a specific Pavlovian stimulus with the chocolate odour. During the test, when the Pavlovian CS was shown the stressed participants squeezed the handle with significantly greater force compared to unstressed controls, showing an enhancement in gPIT. Additionally, Quail et al., (2016) showed that human participants taught to manipulate a virtual vending machine to earn snack foods, such as chocolate or chips, observed a stress-related deficit in both the gPIT and sPIT effect. However, in this study participants were scored using the Depression Anxiety and Stress Scale (DASS), and participants that scored higher on the DASS saw a reduced gPIT and sPIT effect

compared to controls. Instead, high scoring DASS participants responded more during the presentation of a cue associated with a non-reward. These two studies indicate that exposure to either an acute stressor or (at least potentially) a chronic stressor (as represented by higher scores in the DASS) effect PIT performance, and just like in the human studies that investigated outcome devaluation that were discussed earlier (p.54), the effects of chronic stress do translate to humans to affect decision making measures in similar ways. This reinforces the need for further study into the effects of chronic stress on decision making.

These few studies together indicate that PIT is sensitive to stress, affecting both general arousal driven vigour (gPIT) and specific cue driven promotion of action (sPIT). However, specifically within the study of chronic stress, systematic findings are still lacking and those that have been reported are often conflicting; reports indicate increases, decreases and no change, all explicable, but with little consistency. The regions involved in PIT are clear, and the regions affected by stress are becoming more apparent (subchapter 1.4). Further research is required to paint a clearer picture on both the effect of chronic stress on the regions required for PIT and the resultant behavioural effects. Specifically, using a chronic stress model that offers the greatest consistency and reliability for morphological change, might induce more consistent effects on the regions required for PIT.

Reinstatement

It would appear from the literature that chronic stress impairs goal-directed action. With regard to PIT, however, it should be noted that to exhaustively investigate the effect chronic stress has on the ability of cues associated with a specific outcome to guide action selection, we must also consider literature that looks into whether these effects are due to an increase in stimulus control or are secondary to an enhancement in outcome-mediated action selection, i.e. does chronic stress effect action→outcome or outcome→action associations or both? Investigation into this outcome-specific reinstating effect was first conducted by Ostlund and Balleine (2005), where they showed that, after a period of extinction of the instrumental action, the outcome provided specific

information that could engage action selection. Literature on this topic is largely restricted to drug or high fat/sugar seeking behaviour, exclusively so in stress research, in which context the literature indicates that chronic stress induces increased drug and palatable food seeking reinstatement, i.e. after a period of extinction, chronic stress increases the rate at which animals seek drugs or palatable foods after a priming exposure to said outcome (Al-Hasani et al., 2013, Bahi and Dreyer, 2014, Ball et al., 2015, Calu et al., 2014, Chen et al., 2014, Nair et al., 2006). One piece of reinstatement literature does however compare stress-induced reinstatement to outcome-induced reinstatement using food pellets (Ball et al., 2016), showing that chronic stress caused a marginal enhancement of reinstatement, however, this could only be argued to be generally enhanced reinstatement, if chronic stress were found to enhance outcome-specific reinstatement, two outcomes of similar incentive value should be used.

Implications for the Design of Experiment 2

To investigate the effects of chronic stress on decision-making we will use outcome devaluation, contingency degradation, sPIT and reinstatement tests to determine any changes in decision making following chronic stress. We will then compare these changes to changes in neuronal morphology in the aforementioned ROIs. We expect stress to change neuronal morphology in the discussed ROIs in the directions indicated by the summary Fig. 2. And we predict, therefore, a decrease in goal directed behaviour and an increased bias or dependence on habits. As the literature on chronic stress and sPIT is thin, we cannot predict a specific directional effect with this test; however, given the reports of enhanced dendritic length in the BLA, we predict a facilitation in the sPIT effect. Although conflicting with Morgado et al.'s (2012) findings, as already discussed, there are significant issues in interpreting those effects. By investigating these expected changes within neuronal morphology with the novel imaging methods described in subchapters 1.2 and 1.3, we can confirm morphological changes using high resolution imaging of the dendrites and synaptic spines in the key regions known to be involved in the sPIT effect. Jump to Experiment 2 here.

Summary

Stress is a powerful force that allows animals to maintain a healthy homeostasis, however, in particularly severe and chronic situations any beneficial effects of stress are eclipsed by harmful effects. Stress is shown to cause damage and deterioration in many regions of the brain, but also to cause dendritic proliferation within the BLA and nucleus accumbens. This is not a healthy change: it is likely to promote increased activity and output, dysregulating the regions normal activity and the activity of any connected structures. In addition, two distinct processes have been discussed that impact on decision making: (1) Animals make decision based on two dissociable strategies, (i) flexible goal directed actions, where the decision is sensitive to feedback regarding current outcome value and (ii) relatively inflexible habitual actions, where the decision is sensitive to antecedent stimuli; (2) Pavlovian cues can produce a general arousing effect on instrumental performance (gPIT) or elevate performance of actions that earn outcomes shared by Pavlovian predictors (sPIT). As has been discussed in the limited literature, chronic stress appears to affect these decision making processes, making the animal insensitive to changes in outcome value and in the action-outcome contingency. Furthermore, chronic stress impacts on the influence of Pavlovian cues action selection and performance. However, the direction of this change is not clear for either general or specific PIT. With greater insight into stress, goal directed action, PIT and their interactions, we will be able to elucidate the detrimental effects stress has on our decision-making processes.

1.7. Behavioural Control and its Impact on Stress

There is, however, one factor that can mitigate the effects of stress, and that is controllability; how much control over a stressor an animal has appears to exert a powerful impact on the effects the stressor has on the animal. The protective effects of controllability were first observed by Seligman and Maier (1967) who showed that animals developed a certain type complacency or helplessness to their behaviour when the animal learned that it had no control over

an aversive stimulus. Seligman and Maier (1967) used a paradigm where dogs were restrained in a harness which could be repeatedly electrified. One group of dogs, the Escape group, could use their head to press on a lever in front of them to terminate the shock while the other Yoked group, although still presented with the levers, could not terminate this shock themselves but received the same amount of shock as the master animal to which it was yoked. Following this treatment both the Escape and Yoked groups along with a control, unshocked, group, were tested on a new escape/avoidance task. In this task animals are trained to learn that a stimulus, in this case a light, proceeded the electrification of the floor. Dogs could move to the other side of the chamber to avoid the signalled shock. In this escape/avoidance task the Yoked dogs escaped significantly less, compared to the Control and Escape groups, instead choosing to endure the shock. The escapable shock (ES) dogs which had control over the termination of the stressor performed better than the yoked/inescapable shock (IS) dogs, which had no such control. Seligman and Maier (1967) argued that IS dogs learned that shock termination was independent of its responding in the harness and that this learning inhibited subsequent escape responding in the escape/avoidance task. Expanding on this, Seligman and Maier (1967) determined that animals exposed to escape/avoidance training prior to exposure to IS training showed no such inhibition to learning, demonstrating that in this case IS did not reduce escape performance on a subsequent escape/avoidance task. Together these two experiments demonstrate that exposure to control over a stressor protects the animal from the effects of stress. This effect has been shown repeatedly; when an animal learns that their actions no longer have a bearing on a stressor, animals reduce escape performance, however, if animals learn that they can escape a stressor via a response, they do not express this *learned helplessness* (LH) and perform on many measures the same as controls. Maier and Seligman (1976, 2016) reviewed the ES vs. IS and LH studies conducted after the first and fifth decades following Seligman and Maier (1967) and these reviews argue that not only is the cognitive ability of the animal to learn escape actions affected, but also the motivation for the animal to escape reduced, as studies have shown through various manipulations to try to motivate IS animals to escape to no avail. The authors also focussed

on changes in emotional state, as they began to link the psychological phenotypes of LH to those of anxiety and depression (Maier and Seligman, 1976), but also how it is in fact the presence of control, and not the absence of it, that produces the aforementioned effects (Maier and Seligman, 2016). As this thesis is primarily concerned with regional and morphological changes, we will focus continued discussion on controllability in this context.

Neuroanatomy

Using ES vs IS studies, the controllability literature has focussed on the involvement of the PFC and dorsal raphe nucleus (DRN) (Amat et al., 2005, Amat et al., 2006, Maier and Watkins, 2005) on the way that controllability mitigates the effects of stress. This was demonstrated clearly in the Amat et al. (2005, 2006) studies, where authors injected muscimol into the PLC and IFC regions 60 minutes prior to escape learning. In their task, ES rats were required to spin a wheel to terminate shock, the requirements of the wheel spin increasing with success and decreasing with failure. In IS rats, the wheel was present however did not terminate shock. Following this, rats were tested for measures of LH and anxiety using an escape/avoidance task, similar to that previously described, and a conditioned fear test. In the fear test freezing is measured, with increased freezing indicative of increased anxiety. As expected IS rats had reduced escape performance in the escape/avoidance task and increased freezing in the fear test, compared to ES and control rats. However, in the muscimol rats, although the treatment had no further detrimental effect on IS rats, it rendered the performance of ES rats similar to that of IS rats. ES rats given infusions of muscimol into the PLC and IFC region, showed decreased escape performance and increased freezing mirroring that of IS rats. In the 2006 study, the authors replicated this effect, but also showed that the protective effect of prior exposure to controllability was also lost via treatment of muscimol to the PLC-IFC region. Rats given the prior exposure to controllability were immunised to the effects of subsequent IS, presenting the same as ES rats in escape performance and freezing. However, if treated with muscimol during the prior exposure, this effect was lost and a group previously exposed to controllability now presented

as IS rats on the escape and freezing measures. Unfortunately, the PFC has so far not been delineated past the region shared by the PLC and IFC in respect to the protective effect of controllability. Amat et al. (2006) argued that the mPFC (referring to the combination of the PLC and IFC) is required to both process the information about controllability over the stressor and utilize this information to regulate responses to subsequent stressors. The protective effects of controllability may be better understood if it were known whether it was specifically the PLC or IFC involvement because, as previously discussed in subchapter 1.5, the PLC and IFC are heterogeneously involved in the decision making process.

Importantly, other regions important for normal decision-making have been reported to be involved in the protective effects of controllability of stress. Two studies in particular highlight novel ROI in controllability and indicate that behavioural control over a stressor modulates the detrimental effects of stress on behaviour. Amat et al. (2014) demonstrated that rats that were subject to ES performed better, as well as controls, in social exploration tasks compared to IS rats. Social exploration tasks are used as measures of anxiety and depressive-like behaviour, of which we use chronic stress as a model. Measuring *Fos* immunoreactivity, as a measure of neuronal activity, Amat et al. (2014) found that ES rats had higher activity in the DMS and DLS, compared to both controls and IS rats, indicating that the dorsal striatum may be involved in the protective effects control has over the stressor. However, following DMS and DLS antagonism, only silencing of the DMS affected the social exploration task, rendering the performance of ES rats more similar to IS rats. The authors argue, therefore, that the DMS is principle to the protective effect of control. This indicates an interesting potential relationship between stress and decision-making: if it is control over a stressor that dictates how damaging it is, a new approach must be used to understand previous research, and further investigations conducted into the effects of stress on decision making, particularly because the DMS is crucial for that functional capacity. Further to this, using a similar ES vs IS design, it has been shown that prior controllability over a stressor accelerated subsequent cocaine extinction (Baratta et al., 2015). When rats were trained to self-administer cocaine, exposed to ES or IS training

and then placed on extinction where nose poke no longer resulted in cocaine, the IS rats took the longest to extinguish, whereas the ES and control rats took significantly less time, with ES rats extinguishing faster than controls. Additionally, Barratta et al. (2015), silenced IFC neurons via photoinhibition during the prior exposure to ES. This silencing made ES rats present as IS rats in the subsequent cocaine extinction suggesting that prior exposure to controllability or no control, facilitates and inhibits extinction learning respectively, and that the IFC must be involved in the facilitatory effect controllability has over a stressor

These studies together indicate that control over a stressor not only protects against deficits in escape performance (Amat et al., 2005, Amat et al., 2014, Amat et al., 2006), but also promotes extinction learning (Baratta et al., 2015). And the regions reported to be involved are the DMS, IFC and PLC, so it is not unlikely that such effects involve some form of goal-directed learning as these regions are heavily involved in decision making (see subchapter 1.5). In addition to the regions shared with decision-making, these regions are also affected by chronic stress, with chronic stress degenerating morphology across all three regions (see subchapter 1.4). However, before we begin to discuss the relation to chronic stress, we must also discuss the morphological literature surrounding controllability.

Morphology

Shockingly, there are very few studies that have assessed the morphological changes induced by ES vs. IS. Hajszan et al. (2009) showed that rats that develop LH i.e. IS rats, via a forced swim test, (where the rat is forced to swim in a body of water from which it cannot escape) showed reduced synaptic spine density within the HIP, and this remodeling of synaptic spines has been replicated (Hajszan et al., 2010). In another study, Yang et al. (2015) showed that dendritic spine density in IS rats was decreased in the HIP and PLC and increased in the nucleus accumbens, compared to controls and ES rats. Interestingly these morphological changes are similar to those reported following exposure to chronic stress. A decrease in HIP morphology is also an indicator of chronic

stress, and the proliferated morphology within the nucleus accumbens could also be an indicator of chronic stress, if delineated further, with reports of increases in the NaC (see p. 34). The similarities may extend even further, however, currently there is no other literature examining the morphological effects of controllability of a stressor. Clearly this is an area that demands further research.

Predictability

How the protective effects of controllability apply to chronic stress studies is difficult to say. Chronic stress by its nature removes control from the target, so previous research is still consistent with these findings; however, establishing controllability within a chronic stress paradigm obviously cannot then be readily achieved. One possibility might be to manipulate predictable vs. unpredictable chronic stress. In 1971 Weiss showed that ES rats had reduced gastric ulcers compared to their counterparts (Weiss, 1971). Gastric ulcers are used as a measure of stress within the animal, with larger ulcers indicative of a stronger effect of stress. He argued that the lever press was a type of coping strategy which the rat engages in to help mitigate the impact the stressor. Weiss posited predictability would also act as a type coping mechanism, which the rat could use to help it cope with the stressor. And in a similar result to the previous experiment, if a warning signal preceded the shock, rats in the predicted group had reduced ulceration compared to the non-predictive counterparts (Weiss, 1971, Weiss, 1972). This effect on gastric ulcers has been replicated, and has been argued that it specifically indicates a reduced effect of the acute stressor (Tsuda et al., 1983, Goldman and Rosoff, 1968, Konturek et al., 2010). Additionally, rats are shown to have reduced blood pressure and corticosterone concentration when they can predict a shock, indicative of reduced effect of the stressor (Lawler et al., 1993, Pitman et al., 1995). As such, it might be expected that if a chronic stressor were predictable that it would be less harmful than an unpredictable stressor.

Seligman and Meyer (1970) also confirmed this prediction using a similar procedure to the other studies at the time finding that animals given a predictor prior to the stressor had significantly less gastric ulcers and gastric ulcer size compared to the predicted group. Furthermore, the degree of ulceration was measured after 70 sessions of 50 minute trials, and although no other measures were collected, it could be argued to constitute exposure to a chronic stressor. This protective effect of predictability has been replicated in similar models (Gliner, 1972, Sawrey, 1961, Seligman, 1968). Nevertheless, there are equally as many studies that have reported the opposite effect; that predictability of stressors is more harmful than unpredictability. Pare (1964) showed that 23 days of predictable floor shock resulted in significantly reduced body weight and increased adrenal gland weight in the predictable stress rats. And this effect has been replicated as well (Brady et al., 1962, Davis and Levine, 1982, Glavin and Mikhail, 1976). In an excellent review Abbott et al. (1984) collected all the literature on predictability of stressors and found that, on a cursory view, the field is contradictory and inconsistent. However, when viewed in greater detail, Abbott showed that the contradiction comes from (1) the measures used to determine how the harmfulness of the stressor and (2) the number of sessions the stress was applied. Unsurprisingly, the effects reported in studies using single sessions of treatment differ from those using more chronic treatments. If a single session is used the literature is more consistent with predictability protecting against the effects of the stressor; rats exposed to predictable stress have greater body weight, less ulceration and adrenal weight. But in studies where five sessions or greater are applied, this consistency is lost. What is lacking from this research however, is morphological and behavioural analysis which may reveal where predictability does or doesn't protect against the effects of chronic stress. Further what may be confounding this research is the use of a strong physical stressor and variation in stressor intensity between studies, with some studies using much higher amplitude in their shocks than others. A more psychological stressor, such as CRS, may also be helpful in elucidating these effects.

Research in human studies suggests that predictability is protective and not as harmful as unpredictability. Schulz (1976) performed a predictability study on people who permanently live

within an aged care home. Three groups were generated from the population, each to be visited by researchers. A control group had control over when and how long the researchers would visit, the predicted group were told how when and how long they would be visited, and the random group had no control nor predictions and were visited on a completely random schedule leaving at random times. Schulz found that both the predictable and control groups reported significantly fewer feelings of depression and helplessness and increased feelings of wellbeing compared to the random stress group. Schulz argued that the feelings of depression present within the elderly population can be attributed, at least in part, to loss of control that develops with the elderly permanently living within an aged care facility. Although human wellbeing is complex and difficult to compare with measures in the rodent literature, the effect of control and predictability may well translate across species. Indicating that the tangible nature of chronic stress, if mitigated by predictability, could carry across to reduce changes in morphology and decision making.

Investigating the effect of prediction on chronic stress still needs further research to clarify its impact. Most importantly, investigating the effect of prediction of chronic stress on morphology and decision making, particularly in the PLC, IFC and DMS, would reveal whether the protective effects of prediction transfer within more psychological stressors (i.e. CRS) and if so whether this protection maintains healthy morphology and decision making, which has failed to be properly instigated to date.

Implications for the design of Experiment 4

In an attempt to mitigate any effects of chronic stress on neuronal morphology and decision-making, we plan to manipulate, not controllability per se, but the predictability of the chronic stress treatment. Although it is possible – quite likely in fact – that the effects of predictability are in the animals coping behaviour and so in its ability to control the effects of the stressor, it is difficult directly to manipulate those responses in and of themselves. To achieve this, I plan to use the same design as Experiment 2 (p. 58) but to add a third group of rats, the predicted stress group, which will

be exposed to stimuli that allows them to predict upcoming stress exposure to engineer for the rats an environment where they, at least potentially, can exert at least some control over the stressor. A second group will receive random chronic stress rats similar to chronic stress treatment used in Experiment 2, however, the starting time for treatment will be highly variable and more truly random than in Experiment 2. We predict that the predicted stress rats will be at least partially protected against the negative effects of chronic stress on morphology and decision making and that the random stress rats will show a similar or exaggerated treatment effect to that observed in Experiment 2. Additionally, so as to exhaustively examine behavioural changes within chronically stressed animals and their morphological correlates, if any, we plan to add delayed discounting, as described (p. 53) to the battery of behavioural tests. As in Experiment 2 we will use the novel methods developed as described in subchapters 1.2 and 1.3 to take high resolution images with the most complete information about the neurons, to study morphological changes within these two stressed groups of rats. And as before we expect that changes within the morphology will correlate systematically to changes in decision making.

Summary

One of the most important recent findings is the effect of controllability over stress on decision-making. Animals that learn that their actions cannot terminate a stressor develop a symptomology where they are not motivated to escape a harmful stressor nor can they as readily encode the learning required to escape. This LH effect can be immunised against if an animal is exposed to stressor controllability prior to a treatment that would otherwise induce LH. Further, the DMS, IFC and PLC are all reportedly crucial to these effects, with inactivation of these regions leading to animals that would otherwise present healthily, due to prior exposure to control or having control in the stressful situation, as animals without control. In a more recent review Maier and Watkins (2010) suggest that the literature heavily indicates that the control an animal has over stress blunts the negative impact that the stressor. Additionally, controllability and predictability are intertwined,

promoting similar protective effects, albeit in more chronically stressful situations this is less consistent. We will use predicted stress in the hope that, like controllability, predicted stress will protect against the negative effects of chronic stress particularly the changes in morphology, which have been relatively understudied.

1.8. Conclusion

The scope of this thesis is broad and ranges from complex modifications to methodology involving quantum chemistry, to the study of morphology and decision making, and the effects of stress and controllability. In this introduction, I initially discussed how the Golgi-Cox stain could be improved, how it could work within days and within previously restricted conditions, how the compounds within this new URG stain could be energised to emit light, increasing resolution and 3D rendering. I also discussed how chronic stress effects the brain, typically degenerating dendritic arborisation and synaptic spine density across all regions, but uniquely proliferating morphology within the BLA and NaC; how in some regions (NaS and DLS) there is no consensus currently on the direction of change, and in others (InS, CN, LO, VO, MO) there simply was not enough information to make predictions. I also discussed how little research has been conducted on the effects of chronic stress on decision making, often needing to draw analogies from related areas of research. Nevertheless, from this limited literature, I argued that chronic stress should bias an animal towards habits over goal directed action control. I then expanded on the study of decision-making, the tests and measures used, specifically outcome devaluation, contingency degradation and PIT tests. And lastly I discussed how controllability over a stressor can mitigate the damaging effects stress has on animal. And although this literature is limited to acute stress, I argued that if we used prediction as a form of control, which has been shown in some studies to have similar mitigating effects, within a chronic stress paradigm, we would predict similar protective effects. As the title suggests this thesis brings together all of these topics and fields. I will use the novel methodology developed herein to

analyse morphological changes caused by chronic stress to then assess associated changes in an animal's decision-making and any protective effects of stressor predictability.

Chapter 2: Methods

This chapter, unlike Chapter 1, is detailed in chronological order, as in Experiment 1 development of the URG was performed first, then following this Experiment 2 the first behavioural study. It is presented in this fashion to allow the reader to follow and understand the progress of this thesis and understand the steps taken by myself, i.e after the development of the URG stain and its application, I felt the stain could be improved even further and following such was then applied to great success in Experiment 4.

Experiment 1 – Application of an Ultra Rapid Golgi-Cox Method to Modern Brain Clearing Techniques

Animals

A total of 36 adult male hooded Wistar rats (Laboratory Animal Services, University of Adelaide, Australia) weighing approximately 250-350g were used across the development of this method, with 28 rats used in replication of methods. Animals were housed in 12h light/12h dark cycles, with food and water *ad libitum*. All methods and protocols were performed in accordance with The University of Sydney Animal Ethics Committee (AEC No. 5960).

Ultra Rapid Golgi (URG)

Golgi stock solution was prepared 1 day before incubation. The solution may be stored for up to 6 months. The final solution is mercury chloride, potassium chromate and potassium dichromate, each at 1% concentration. A stock solution of 500mL would be made as follows; 5g mercury chloride is first dissolved in 100mL of distilled water at 80^{o}C, allowed to cool, 100mL of 5g potassium dichromate is dissolved in distilled water and added while stirring to the above solution. The

resultant solution is then added while stirring to 5g potassium chromate dissolved in 300mL distilled water. Ensuring the solution is protected from light.

The animals were deeply anesthetized (Lethalbarb ™ 325mg Pentobarbital Sodium/mL) and transcardially perfused with either 0.9% saline (250ml) followed by 4% paraformaldehyde (pH 7.4, 200ml) followed by 0.1 M PBS (300ml), or with 0.9% saline (300ml) followed by 0.1 M PBS (250ml). Brains were removed and placed in light sensitive (100% UV blocked) polypropylene 50mL tubes with 20mL of URG solution, ensuring the samples are completely submerged. The containers were then placed in an incubator set to 37°C or 42°C for PFA fixed brains, and left for 36hrs. Brains were then rinsed twice in distilled water for 5 minutes each, then left in 33% Ammonium Hydroxide for 20 minutes. Brains were rinsed for another 5 minutes in distilled water before being left in a 10% Sodium Thiosulfate solution for 20 minutes. Development was performed in the in a dimly lit room, with aluminium foil used to keep samples in the dark. Development was performed either immediately following incubation or following clearing. In which case after incubation brains were cleared following the protocols described below, and then developed the same as above.

URG may be used without a clearing technique as follows, after incubation, samples were cut into 200um sections on a vibratome (Leica VT1200s) in an ice chilled bath of 0.1 M PBS, and placed onto gelatinised slides. Prior to sectioning the sample may be placed in 30% sucrose solutions for up to 5 days. Filter paper was used to remove excess PBS from the slides, which were left in the dark for 10 minutes to ensure strong adhesion, in other circumstances an incubator set to 37°C was also used for 5 minutes offering similar adhesion results. Slides were then developed as above. Following this slides were dehydrated in a series of ethanol solutions each rinsed for 4 minutes; 50%, 75%, 90% 100%. Lastly, slides were rinsed in xylene twice for 4 minutes each. Slides were coverslipped with Entellan ™.

Clearing Techniques

For both CLARITY and CUBIC clearing, brains were cubed into the following cuts: 5 x 5mm, 5 x 10mm and 10 x 10mm as well as in half coronally, horizontally and sagittally and left as a whole brain.

CLARITY

Brains were CLARITY cleared using the following method. A hydrogel solution was first prepared via combination of 40ml of acrylamide (40%), 10 ml of bis-acrylamide (2%), 1g of VA-044 initiator (10% wt), 40 ml of 0.1M PBS, 100 ml of 16% paraformaldehyde and 210 ml of dH_2O (cf. Chung et al., 2013), tissue was then submerged in hydrogel solution, within 50ml light sensitive tubes as described above, ensuring the brains are completely covered and in the dark, and stored at 4^{o}C for 3 days. Following this hydrogel was embedded into the tissue within a desiccation chamber under a fume hood. This process removed as much oxygen from the tube as possible. The 50ml tubes were placed on a rack in the desiccation chamber. I twisted cap of the 50-ml conical tube off to allow gas exchange, then turned on the nitrogen tank and adjusted the control valve such that the inlet to the desiccation chamber filled with nitrogen gas and switched the desiccation chamber valve from flow to the vacuum and then turn on the vacuum pump. I kept the vacuum pump on for 20 min. Then to extract the tubes, I turned the flow of nitrogen back on, while opening the chamber enough to quickly reach the tubes and re-seal their lids. Following this, the tubes were placed within a rotating incubator set to 37^{o}C for 3 hours. Finally, under fume hood, I extracted the now embedded tissue from the gel and washed it in a 4% solution of sodium dodecyl sulphate (SDS). Under passive clearing conditions, tissue was left in said SDS solution for 48 hours in a rotating incubator at 37^{o}C, and tissue was clear. For acitive clearing tissue was instead transferred to a electrophoretic tissue clearing (ETC) chamber, one was constructed using the standard designs laid out (http://clarityresourcecenter.org/docs/ETC_Chamber_Designs/). Tissue was placed in said ETC chamber, with circulating 40 ^{o}C SDS solution described above. 20 V was applied through the tissue as circulation continued for 48 hours.

After 48hrs the tissue was clear. If tissue needed to be developed, described protocol above was followed, otherwise I proceeded to prepare the brains for imaging; Tissue was incubated at 37^{o}C in 85% glycerol in water for 24hrs. Images were taking from brains free floating in a small petri dish surrounded by 85% glycerol and from Blu-Tak ^{TM} sealed coverslipped slides.

CUBIC

Brains which were cleared via the CUBIC methods instead followed this protocol. Beginning with the preparation of Sca/eCUBIC-1 (reagent 1) solution which was a solution of 25 wt% urea, 25 wt% N,N,N',N'-tetrakis (2-hydroxypropyl) ethylenediamine , and 15 wt% polyethylene glycol mono-p-isooctylphenyl ether/Triton X-100 (cf. Susaki et al., 2014, Susaki et al., 2015). No reagent-2 was prepared, as it was not required, and prevented the rapid clearing and URG stain adherence. Tissue were submerged in enough reagent 1 for it to be completely covered, and placed on a rotating incubator at 37^{o}C for 48 hrs. Tissue was clear after 48hrs. If tissue needed to be developed, I followed protocol described above, otherwise images were taking from brains free floating in reagent 1 in a small petri dish or sealed between a microscope slide and a coverslip in Olympus immersion oil nd refractive index of 1.516. Samples may be stored in reagent 1 or immersion oil for up to 2 weeks before stain deterioration or fading began to occur. All imaging was performed on a ZEISS laser Scanning Microscope 710 (LSM710) and processed using the Zen 2015 software package.

Imaging

Confocal

Confocal imaging was performed on a ZEISS laser Scanning Microscope 710 (LSM710) with an argon laser and processed using the Zen 2015 software package. Samples were prepared as described above for confocal imaging. Z-stacks were captured using a 20x objective for both

brightfield and reflective imaging. Reflective imaging was achieved using the 488nm wavelength. For both brightfield and reflective imaging filters were removed.

Lightsheet

The Lightsheet Z.1 (Carl Zeiss) setup equipped with an EC Plan-Neofluar 5x/0.16 detection and a 5×/0.1 illumination object was used in this study. Samples were attached to one end of a glass capillary and inserted into the chamber filled with 85% glycerol in H_2O from the top. The sample was moved continuously through the lightsheet along the z-direction. The scattering of the metal particles as the sample travels through the lightsheet was detected by inserting a 100% reflective mirror in the front of the camera. 3D reconstruction of the z stacks was undertaken by Huygens and Volocity software. Jump to Results of Experiment 1 here

Experiment 2 – The Effects of Chronic Stress on Decision Making and Neuronal Morphology.

Subjects and apparatus.

32 Male Long-Evans rats (250-350g) (divided into 2 groups; n=16) served as experimental subjects. All treatments, examination and culling of these rats were performed in accordance with the guidelines stipulated by the University of Sydney Animal Ethics Committee (AEC No. 5960) and as approved by the committee. Animals were housed in groups of 4 or less, in 12hr light/12hr dark cycles (lights on at 7 a.m.); with ad libitum access to food and water and the temperature set at approximately 23°C. The rats were received at 5 weeks of age, and left to habituate to the cage for 1 week, during which rats were handled by the experimenter for at least 2 min a day for 3-4 days consecutively to reduce stress induced by handling, after which treatment began. Rats were maintained at 85% of their free feeding weight by restriction of food intake.

Training and testing took place in identical operant chambers (24 × 21 × 30 cm; Med Associates) housed within sound-attenuating cubicles. Each operant chamber was equipped with a pellet and sucrose dispenser that delivered a 45-mg pellet or and 0.1mL of 20% sucrose solution when activated into a receptacle that was positioned in the centre of one wall. In addition, each chamber contained two retractable levers located on the left and right sides of the receptacle. A 24-V/3-W house light mounted within the chamber. The speaker that delivered the auditory conditioned stimuli was mounted on the wall opposite to the levers and the receptacle. Two auditory stimuli (white noise and a tone) served as conditioned stimuli. A computer system (MedPC Software; Med Associates) controlled the equipment and recorded the data. For chronic stress, Perspex restrainer tubes (Able Scientific, 21cm in length and 6 cm in diameter) closed at one end (where the nose of the rat was placed) with small holes to allow adequate ventilation and with an adjustable closure on the other end of the tube to accommodate the rat with a small hole to allow the tail to protrude were used.

Group assignment and histological controls

Of the 32 rats, 16 were assigned to a chronic stress group and 16 to an unstressed control group. Of the 16 animals in each group 6 rats served as histological controls. Generally, our aim was to relate the behavioural effects of chronic stress exposure to any changes in neuronal morphology induced by that treatment. However, it is possible that the behavioural training and testing interacted with the stress treatment compromising the histological results. To evaluate this possibility 6 histological control rats were used in each group. These rats went through all of the procedures of their group counterparts except they did not undergo any behavioural training or testing. These rats were used to establish whether the effects of stress on training and testing caused changes in dendritic morphology induced by the behavioural testing. If, after morphological analysis, any differences observed between groups were similar in these rats to those given the behaviourally training and testing, then we can conclude that the training did not further alter neuronal

morphology. Given this outcome, these additional histological results will be combined with the scores of the rats that went through training.

Chronic Stress

Treatment involved placing the rats individually within a clear Perspex restrainer tube (Able Scientific, 21cm in length and 6 cm in diameter) closed at one end (where the nose of the rat was placed) with small holes to allow adequate ventilation and with an adjustable closure on the other end of the tube to accommodate the rat with a small hole to allow the tail to protrude. The rats were placed under restraint beginning between 8am and 2pm and ending after 6 hours every day for a total of 21 days. Immediately, prior to and following stress, each rat was weighed. During treatment, the restrainers were placed individually in cages and in a room with the lights on, control rats were left in the group cage without access to food or water and also in a room with the lights left on. At the end of this treatment period, on the morning of the 22nd day, behavioural training started.

Behavioural Procedures

For a breakdown of the sequence of behavioural training and testing see Fig. 3.

Magazine training

For the first 2 days all rats were placed in the operant chambers for 20 min. During magazine training both the pellet and sucrose outcomes were delivered on independent 60-s random time schedules with the levers retracted. The house light was illuminated at the start of each session and turned off when it ended.

Pavlovian Training

Rats were given eight sessions of Pavlovian training one each day for eight days. Each session consisted of 8 presentations each of the white noise and the clicker stimuli each for 2 min (CS).

During each of these presentations either the pellets or sucrose outcomes were delivered into the magazine. For half of the rats the pellets were delivered during the noise and the sucrose during the clicker whereas for the other half these stimulus-outcome associations were reversed. Outcomes were delivered on a random time 30s schedule during the stimuli. The stimuli were presented in a pseudo-random order, which changed each day. A variable inter-trial interval (ITI) was used with a mean of 4 min. Magazine entries were recorded during each session which lasted approximately 1 hr.

Instrumental Training

After Pavlovian training the rats received eight sessions of instrumental training over the next 8 days. Rats were trained to perform lever press responses on the left and on the right lever. Each lever was associated with one of the two outcomes, either the pellet or the sucrose. For half of the rats the left lever delivered pellets and the right lever sucrose whereas for the remainder this lever-outcome association was reversed. Counterbalancing of the lever-outcome associations made with respect to stimulus-outcome counterbalancing during Pavlovian conditioning.

For instrumental training the outcomes were delivered on a continuous reinforcement schedule for the first 2 sessions during which each lever press resulted in an outcome. For the next three sessions the outcomes were delivered on a random ratio 5 (RR5) schedule during which each lever press had a 0.2 probability of delivering an outcome. Outcomes were then delivered on an RR10 schedule for the final 3 days of training during which each outcome was delivered with 0.1 probability after the appropriate lever press response. Each session lasted for 50 min and consisted of two 10 min sessions on each lever (i.e., 20 min on left lever and 20 min on right lever in total) separated by a 2.5 min time-out period in which the levers were retracted and the houselight was turned off. The order of presentation of each lever was pseudorandom.

Pavlovian-Instrumental Transfer Test

After the instrumental training was completed the rats received a Pavlovian-instrumental transfer test. This test was conducted in extinction; i.e., no outcomes were delivered at any time. During this test the rats were able to lever press while various stimuli were presented. No stimuli were presented for the first 4 min, following which there were eighteen 2 min periods during which there were eight 2 min stimulus trials, 4 of each stimulus, separated by 2min ITIs. Lever pressing was recorded during each bin. The stimuli were presented in an ABBABAAB order; i.e., clicker, noise, noise, clicker, noise, clicker, clicker, noise. The test last for 45 min.

Retraining

Rats received 1 session of RR10 lever press retraining following each test to ensure strong and consistent lever press performance. After the PIT test and this retraining session an outcome devaluation test was conducted.

Outcome Devaluation

The day following retraining, rats were given free access to either the pellet or the sucrose outcome for 1 hour. The aim of this treatment was to induce sensory-specific satiety for one or other of the two outcomes (cf. Balleine & Dickinson, 1998). After this pre-feeding period the rats were given a 10 min choice extinction test in the operant chambers with both levers extended with lever presses being recorded. The next day the rats were pre-fed on the other outcome for one hour (if, on day one they were pre-fed pellets then on day two they were pre-fed the sucrose and vice versa) and then given a second 10 min choice extinction test on the two levers.

Outcome Specific Reinstatement

Following contingency degradation, rats were given 3 sessions of RR10 retraining to re-establish their action-outcome contingencies, following this an outcome specific reinstatement test

to assess their ability to use the outcome to select a specific action; a test of cued recall based on the specific action-outcome associations (cf. Balleine & Ostlund, 2007). For the reinstatement test rats are first given a period of extinction followed by the delivery of one of the two outcomes and then are given a choice between the two levers to assess whether pressing on the lever associated with that outcome is elevated. Rats received two sessions of reinstatement, one for each outcome. Each session began with a 20-minute extinction period to suppress responding, followed by noncontingent delivery of one of the two outcomes (pellets or sucrose), followed by a 3-minute period of reinstatement assessment in which lever pressing was recorded. Outcome delivery was counterbalanced across both groups, with half receiving pellets for the first test and sucrose for the second, and the other half receiving the opposite test-outcome assignment.

Contingency Degradation

After a further session of retraining on the RR10 schedule the rats began contingency degradation training. During a 6-day period rats continued to receive RR10 lever press training, however, one of the two outcomes was also delivered non-contingently. For half the rats the non-contingent outcome was pellets whereas for the remainder it was sucrose. This non-contingent schedule was achieved by dividing the session into 1-sec bins and then arranging that one of the outcomes was delivered at the same probability in each second in which no-response was performed as in each second in which its associated lever response was performed for example, if the pellet lever is degraded then the pellets will be delivered at a probability of 0.1 in each second in which the appropriate lever press response is performed and at a probability of 0.1 in each second in which no lever press is performed. In this example, the sucrose outcome continues to be delivered at a probability of 0.1 in each second in which its appropriate action is performed. Rats were given two sessions each day, one on each lever with each lasting 20 minutes separated by at least a 1-hour break. Rats were given 5 days of this contingency degradation training after which, on Day 6, they were given a 10 min choice extinction test with both levers presented. Rats were able to press freely

on either lever with lever presses recorded across the session. After this test, the following day the rats were culled.

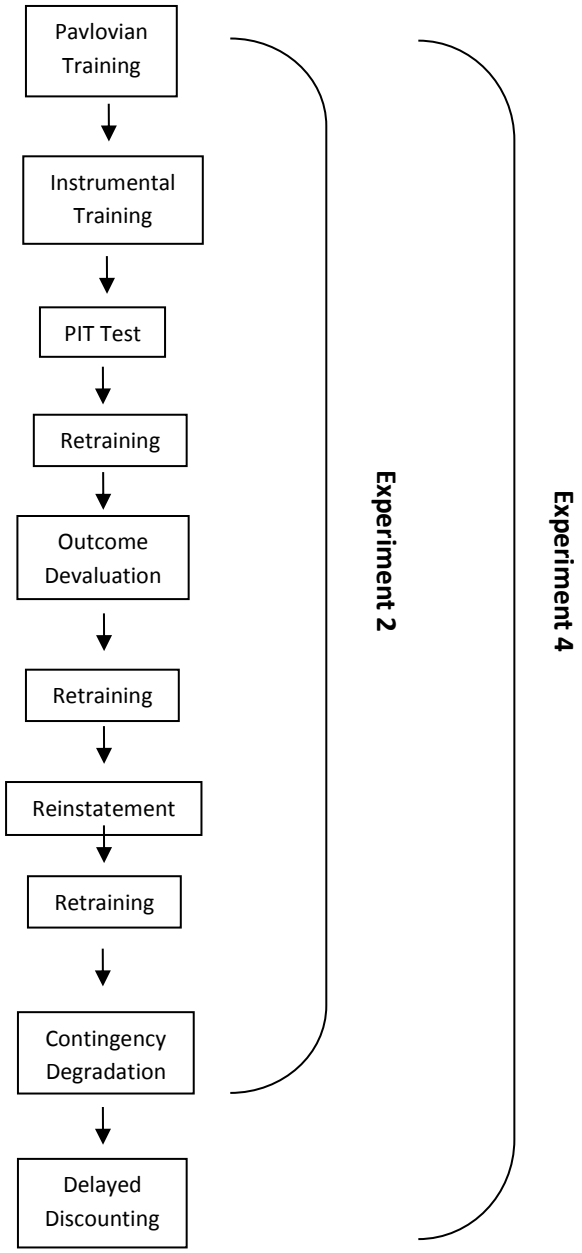

Figure 3. Diagram illustrating the tests used and sequence of behavioural modification in Experiments 2 and 4. Showing where the various retraining days are in the sequence.

Histology

Animals were deeply anesthetized (Lethalbarb ™ 325mg Pentobarbital Sodium/mL) and following no pulse, brains were removed, and the URG protocol described in Experiment 1 was used to stain the neurons.

Imaging and Quantification

Dendrites and synaptic spines were captured on a ZEISS laser Scanning Microscope 710 (LSM710) with an argon laser and processed using the Zen 2015 software package. Z-stacks were captured using a 20x and 40x objective for brightfield imaging with imaging filters removed. The following ROIs were selected according to the atlas of Paxinos & Watson (2006): PLC, IFC, DMS, DLS, BLA, CN, NaC, NaS, LO, VO and ACC. Six neurons per ROI per rat were selected, with 6 samples per neuron for branch orders 1 to 5 sampled for synaptic spine measurement. Together this generated a total of 10560 measurements. Using the Neurolucidia and Neurolucidia Explorer software (MicroBrightField, Colchester, VT), dendritic lengths and synaptic spine density was recorded. All protrusions were counted if they were less than 3µm in length and 1.5µm in width. Slides were coded before quantification and the experimenter was blind to the condition. Jump to Results of Experiment 2 here.

Experiment 3 – Quantifying Morphological Changes in Golgi Stained Neurons Using Novel Photoluminescence caused by Multi-Photon Excitation

Animals

A total of 24 Adult male hooded Wistar rats (Laboratory Animal Services, University of Adelaide, Australia) weighing approximately 250-350g were used across the development of this method, with 14 rats used in replication of methods. Animals were housed in 12h light/12h dark

cycles, with food and water *ad libitum*. All methods and protocols were performed in accordance with The University of Sydney Animal Ethics Committee (AEC No. 5785).

Ultra Rapid Golgi

Animals were deeply anesthetized (Lethalbarb ™ 325mg Pentobarbital Sodium/mL) and following no pulse, brains were removed, and the URG and clearing protocols developed and described in Experiment 1 were used to clear the tissue and stain the neurons.

Optimisation and Imaging

The imaging of whole and sectioned brains (as described in Experiment 1) were performed using a Zeiss LSM 710 confocal microscope with a Mai Tai Chameleon multiphoton laser attachment. To find the optimal excitation, imaging was varied via, excitation wavelength, collection wavelength and laser strength. Two-photon excitation was performed between 690nm – 1100nm, in 10nm increments, similarly collection was gathered in 50nm brackets starting from 200nm to 1100nm, ensuring to leave a 20nm gap between collection and excitation. Further at each excitation, laser strength was varied between 1 to 15 intensity levels, with increasing increments of 1 intensity, this created a sampling process by which at each 10nm increment, every 50nm bracket was collected from and collected against a laser strength that increased from 1-15 intensity, creating a total of 43050 imaged samples. Intensity was measured using a Thor Labs pm100d power meter in the same conditions and range as was applied to the samples, intensity levels 1-15 represented a power level between 0.3mW to 37mW. Once the most optimal setting was collected, optimal image collection was replicated to ensure reliability. Replication consisted of using a 690nm laser, at intensity 10 (0.3mW), to excite the mercury impregnated tissue and then excitation was collected between 620nm-670nm using the internal photodetectors. Simultaneously, transmitted light was collected to correlate the new imaging method to traditional brightfield analysis of a Golgi-stain. Images were acquired at 2048 x 2048 pixels through the tissue, taking images every 0.1um in the Z-direction.

Images were processing using Zen 2015 software package, Image J/Fiji, Huygens, Volocity software.

Jump to the Results of Experiment 3 here

Experiment 4 – The effects of Predictable Chronic Stress on Neuronal Morphology and Decision-making

Subjects and Apparatus.

48 Male Wistar Rats (250-350g) served as subjects in this experiment. All treatments, examination and culling of these mice were performed in accordance with guidelines agreed upon by the University of Sydney Animal Ethics Committee (AEC No. 5785) and were approved by the committee. Animals were housed in groups of 4 or less, in 12hr light/12hr dark cycles (lights on at 7 a.m.); with ad libitum access to food and water and the temperature set at approximately 23^{o}C. The rats were received at 5 weeks of age, and left to habituate to the cage for 1 week, during which rats were handled by the experimenter for at least 2 min a day for 3-4 days consecutively to reduce stress induced by handling, after which treatment began. Rats were maintained at 85% of their free feeding weight by restriction of food intake.

Training and testing took place in identical operant chambers (24 × 21 × 30 cm; Med Associates) housed within sound-attenuating cubicles. Each operant chamber was equipped with a pellet and sucrose dispenser that delivered a 45-mg pellet or and 0.1mL of 20% sucrose solution when activated into a receptacle that was positioned in the centre of one wall. In addition, each chamber contained two retractable levers located on the left and right sides of the receptacle. A 24-V/3-W house light mounted within the chamber. The speaker that delivered the auditory conditioned stimuli was mounted on the wall opposite to the levers and the receptacle. Two auditory stimuli (white noise and a tone) served as conditioned stimuli. A computer system (MedPC Software; Med Associates) controlled the equipment and recorded the data. For chronic stress, Perspex restrainer tubes (Able Scientific, 21cm in length and 6 cm in diameter) closed at one end (where the nose of the

rat was placed) with small holes to allow adequate ventilation and with an adjustable closure on the other end of the tube to accommodate the rat with a small hole to allow the tail to protrude were used.

Group Assignment and Histological Controls

Of the 48 rats, 16 were assigned to a random chronic stress group, 16 to a predicted chronic stress group and 16 to an unstressed control group. Of the 16 animals in each group 6 rats served as histological controls. Generally, our aim was to relate the behavioural effects of random and predicted chronic stress exposure to any changes in neuronal morphology induced by that treatment. However, it is possible that the behavioural training and testing interacted with the stress treatments compromising the histological results. To evaluate this possibility 6 histological control rats were used in each group. These rats went through all of the procedures of their group counterparts except they did not undergo any behavioural training or testing. These rats were used to establish whether the effects of stress on training and testing caused changes in dendritic morphology induced by the behavioural testing. If, after morphological analysis, any differences observed between groups were similar in these rats to those given the behaviourally training and testing, then we can conclude that the training did not further alter neuronal morphology. Given this outcome, these additional histological results will be combined with the scores of the rats that went through training.

Chronic Stress

Treatment involved placing the rats individually within a clear Perspex restrainer tube (Able Scientific, 21cm in length and 6 cm in diameter) closed at one end (where the nose of the rat was) with small holes to allow adequate ventilation and an adjustable small hole to allow the tail to protrude. The rats were divided into three groups, predicted stress group and random stress group and group receiving no stress. Predicted stress rats started stress every day at 10am, at 20min

preceding their housing boxes were removed from the housing facility and placed in a separate waiting room, 5 minutes preceding they were exposed to a 200 lumens light (Colemans Australia) into the boxed for 1 minute. Random stress rats started stress every day between 6am and 8pm, creating a 14-hour bracket through which random stress could begin, a random number generator was used to determine time, with at least 1 day starting a 6am, one at 8pm and one at 8pm followed by one at 6am. All stress sessions lasted 6 hours and continued once a day for 21 days. Immediately, prior to and following stress, each rat was weighed. During treatment, restrainers were placed individually in cages and in a room with the lights on. Control rats were left in the group cage, without access to food or drink and also in a room with the lights left on. At the end of the treatment period on the morning of the 22nd day the rats began behaviourial training.

Behavioural Procedures

For a full breakdown and sequence of the behavioural modifications used see Fig. 3. The behavioural procedures in this experiment closely followed those used in the Experiment 1. The following is a brief description of the methods.

Magazine training

2 days of magazine training, both pellet and sucrose outcomes were delivered on independent 60-s random time schedules with the levers retracted.

Pavlovian Training

Rats were given eight daily sessions of Pavlovian training. Each session consisted presentations of either a white noise or clicker sound for 2 min (CS). During CS presentations either pellets or sucrose were delivered. Half of the rats were given a noise-pellet/clicker-sucrose association, and the other half the opposite. Outcomes were delivered on a random time 30s schedule.

Instrumental Training

Rats were given eight daily sessions of instrumental training. Rats were trained to perform on two lever press responses. Each of the two levers was associated with either pellet or sucrose delivery. Sessions followed an increasingly reduced probability ratio for outcome delivery (CRF→RR5→RR10).

Pavlovian-Instrumental Transfer Test

After training was completed the rats received a PIT test. This test was conducted in extinction. During this test the rats were able to lever press while various stimuli were presented. There were eighteen 2 min periods during which there were eight 2 min stimulus trials, 4 of each stimulus, separated by 2min ITIs. Lever pressing was recorded during each bin.

Retraining

Rats received 1 session of RR10 lever press retraining following each test to ensure strong and consistent lever press associations.

Outcome Devaluation

Rats were given free access to either the pellet or the sucrose outcome for 1 hour. Aiming to induce sensory-specific satiety for one or other of the two outcomes (cf. Balleine & Dickinson, 1998). After pre-feeding period rats were given a 10 min choice extinction test with both levers extended with lever presses being recorded. The next day the rats were pre-fed on the other outcome for one hour and then given a second 10 min choice extinction test on the two levers.

Outcome Specific Reinstatement

Outcome specific reinstatement was used to assess their ability to use the outcome to select a specific action. Each session began with a 20-minute extinction period to suppress responding, followed by noncontingent delivery of one of the two outcomes (pellets or sucrose), followed by a 3-minute period of reinstatement assessment in which lever pressing was recorded. Outcome delivery was counterbalanced across both groups, with half receiving pellets for the first test and sucrose for the second, and the other half receiving the opposite test-outcome assignment.

Contingency Degradation

During a 6-day period rats continued to receive RR10 lever press training, however, one of the two outcomes was also delivered non-contingently. This non-contingent schedule was achieved by dividing the session into 1-sec bins and then arranging that one of the outcomes was delivered at the same probability in each second in which no-response was performed as in each second in which its associated lever response was performed. Rats were given 5 days of this contingency degradation training after which, on day 6, they were given a 10 min choice extinction test with both levers presented. Rats were able to press freely on either lever with lever presses recorded across the session.

Delayed Discounting

Following a retraining day in which both levers earned pellets, rats begin delayed discounting training were one lever offers the same amount of reward as before with no delay and the other a greater reward however with a delay. Levers were counterbalanced so that for half the rats the delayed lever was on the left whereas for the remainder it was on the right. A choice between levers could be made every 60 sec. If no choice was made the levers retracted and the light extinguishes until the next trial. The session lasted an hour. During training choosing the non-delay lever delivered one pellet whereas choosing the delayed lever delivered 5 pellets. For the first three days of training,

the delayed lever delivered the 5 pellets with no delay and the animals quickly learned that the delayed lever was most optimal. On the next day a delay of reward delivery was imposed of 3 secs, on the next day 5 secs, then 7, 10, 15, 20 and 30 secs respectively on each subsequent day. Lever pressing on both the delay lever and the non-delay lever were recorded across all days. The day after the final session of delay discounting testing the animals were culled for histology.

Histology

Animals were deeply anesthetized (Lethalbarb ™ 325mg Pentobarbital Sodium/mL) and following no pulse, brains were removed, and the URG protocol used in Experiment 1 and the Two-Photon protocol in Experiment 3 were used to stain and visualise the neurons.

Imaging and Quantification

Dendrites and synaptic spines were captured on a ZEISS laser Scanning Microscope 710 (LSM710) with an argon laser and processed using the Zen 2015 software package. Z-stacks were captured using a 20x and 40x objective for brightfield imaging with imaging filters removed. The following ROIs were selected according to the atlas of Paxinos & Watson (2006): PLC, IFC, DMS, DLS, BLA, CN, NaC, NaS, LO, VO, MO, InC, ACC and HIP. Six neurons per ROI per rat were selected, with 6 samples per neuron for branch orders 1 to 5 sampled for synaptic spine measurement. Together this generated a total of 20160 measurements. Using the Neurolucidia and Neurolucidia Explorer software (MicroBrightField, Colchester, VT), dendritic lengths and synaptic spine density was recorded. All protrusions were counted if they were less than 3µm in length and 1.5µm in width. Slides were coded before quantification and the experimenter was blind to the condition. Jump to the Results of Experiment 4 here

Chapter 3: Results

Experiment 1 - Application of Ultra Rapid Golgi-Cox Method to Modern Brain Clearing Techniques

Development

The successful development of the URG stain was first observed in fresh and PFA fixed tissue. Fig. 4 illustrates the comparison between URG and traditional Golgi-Cox stained neurons in 200um sections. In this Figure the left-hand column of images was captured at 5x objective and the right-hand column at 20x. Fig. 4a and b show traditional Golgi-Cox stained neurons in fresh tissue and comparable URG stained neurons in Fig. 4c and d. It can be seen that the neurons in Fig. 4c and d are more isolated, there is less background staining and less artefact (the dense black blotches of indistinguishable stain). This allows for easier identification, visualisation and analysis of morphology. Furthermore, with the increase in incubation temperature to 42^{o}C, neurons in PFA fixed tissue are significantly more visible. Fig. 4e and f show traditional Golgi-Cox stained neurons in PFA fixed tissue, compared to the URG neurons stained in Fig. 4g and h. Again, it can be seen that the neurons are far more isolated and visible and, additionally, although there is artefact still present within URG stained tissue, it is significantly less present compared to traditional Golgi-Cox staining, see Fig 6 for an illustration. Across a selection of 80 images, 20 per group, for fresh and fixed tissue stained via traditional and URG, artefact number in a 200x200x33um selection were counted, after performing an Abercrombie correction (1946), the scores were compared via ANOVA, to reveal that in both groups, fresh and fixed, URG had significantly less artefact present ($F(1,38)=242.43$, $p<0.0001$; $F(1,38)=184.82$, $p<0.0001$, see Sup. Fig. 6 for further information). Current literature does not formalise the classification requirements for identifying artefact, so I defined by the following: 1, object does not have synaptic spines; 2, does not have discernible dendrites or axons; 3, object is not smaller than 30um; 4, if object is smaller than 30um, query if glia, if not object is artefact.

The use of the URG in tissue was repeated and shown to be reliable in staining neurons within cortex. It was revealed that for reliable staining of sub-cortical neurons, incubation times should be increased to 72 hrs instead of 48hrs. See Fig. 7 Additionally, it was revealed that URG stain is far more effective than a traditional Golgi stain in staining pre-sliced tissue, i.e., usually a Golgi-Cox stain is applied to whole brains or thick brain sections which are then submerged in Golgi solution (Levine et al., 2013). We report here however, that the URG can be effectively applied to pre-sliced tissue, see Fig. 8. Lastly for completeness, a selection of photomicrographs showing temperatures need to be specific, and submersion time can dramatically change the stain, in that, should fresh or fixed tissue be incubated at 40^{o}C instead of 37 and 42 respectively, its will fail to stain, Fig. 5, furthermore, should the stains be left for more than a week in incubation, the stain will be too heavy to be viable. Fig. 5

With the repeated success within fresh and PFA fixed tissue the URG stain development was extended to applications within modern clearing techniques. The CLARITY and CUBIC techniques were chosen for their different approaches to clearing tissue, and their results are presented below.

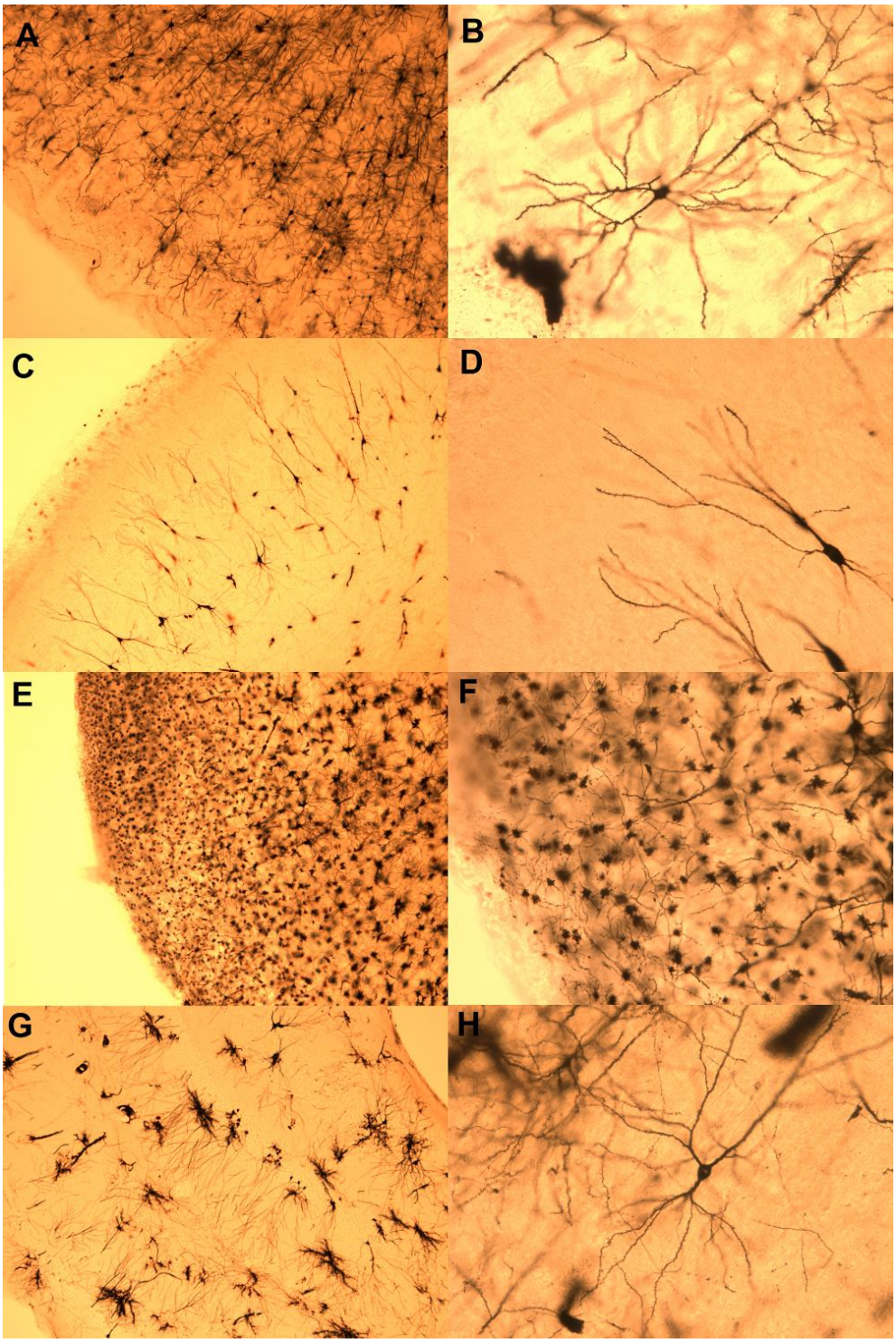

Figure 4: Photomicrographs illustrating a comparison, within 200um sectioned tissue, between traditional Golgi-Cox and URG stained neurons. Images in the Left-hand column were caught at 5x objective, figures on the right in 20x. **A** and **B** traditional Golgi-Cox within fresh tissue, **C** and **D** URG in fresh tissue, **E** and **F** traditional Golgi-Cox stained neurons in PFA fixed tissue, **G** and **H** URG stained neurons in PFA fixed tissue.

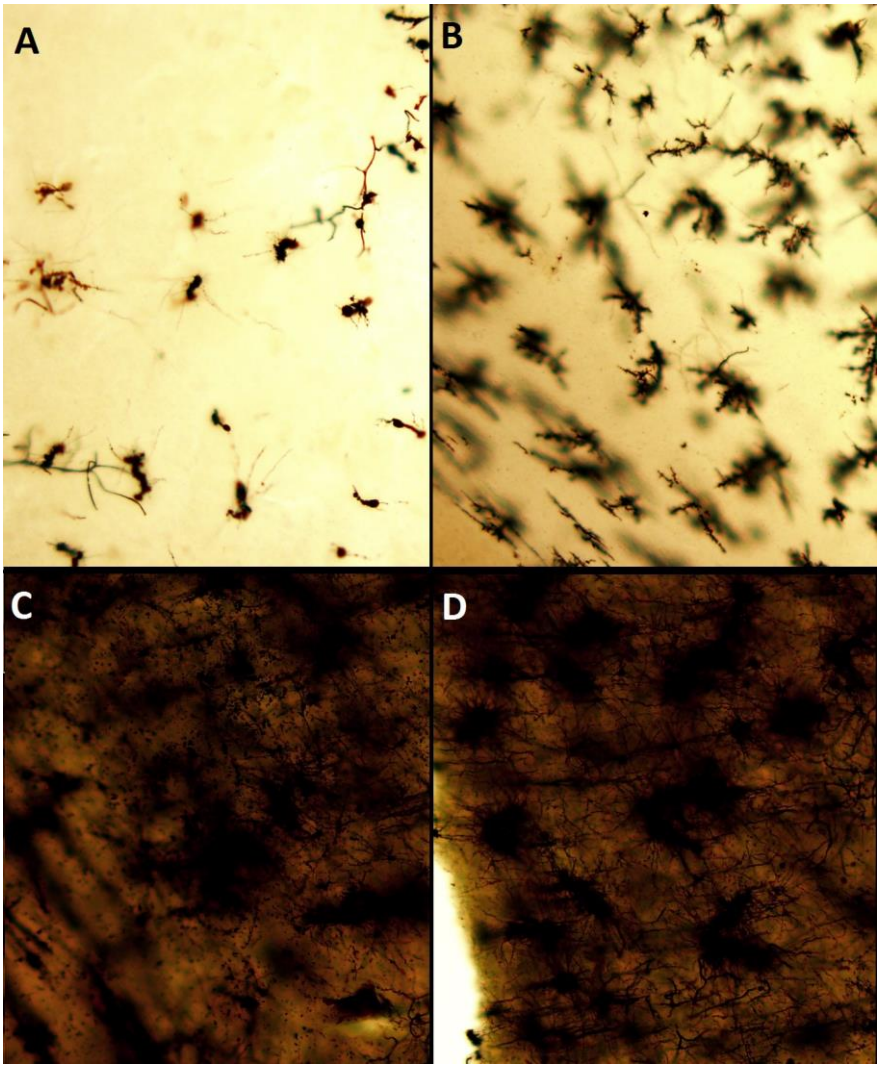

Figure 5: Photomicrographs taken at 10x objective, illustrating fresh **A** and fixed **B** tissue incubated at 40°C instead of 37°C and 47°C respectively, further if fresh **C** and fixed **D** tissue is left to incubate for more than a week.

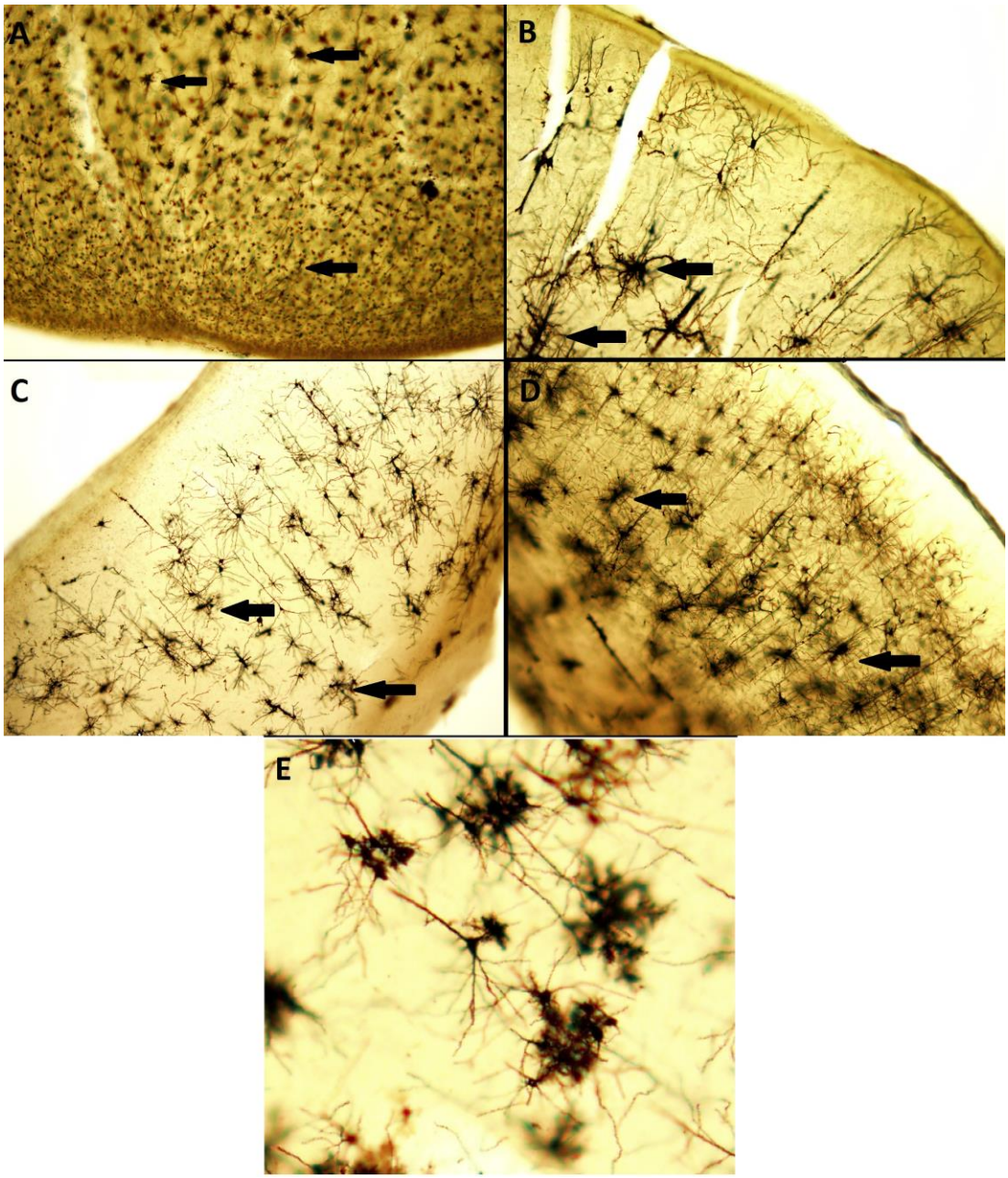

Figure 6: Photomicrographs taken at 10x objective, showing for illustrative purposes the various densities of artefact across fixed and fresh tissue in both traditional and URG. **A** PFA traditional, **B** PFA URG, **C** fresh URG, **D** fresh traditional. Arrows point to evidence of artefact in each picture to help the reader distinguish. **E** Close up of a pyramidal neuron surrounded by artefact.

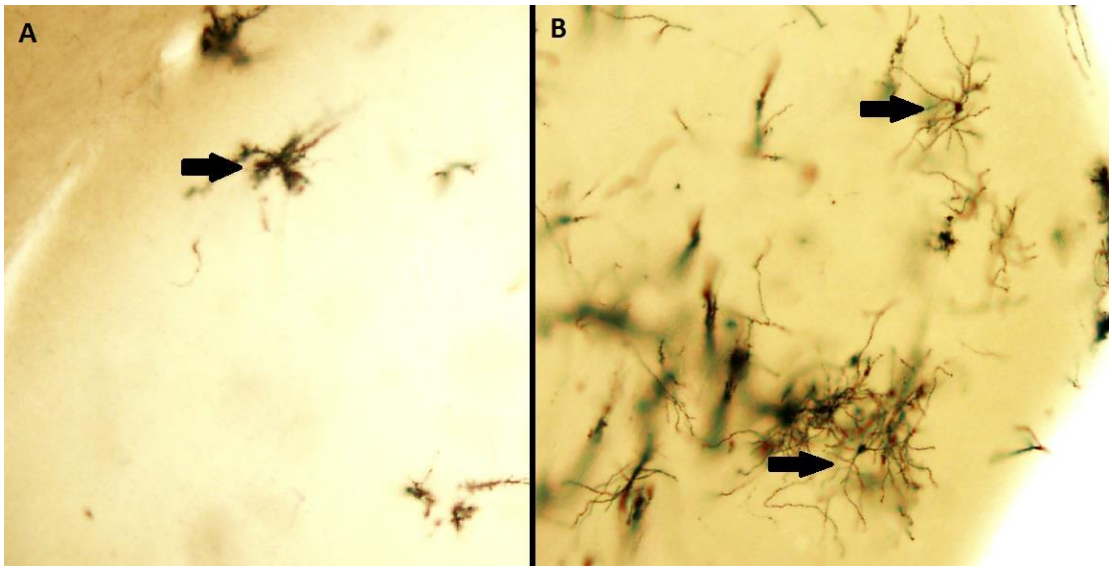

Figure 7: Photomicrographs taken at 10x objective, illustrating the difference between neuron stain visibility within the striatum when the stain is incubated for 48 **A** or 72 hours **B**.

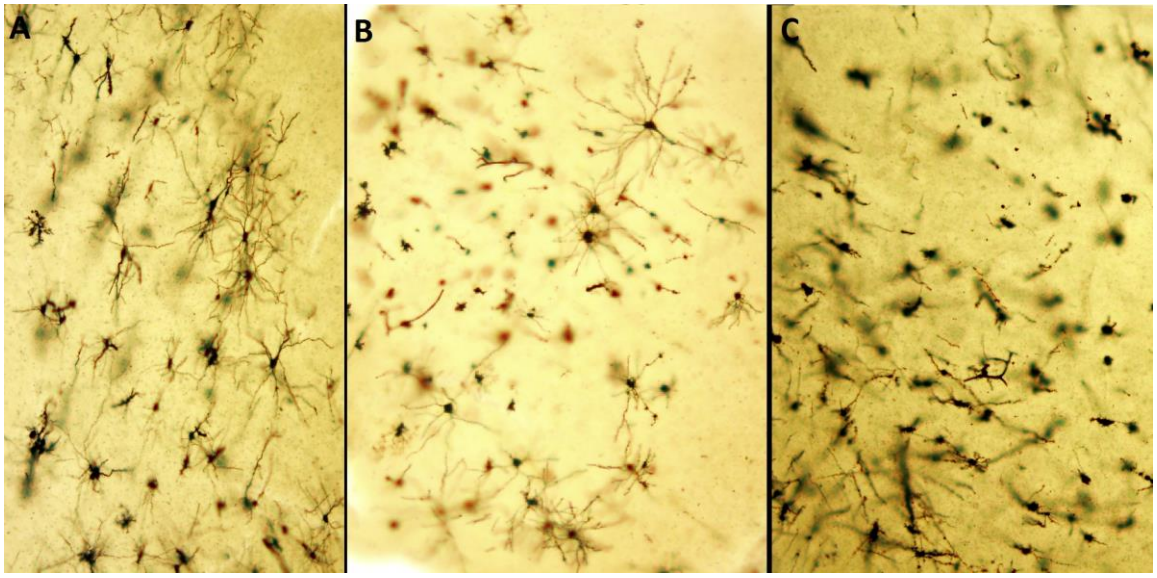

Figure 8: Photomicrographs taken at 10x objective, illustrating the stain effectiveness of URG when applied to pre-sliced tissue, cut at 200um **A** and 40um **B**. **C** is pre-sliced tissue, cut to 200um and stained with traditional Golgi.

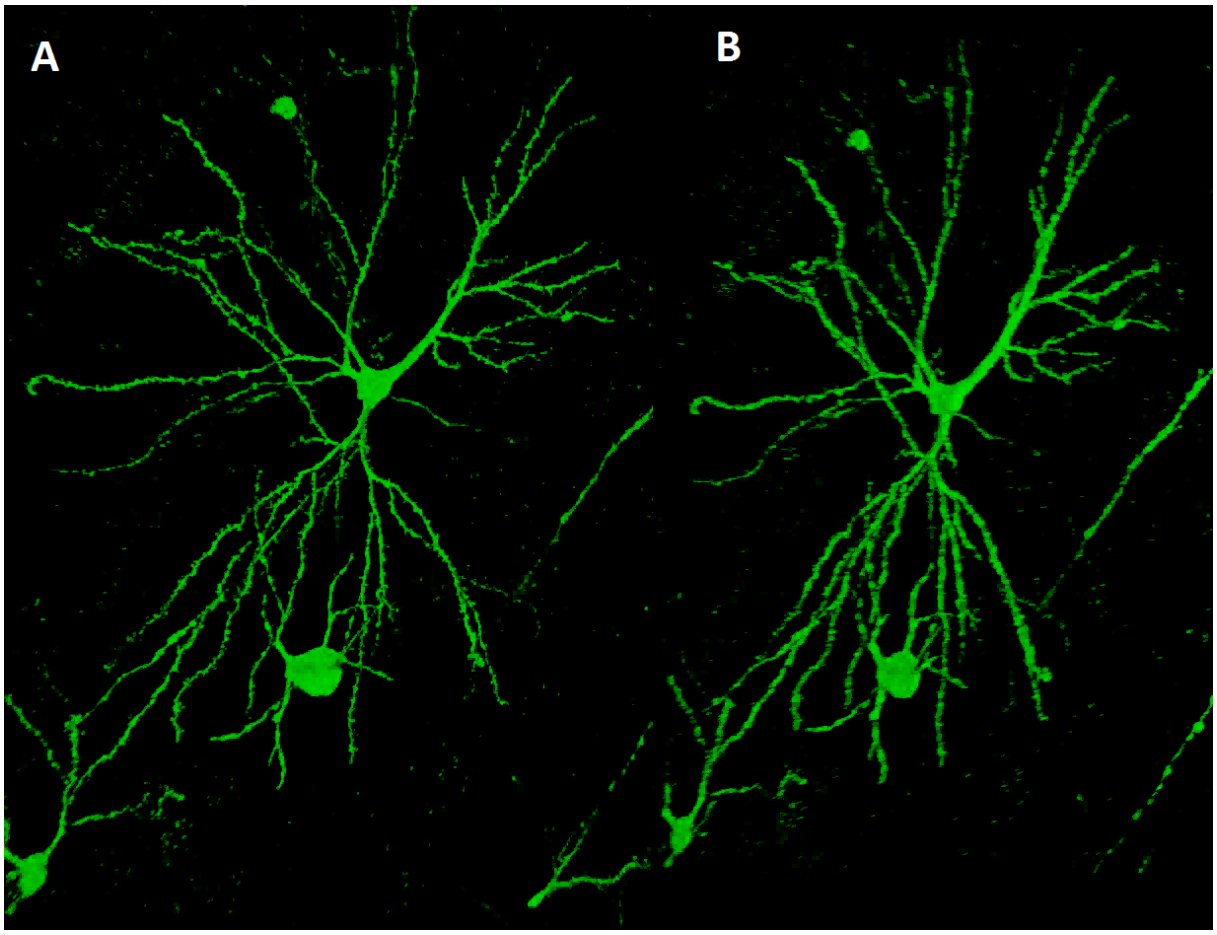

Figure 9: Snaps taken from a neuron stained via URG and rendered in 3D via Volocity software. **A** is a snap taken at from the direction parallel to the objective and **B** is a snap taken from the 3D render as it is rotated. These snaps are shown here for convenience, for a video see Sup. Fig. 1

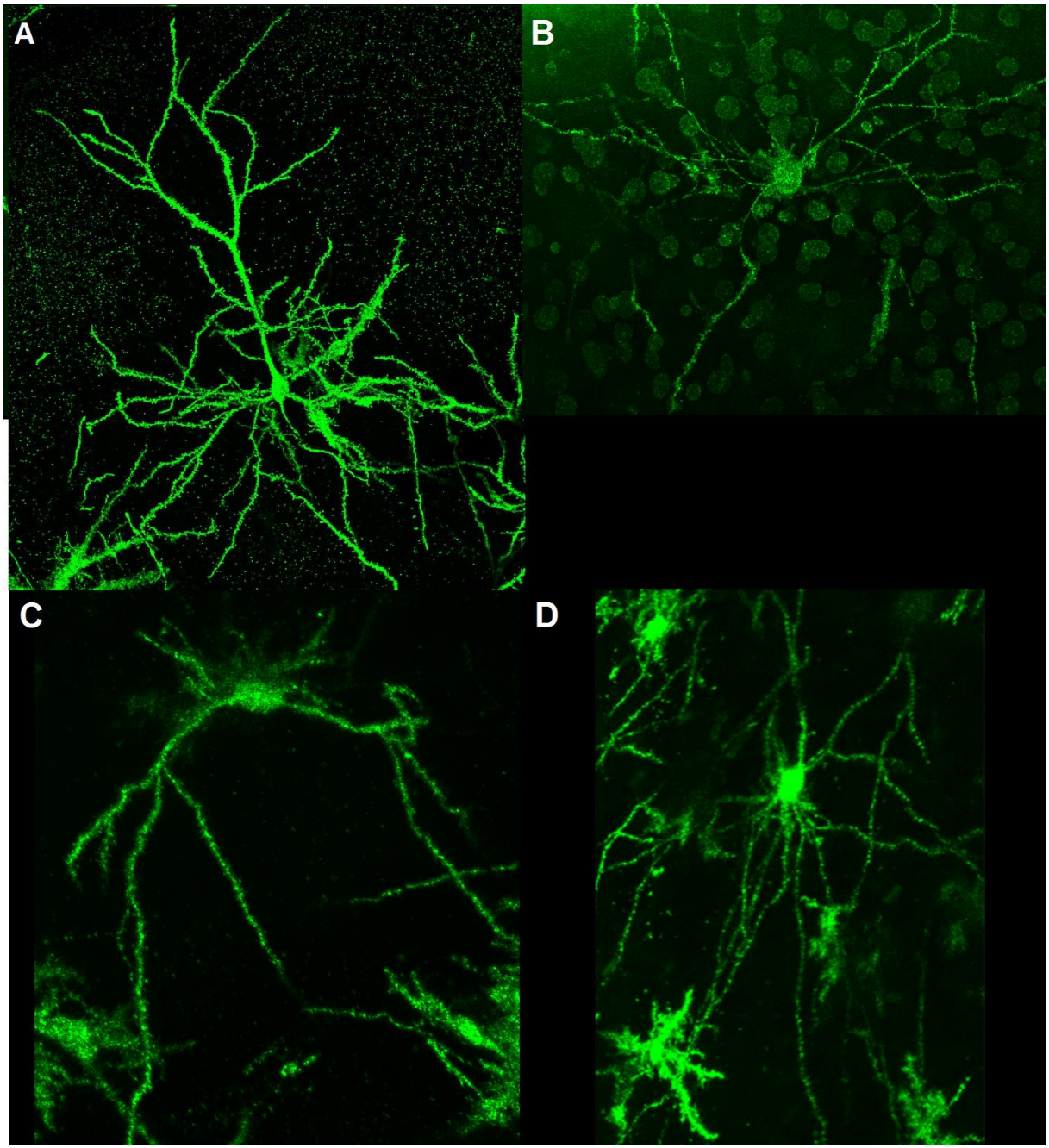

Figure 10: Photomicrographs illustrating the staining effectiveness in PFA fixed URG stained rat brains cleared with CLARITY between different methods of development and clearing. **A** Section developed then cleared via passive. **B** Section developed then cleared via active. **C** Section cleared via passive then developed. **D** Section cleared via active then developed.

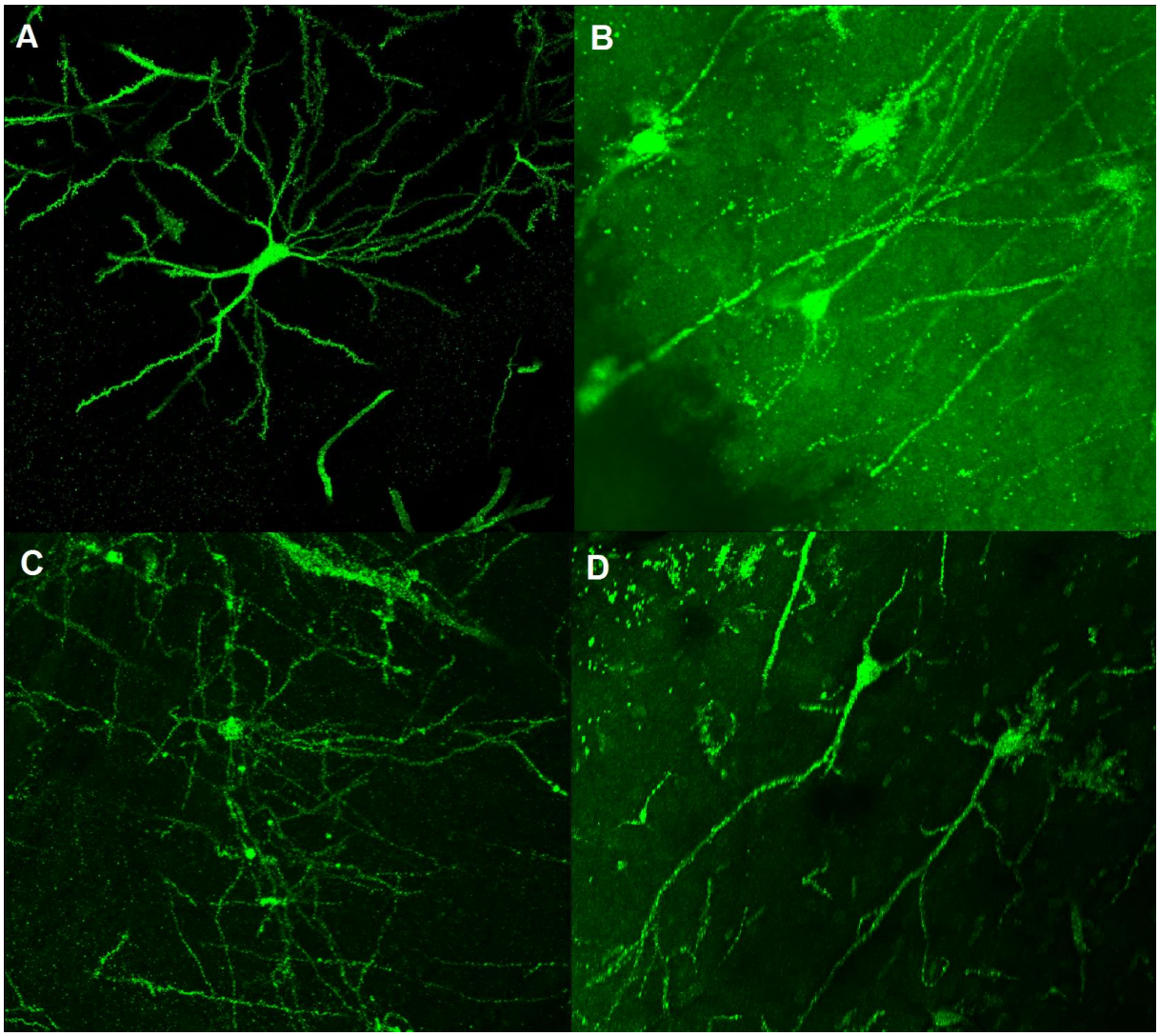

Figure 11: Photomicrographs illustrating the staining effectiveness in fresh non-fixed URG stained rat brains cleared with CLARITY between different methods of development and clearing. **A** Section developed then cleared via passive. **B** Section developed then cleared via active. **C** Section cleared via passive then developed. **D** Section cleared via active then developed.

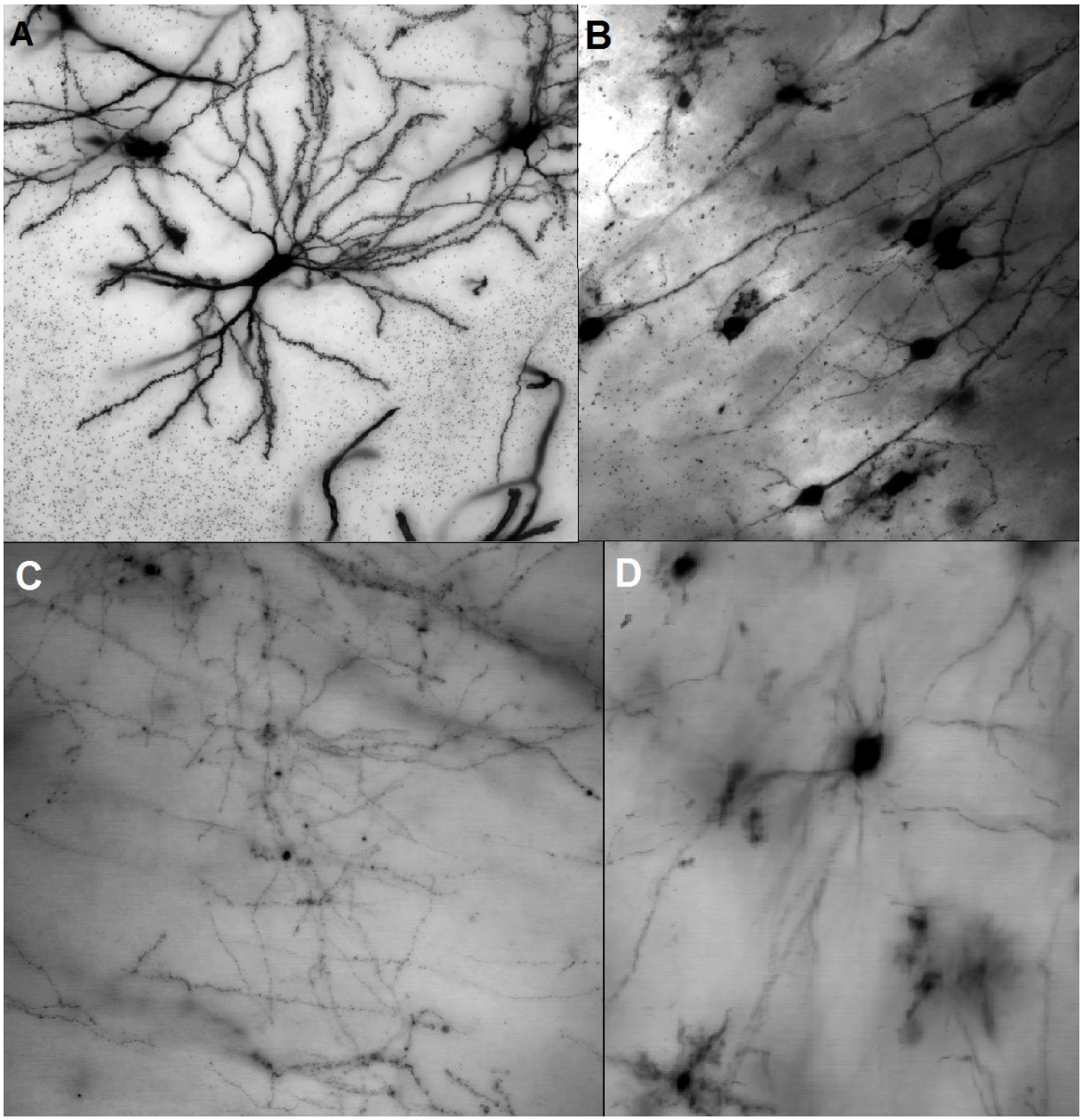

Figure 12: Brightfield photomicrographs of Fig. 6a, b, c and Fig. 5d illustrating the changes in imaging across methods, where **A** and **B** show equal or greater neuronal visibility via bright field and **C** and **D** show less.

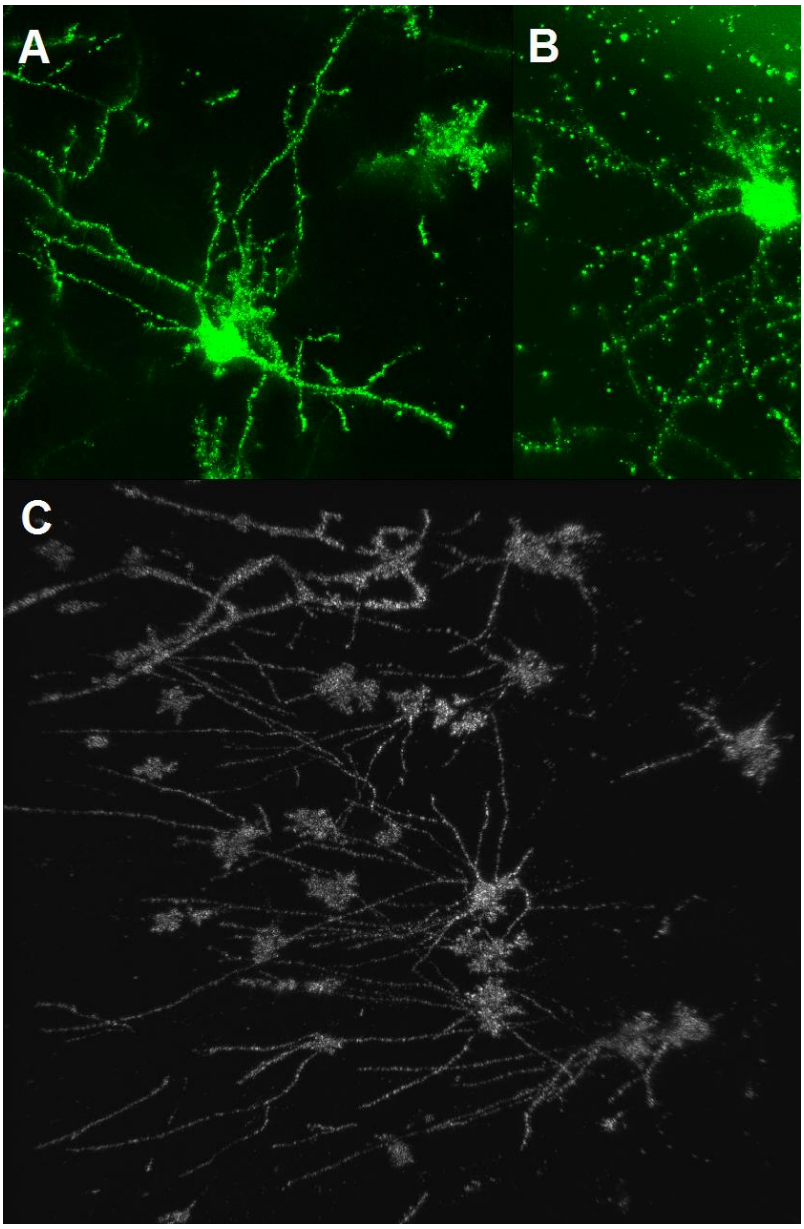

Figure 13: Photomicrographs illustrating the staining effectiveness in PFA fixed (**A**) and fresh non-fixed (**B**) URG stained rat brains cleared with CUBIC. **C** Light sheet image of CLARITY cleared brain taken from a fresh non-fixed Golgi stained brain developed before passive cleared. As with Fig. 9, for this snap is shown here for convenience for a video see Sup. Fig. 2.

CLARITY

URG staining within CLARITY cleared tissue was successful, a snaps taken from a 3D render are seen in Fig. 9, where the neuron is visualised to a level of detail rivalling injection staining. Within CLARITY all eight methodological conditions showed adequate staining via URG, those being fixative or fresh, development prior or post clearing and whether the stain was cleared via active of passive CLARITY clearing. Illustrated in Fig. 10 & 11 we see that all eight conditions show a level of detail that delineates neuronal soma, dendrites and synaptic spines via both reflective imaging and bright field imaging (Fig. 12). The precision of each of the eight methods however does differ. Fig. 10a, b, c and Fig. 11a, c shows the greatest level of detail of synaptic spines, with the least transparent dendrites. All of the aforementioned conditions except Fig. 10b were developed either prior or following passive CLARITY clearing, indicating a processing preference for passive cleared tissue. Of all these methods, Fig. 9 and 10a shows the clearest picture, and assuming there are no methodological restrictions imposed, passively cleared PFA fixed CLARITY tissue developed prior to clearing is the best result.

The clearing methods collected and visualised in Fig. 12, specifically fresh tissue cleared prior and post development under active and passive condition and PFA fixed tissue actively cleared and developed post, offer differing levels of delineation based on brightfield or reflective imaging. Fig. 12a and b are better visualised under brightfield, while Fig 12c and d and better visualised by comparison under reflective imaging. The difference between these two sets of images appears to be when development occurred. In Fig 12a and b these images, as the legend points out, are bright field images of the same neurons from Fig 11a and b respectively, both of which were developed prior to clearing, and comparatively Fig 12c and d are both taken from neurons developed post clearing. Neurons developed prior to clearing offer better equal to or better visibility under brightfield imaging, and neurons developed following clearing offer better visibility under reflection imaging.

CUBIC

Cubic cleared brains were only investigated comparing PFA and fresh tissue, with both conditions developed prior to clearing, CUBIC clearing does not have an active or passive component. Fig. 13a, b show URG stained neurons developed prior to CUBIC clearing. Fig. 13a PFA fixed tissue shows the greatest detail delineating the neuronal soma, dendrite and synaptic spines. Fig. 13b Fresh non-fixed tissue is far more transparent, with the soma delineated and the synaptic spines illustrated via brighter spots of light, the dendrites are barely visible. Furthermore, Neurolucidia software was unable to reconstruct said neuron nor gather adequate morphological information. From these it would be clear that the best method would be to use PFA fixed tissue and develop prior to clearing via CUBIC.

Lightsheet

Lightsheet offered a great level of detail in a very large scope best illustrated in 3D rendered video, see Fig 13c for a snap and Sup. Fig. 2 for a video. Fig. 13c shows in Fresh non-fixed tissue, URG stained neurons, developed prior to passive CLARITY clearing, a level of detail that delineates neuronal soma, dendrites and synaptic spines. The indiscernible blotches in the images are artefacts, common in Golgi staining, and reduced in the URG method, as reported above; they are easily ignored during analysis, not sharing any features with neurons, astrocytes or oligodendrocytes. Neurolucidia software was able to ignore these during analysis.

Experiment 2 – The Effects of Chronic Stress on Decision Making and Neuronal Morphology.

Changes in Body Weight Following Stress

ANOVA revealed that chronic restraint stress significantly decreased the body weight of rats. Main effect of stress ($F(1, 30) = 57.45$; $p < 0.0001$) and day of paradigm ($F(20, 600) = 13.88$; p

<0.0001), additionally there was a significant interaction of stress and time ($F(40, 600) = 36.95$; p <0.0001). This represents a decrease of 21% body weight by day 21 of stress and indicates that stress continued to decreases weight across the paradigm. Fig. 14 illustrates the clear change in body weight across CRS. The observed weight loss within the stress group suggests that the group was chronically stressed and is consistent with previous studies (Sousa et al., 1998, Watanabe et al., 1992b, Coburn-Litvak et al., 2003, Conrad et al., 2004, Kassem et al., 2013).

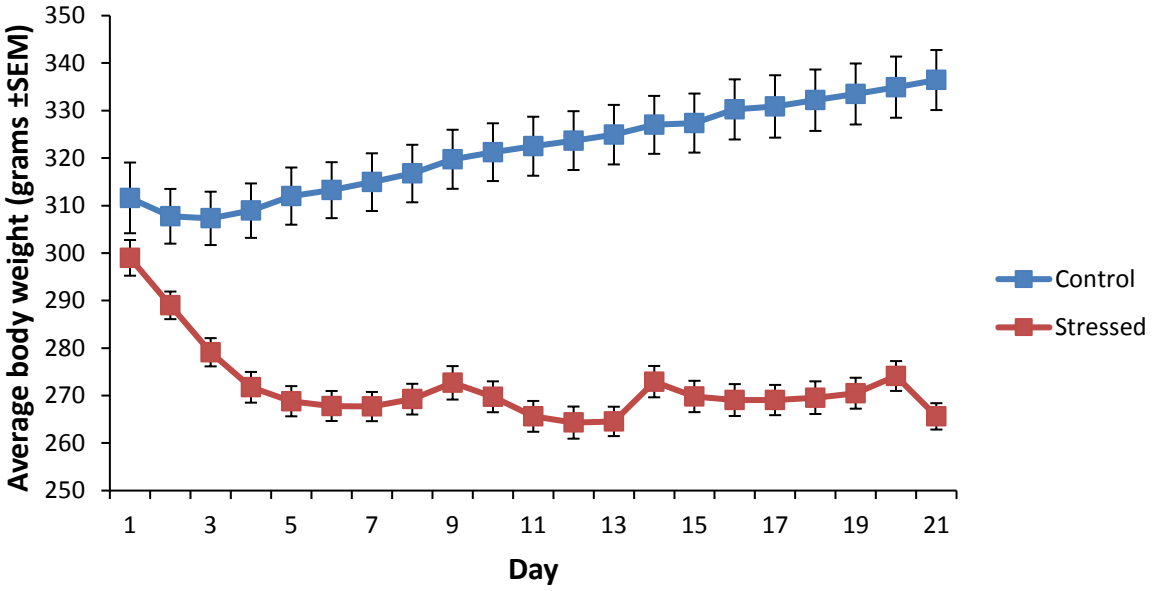

Figure 14: Illustration of the loss of body weight in stressed animals across the chronic stress paradigm compared to controls. From day 3, there was a significant loss in body weight.

Histological controls

As described in the methods an additional 6 rats per group were not subject to any behavioural training or testing and the morphological changes in this group were used to assess any confounding effects of behavioural training. We found that the histological measures taken in these subgroups of rats did not differ significantly from the behavioural counterparts (p values across main effects and of the treatment and all interactions across and all ROI > 0.3). We concluded, therefore, that behavioural testing did not significantly affect dendritic length or spine density in either the

chronically stressed or unstressed groups. The data from the histological controls were, therefore, collapsed into their respective groups.

Changes in Dendritic Length

The cumulative length of the dendrites of individual neurons was measured in a battery of regions for control and stressed rats. ANOVA revealed significant differences in dendritic length in all ROI (Tab. 1) except the DLS and CN, however the DLS showed a marked trend towards a decreases ($F(1, 30) = 3.88$; $p < 0.06$). See Fig. 15 and 16 for illustrative changes and Fig. 20 for a summary of the changes across all ROI. Note that, where most regions showed a decrease in dendritic length, as predicted from previous research the NaC and BLA showed an increase on this measure. Additionally, as shown by Fig. 20b, there were changes of up to 45% in either direction and that chronic restraint stress causes decreases in all ROIs except the DLS, CN and increases in the BLA and NaC.

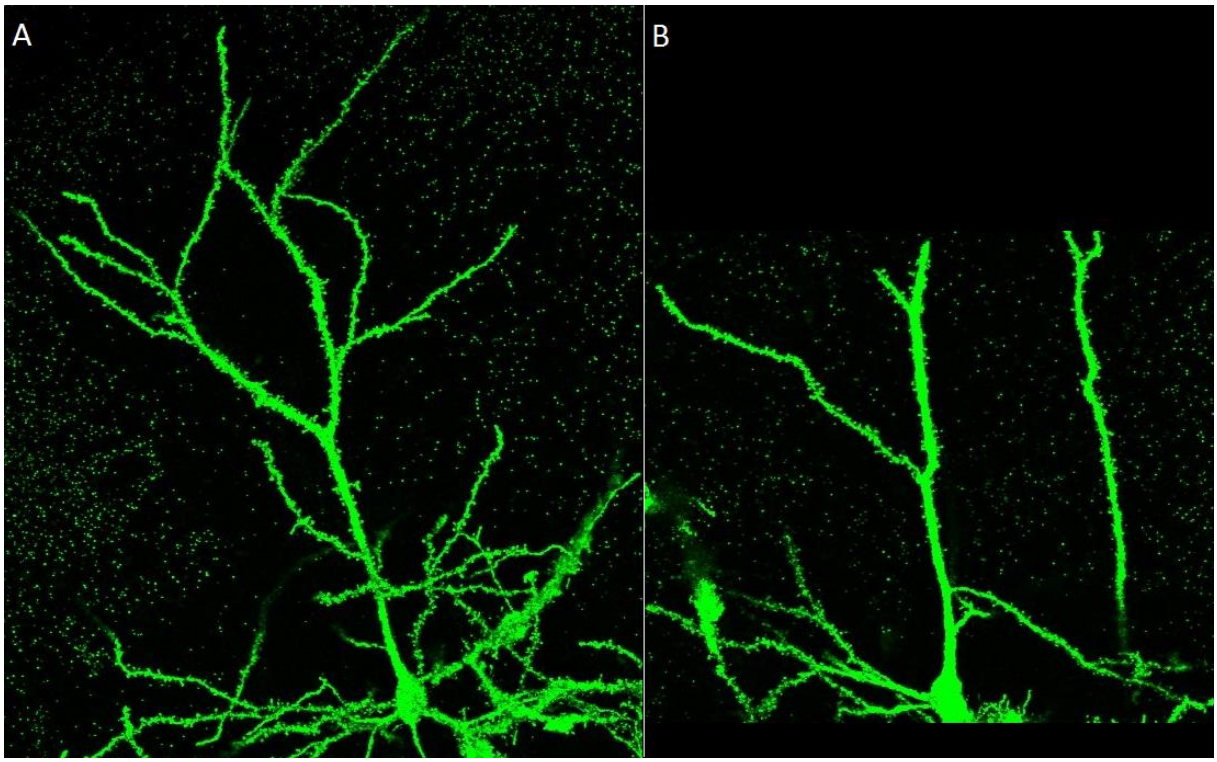

Figure 15: Photomicrographs depicting apical dendritic deterioration within pyramidal neurons in the ACC. Illustrating the decreases in dendritic length caused by chronic stress. **A** Healthy non-stressed neuron. **B** Chroncially stressed neuron.

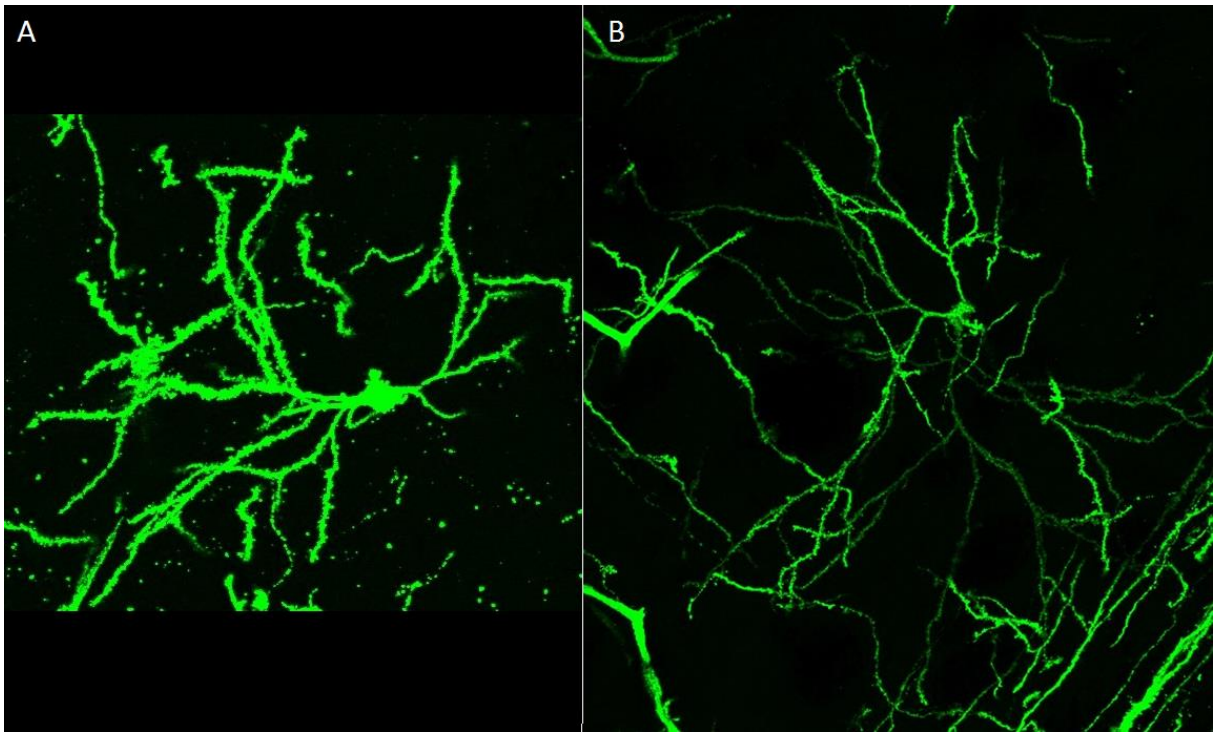

Figure 16: Photomicrographs depicting dendritic proliferation within medium spiny neurons in the BLA. Illustrating the increase in dendritic length caused by chronic stress. **A** Healthy non-stressed neuron. **B** Chroncially stressed neuron.

Changes in Synaptic Spine Density

Synaptic spine density was measured in each ROI with the measurements separated by dendritic order. Dendritic orders 2-5 were measured and the density changes are illustrated in Fig.17 and shown for al ROI's in Fig. 21. ANOVA revealed significant changes in all ROIs (Tab. 1). Furthermore, the changes in spine density in each ROI largely matched the changes in dendritic length. Dendritic order 1 was is not shown as no significant changes in any ROI were found (*p* values > 0.1); as has been previously observed, spine density changes are rarely found on order 1 dendrites (Kassem et al. 2012, Padival et al. 2013). Importantly, the changes reported in Fig. 21 indicate that chronic restraint stress causes a decrease in synaptic spine density in all ROIs except the BLA and NaC where increases in synaptic spine density were observed.

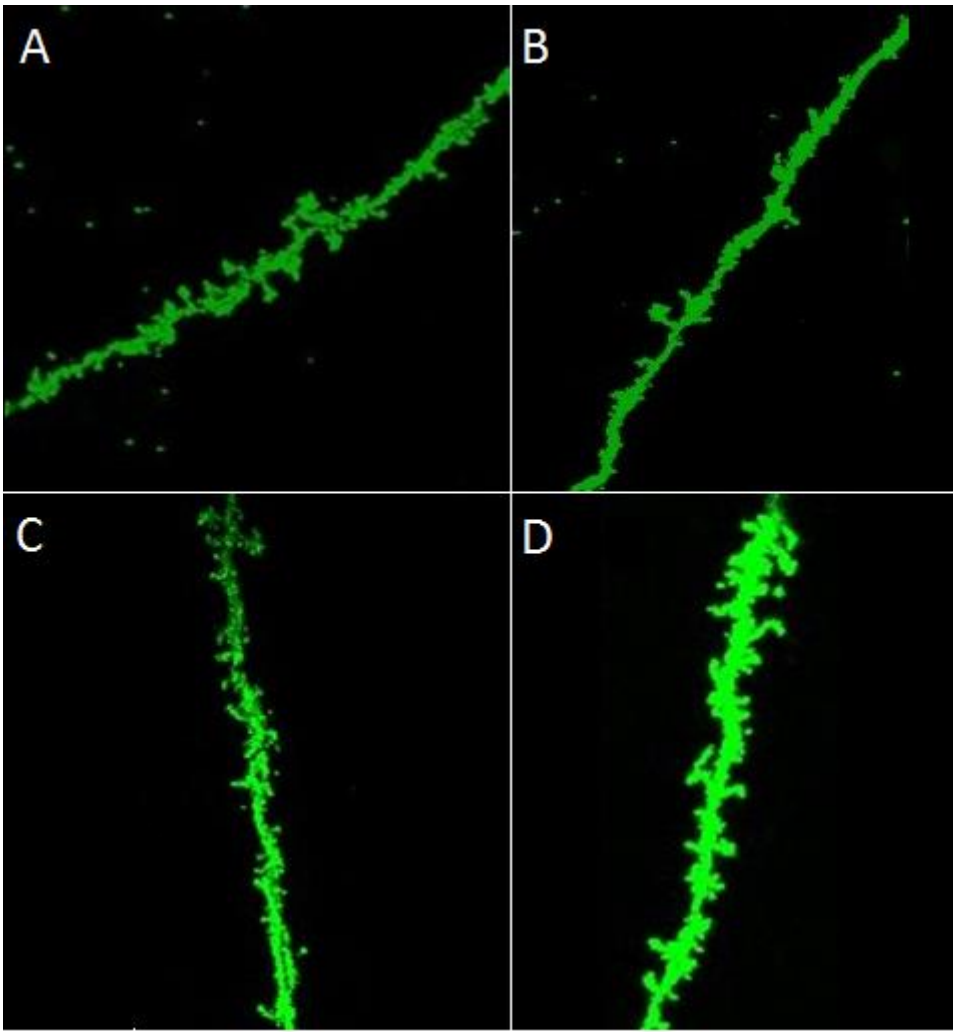

Figure 17: Photomicrographs depicting synaptic spine changes within chronically stressed rats. **A** & **B** illustrate the decrease in synaptic spines caused by chronic stress, depicted in the HIP, **A** Healthy synapic spine density. **B** Spine density decreased in chronically stressed rat. **C** & **D** illustrate the increase in synapitc spine denisty caused by chronic stress, depicted in the BLA **C** Healthy synapic spine density. **D** Spine denisity proliferation in chronically stressed rats.

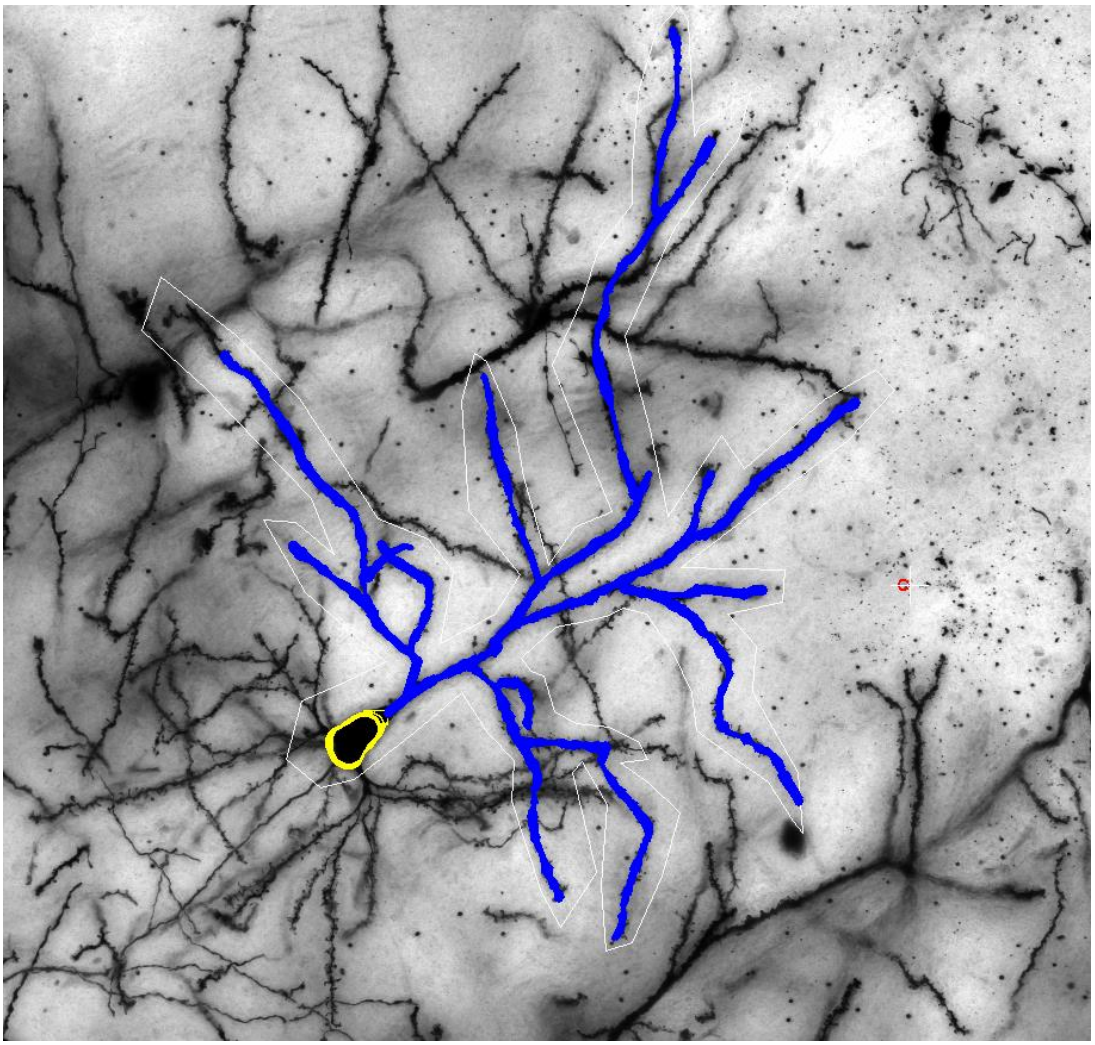

Figure 18: Photomicrograph illustrating the use and precision of Neurolucidia. Neurolucidia overlays the z-stacked image, deliniating the soma and specifically in this example the apical dendrites of a pyramidal neuron in the HIP.

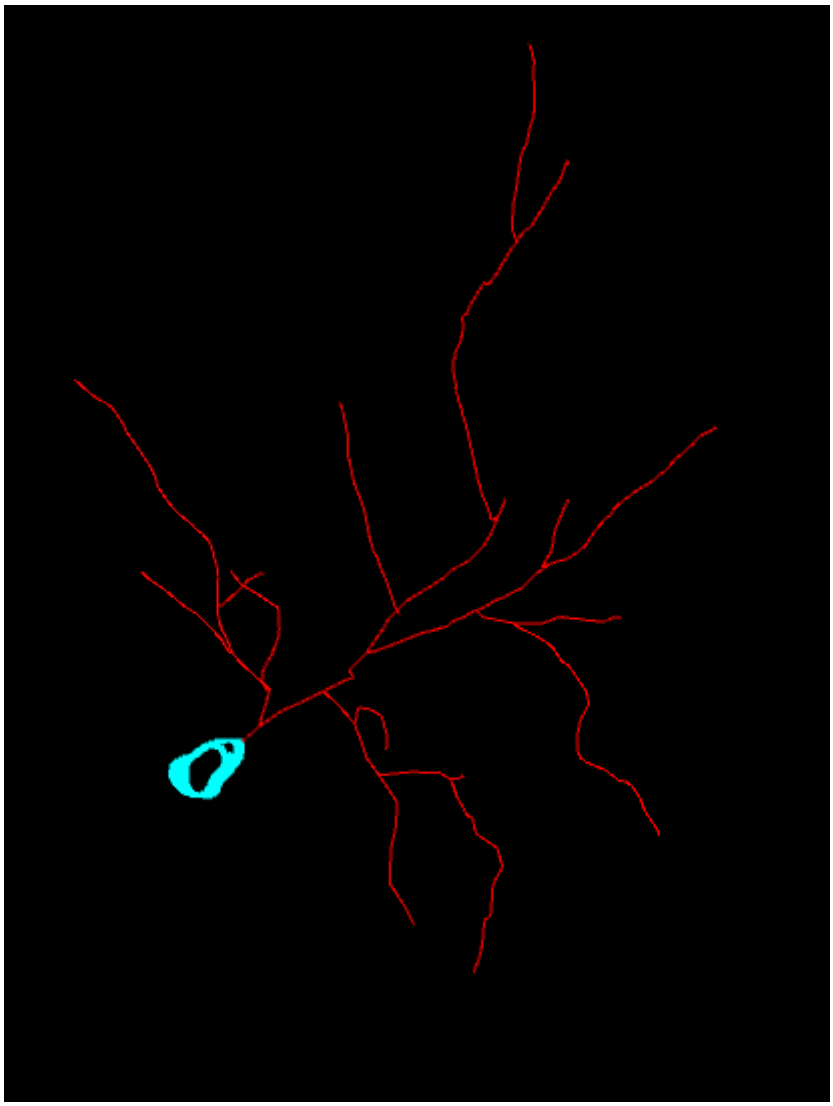

Figure 19: 3D render, of neuron in Fig. 18, in Neurolucidia created for morphology analysis. See Sup. Fig. 3 for a video.

Behavioural Measures

Pavlovian training

To determine whether the animals learnt the association between the stimulus and the outcome, magazine entries were measured during the stimulus and during a prestimulus interval of equal length. Comparing stimulus and outcome types showed no difference between either group, and so responding was collapsed across the two outcomes (*p* values > 0.25). Both control and

stressed animals responded significantly more during the stimulus period (Control $F(1, 18) = 132.33$; $p < 0.0001$, Stress $F(1, 18) = 67.28$; $p < 0.0001$), and this is illustrated in Fig. 22a. ANOVA revealed a main effect of session ($F(7, 126) = 38.36$; $p < 0.0001$), stress ($F(1, 18) = 6.18$; $p < 0.023$), and stimulus $F(1, 18) = 16.78$; $p < 0.002$) but no 3-way interaction between these variables ($F(28,126) = 1.23$; $p > 0.268$), nor an interaction between stress and session ($F(14, 126) = 0.77$; $p > 0.613$), nor stress and stimulus ($F(2,18) = 1.03$; $p > 0.602$), but there was an interaction between stimulus and session ($F(14, 126) = 8.77$; $p > 0.0001$). This indicates that as training progressed responding increased, during the CS period and not during the prestimulus and an effect of stress indicates that stressed animals responding more than controls, however as there were no interactions with stress nor a 3-way interaction.

Instrumental training

Rats acquired lever pressing similarly for the two outcomes across both groups (p values > 0.2) and so we collapsed across actions to analyses the training data. The mean number of lever presses across sessions for both groups is presented in Fig. 22b. Two way ANOVA (stress x training day) revealed that there was a main effect of training day ($F(7, 126) = 109.4$; $p < 0.0001$) but no main effect of stress ($F(1,18) = 3.12$; $p > 0.094$) and no interaction between these factors ($F(14, 126) = 1.43$; $p > 0.2$). This indicates that as training progressed responding increased, and, although the chronically stressed rats responded at a numerically higher rate, that chronic stress did not impact on the acquisition of instrumental conditioning.

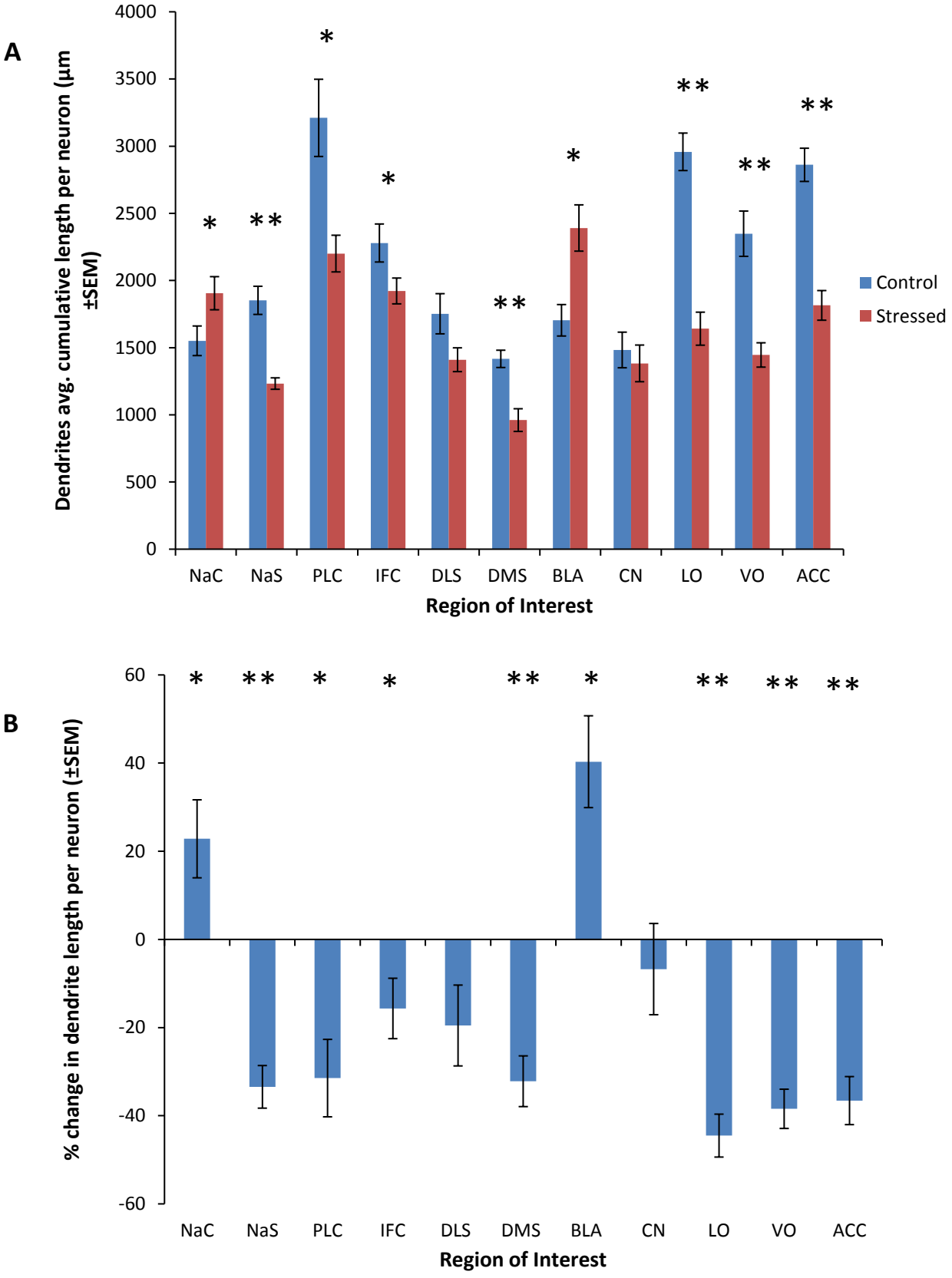

Figure 20. Changes in dendritic length due to chronic restraint stress. **A** Comparison between controls and stressed rats within regions of interest. **B** Percentage changes within stressed animals compared to controls. * indicates significant difference p <0.05, ** p< 0.001, refer to Tab. 1

NaC		NaS		PLC		IFC		DLS		ACC	
F(1, 30)	p	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Dendrite											
4.58	<0.041	30.00	<0.001	10.10	<0.003	4.36	<0.045	3.88	0.06	39.86	<0.001
Order 2											
15.00	<0.001	0.72	0.40	5.94	<0.021	12.15	<0.002	19.10	<0.001	18.70	<0.001
Order 3											
85.79	<0.001	14.18	<0.001	34.66	<0.001	46.74	<0.001	6.77	<0.014	12.80	<0.001
Order 4											
36.52	<0.001	1.43	0.25	25.02	<0.001	205.88	<0.001	16.69	<0.001	14.53	<0.001
Order 5											
1.09	0.32	27.49	<0.014	48.89	<0.001	101.97	<0.001	74.50	<0.001	61.75	<0.001
BLA		CN		LO		VO		DMS			
F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*		
Dendrite											
10.88	<0.003	2.75	0.60	50.10	<0.001	22.23	<0.001	18.43	<0.001		
Order 2											
37.00	<0.001	9.50	<0.004	4.74	<0.038	16.92	<0.001	19.80	<0.001		
Order 3											
17.82	<0.001	35.03	<0.001	33.72	<0.001	24.88	<0.001	11.41	<0.002		
Order 4											
21.26	<0.001	40.77	<0.001	24.84	<0.001	111.90	<0.001	43.10	<0.001		
Order 5											
4.98	<0.035	21.67	<0.001	42.10	<0.001	100.33	<0.001	51.98	<0.001		

Table 1. Quantitative changes between controls and chronic stress rats, revealed by ANOVA in dendritic length in the above ROIs.

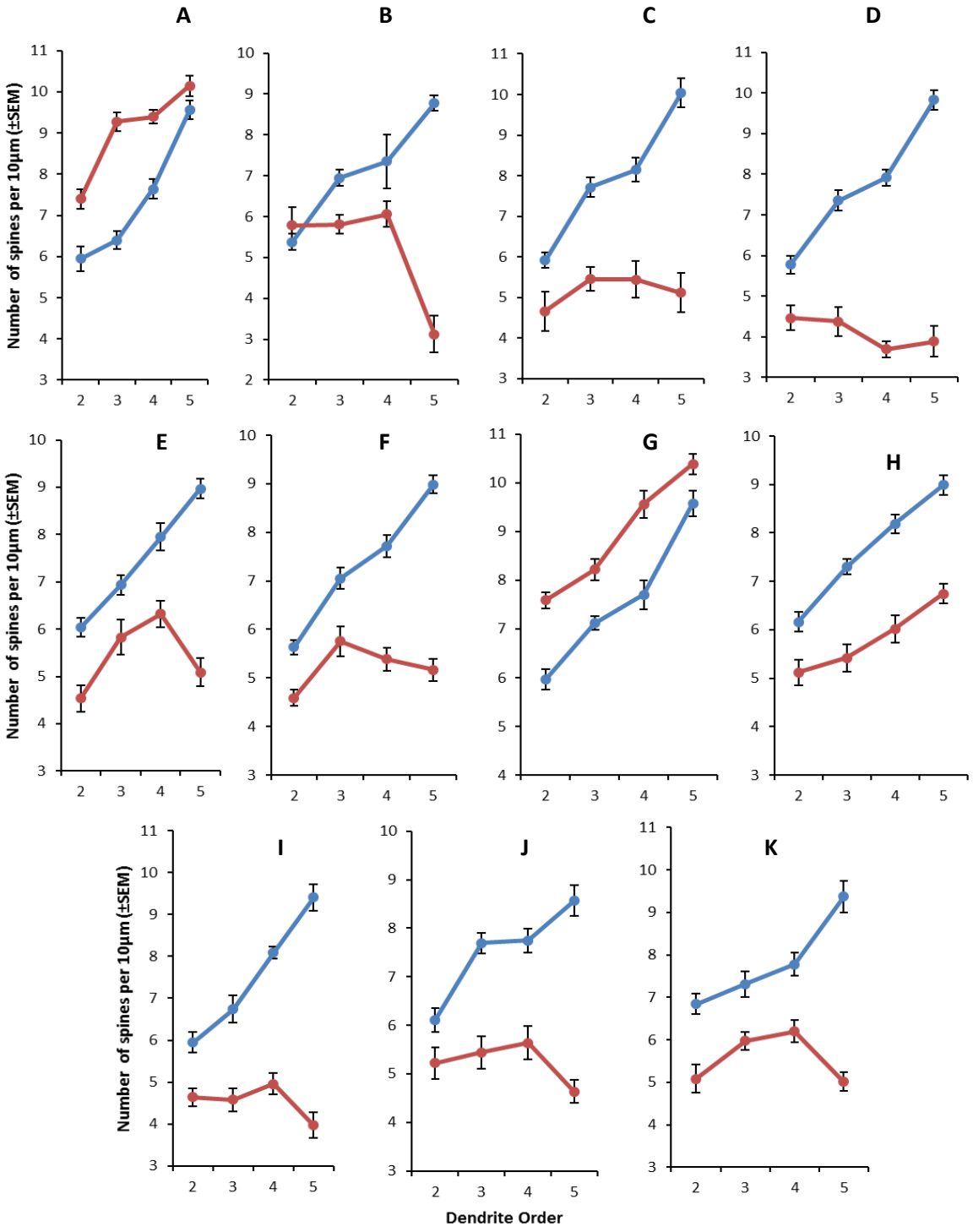

Figure 21. Changes in synaptic spine density due to chronic stress across. Stress is represented in red, Control in blue. **A** NaC, **B** Nas, **C** PLC, **D** IFC, **E** DLS, **F** DMS **G** BLA, **H** CN, **I** LO, **J** VO and **K** ACC. Significant changes are seen at every dendritic order across all ROIs. Refer to Tab. 1 for values.

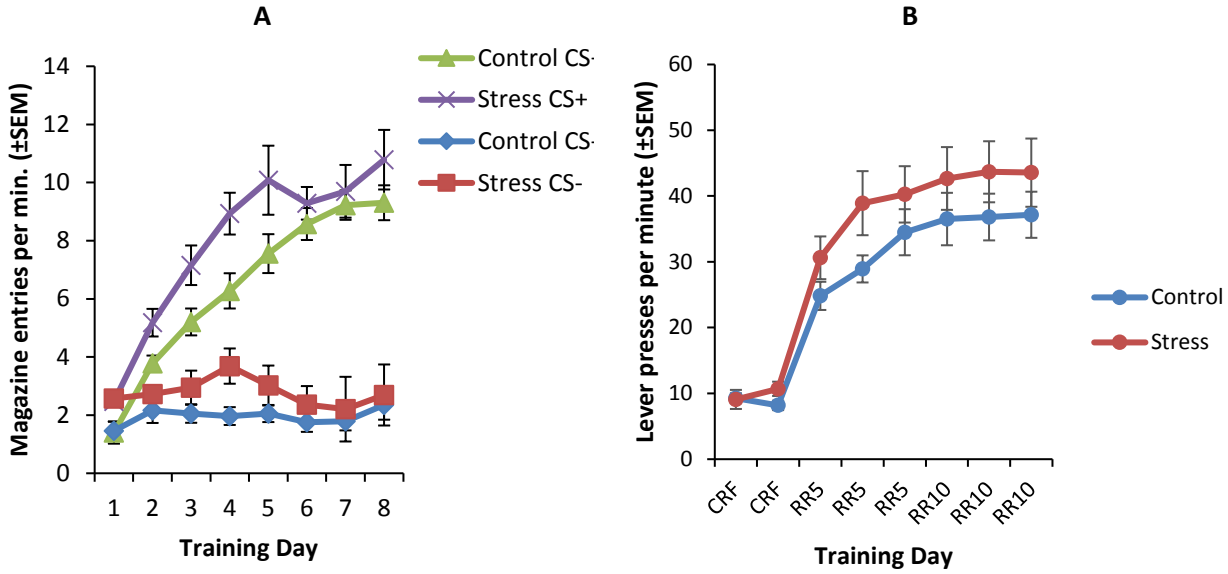

Figure 22. Both control and stressed animals learnt stimulus outcome and action outcome associations. **A** Stressed rats responded significantly greater on days 2 to 5, having higher magazine entries during CS+ than controls. **B** There was no difference between controls and stressed animals during instrumental training, both accelerated lever pressing as training increased. CS+ represents the period when the stimulus is presented and CS- during the prestimulus interval.

Pavlovian-Instrumental Transfer

The object of PIT was to test the impact of specific Pavlovian predictors on instrumental action selection. Each lever was associated with a specific outcome, pellets or sucrose, and this association was shared by a specific Pavlovian conditioned stimulus; i.e., the clicker and white noise were paired with either pellets or sucrose. The action paired with the outcome predicted by the stimulus in any stimulus situation was designated 'Same' and the other action was designated 'Different' in both the stimulus and the pre-stimulus period (e.g. If the left lever was paired with sucrose and the clicker was paired with sucrose then left lever performance would be classified as 'same' and the right lever performance as 'different' for that specific stimulus and so on). Fig. 23a presents the data showing pressing on the 'same' vs. 'different' lever collapsed across stimuli (no differences were found under the different stimuli; $p > 0.3$). ANOVA revealed a main effect of response ($F(1, 18) = 200.34$; $p < 0.001$) but no main effect of stress ($F(1, 18) = 2.51$; $p < 0.13$), however

an interaction effect between response and stress was found $F(1, 18) = 4.85$; $p < 0.041$). Simple effects revealed that both control ($p < 0.001$) and stressed ($p < 0.001$) animals pressed significantly more on the same lever than the different, this indicates that both groups saw enhanced lever pressing due to a paired Pavlovian stimulus and that chronic stress may facilitate a greater enhancement of lever press from PIT. As seen in Fig. 23a, the sPIT effect was larger in stressed than unstressed rats. For full breakdowns of the ANOVA refer to Tab. 2.

Outcome Devaluation

The objective of the outcome devaluation test is to assess the degree to which the rats's instrumental performance is goal-directed; i.e. to what degree the action-outcome associations to which the rats were exposed during training control their instrumental performance. To achieve this, the value of one outcome was reduced, in this case by specific satiety, relative to the other outcome and the rats are given a choice between the actions that in training delivered the devalued and the non-devalued outcomes. The results of this test are presented in Fig 23b for the control and the stressed rats. As is clear from the figure, although the control rats showed a clear outcome devaluation effect, responding less on the action that in training delivered the now devalued outcome than the other action, this effect was not as clearly observed in the stressed group, a description that was confirmed by the statistical analysis. Two-way ANOVA was conducted using factors response (devalued vs. non-devalued) and stress (control vs. chronic stress) revealed main effects of both devaluation, $F(1,18)=47.75$, $p<0.001$, and of stress, $F(1,18)=7.63$, $p<0.013$, and, more importantly, a significant interaction between response and stress, $F(1,18)=6.7$, $p<0.019$. The full ANOVA table is presented in Tab. 2. It is clear from Fig. 23b that the stressed group pressed less on the valued lever than the unstressed control group. These results demonstrate, therefore, that although the stressed group were able to show a degree of control by the action-outcome association after the value of one of the outcomes was devalued, this control was less than that

observed in the unstressed control group. It appears, therefore, that the chronic stress treatment significantly blunted goal-directed control relative to the control treatment.

Reinstatement

Although chronically stressed rats have a clear deficit in the acquisition of goal-directed action control, they were enhanced in their ability to use predictive cues associated a specific outcome to guide action selection in the test of specific PIT. We do not know from this test whether this enhancement was due to an increase in stimulus control or was secondary to an enhancement in outcome-mediated action selection *per se*. To assess this question we used outcome-specific reinstatement test. The objective of this test is to assess the ability of the rats to use the outcome itself, rather a stimulus paired with the outcome, to drive action selection. To achieve this, we first gave a period of extinction on both actions and then assessed the ability of delivery on one or other outcome to reinstate performance of the action with which it was associated during training, i.e., the 'same' action, relative to performance of the other, or 'different' action. The data from this test are presented in Fig 23d. As is clear from this figure, outcome-specific reinstatement appeared to be similar in both the stressed and the control groups. In line with this suggestion, two way ANOVA conducted on these data, using factors of stress condition (stressed vs. unstressed groups) and response (same vs. different), found a main effect of response, $F(1,18)=96.64$, $p<0.001$, but neither an effect of group nor any interaction between these factors ($F(1,18)=0.6$, $p>0.45$; $F(1,18)=0.07$, $p>0.797$). The full ANOVA table is presented in Tab. 2). These data suggest that, although the effect of predictive stimuli on action selection was enhanced after chronic stress, this was unlikely to have been due to an enhancement in outcome-mediated control of action-selection *per se*. The implications of these data will be addressed in the general discussion.

Contingency Degradation

The object of the contingency degradation training and test is to assess whether the rats are sensitive to changes in action-outcome contingency; in this case we degrade one action-outcome contingency whilst another contingency remains intact. Across groups, which of the two training contingencies was degraded was counterbalanced and, as the counterbalancing did not interact with the effects observed ($p>0.45$) we collapsed across this factor for the presentation of the data and for the overall analysis. Contingency degradation training produced a clear reduction in the performance of the degraded action relative to non-degraded action in the control group. When all training days were analysed via repeated measures ANOVA, a main effect of response ($F(1,18)=8.78$; $p<0.001$) and training day ($F(4,108=5.21$; $p<0.0007$) and group ($F(1,18)=5.6$; $p<0.03$) were found, furthermore, an interaction was found between group x response ($F(1,18)=4.56$; $p<0.047$) and response x training day ($F(4,108)=14.56$; $p<0.0001$). Simple effects used to establish the source of these effects revealed that control groups significantly pressed more for the non-degraded lever over the degraded lever (Control $F(1,9)=7.48$; $p<0.023$), while the chronic stress group did not ($F(1,9)=2.34$; $p>0.16$). These indicate that as training progressed control rats significantly pressed more for the non-degraded lever over the degraded lever, while random stress rats did not. The data from the contingency degradation test are presented in Fig. 23c. As is clear from this figure, although the control group demonstrated sensitivity to the specific degradation training, reducing performance on the degraded vs. the non-degraded action, the chronically stressed rats failed to distinguish between the degraded and non-degraded contingencies, responding on both actions at a similar rate during the test. Although two-way ANOVA revealed no main effect of response (degraded vs. non-degraded) ($F=1.12$) it revealed a main effect of stress (stressed group vs. the unstressed control), $F(1,18)=27.09$, and, more importantly, a significant stress by response interaction, $F(1,18)=4.43$, $p<0.05$. Simple effects analysis conducted to establish the source of the interaction revealed that, whereas there was a significant contingency degradation effect in the un-stressed controls, $F(1,18)=5.14$, $p<0.036$, there was no effect in the stressed group ($F(1,18)=0.07$; $p>0.796$). The full ANOVA table is presented in Tab.

2. These data suggest that the chronic stress treatment rendered rats unable to encode the action-outcome contingency and, together with the effects in the outcome devaluation test, confirm that chronically stressed rats have a deficit in their ability to acquire goal-directed actions.

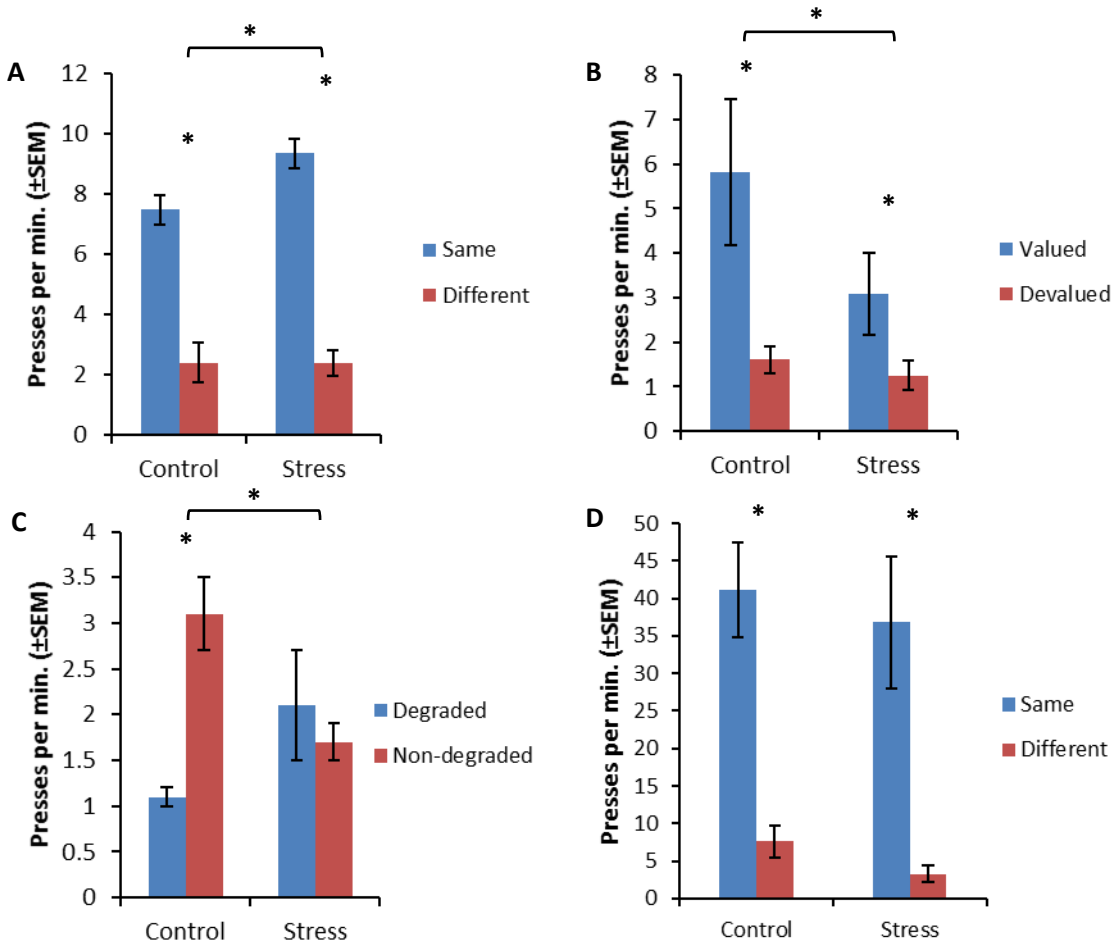

Figure 23. The four behavioural tests performed represented as histograms **A** PIT, **B** Outcome Devaluation, **C** Contingency degradation and **D** Reinstatement. Scores are means averaged across animals in each group and as lever presses per minute. * indicate a significant changes see Tab. 2 for further information.

PIT

	Simple Effects		Main Effects		
	Control	Stress	Response	Stress	Interaction
F (1, 18)	54.25	86.3	200.34	2.51	4.85
P	<0.001	<0.001	<0.001	<0.13	<0.041

Outcome Devaluation

	Simple Effects		Main Effects		
	Control	Stress	Response	Stress	Interaction
F (1, 18)	23.79	10.16	41.75	7.63	6.7
P	<0.001	<0.025	<0.001	<0.013	<0.019

Contingency Degradation

	Simple Effects		Main Effects		
	Control	Stress	Response	Stress	Interaction
F (1, 18)	5.14	0.072	1.12	27.09	4.43
P	<0.036	<0.796	<0.303	<0.001	<0.05

Reinstatement

	Simple Effects		Main Effects		
	Control	Stress	Response	Stress	Interaction
F (1, 18)	52.27	25.58	96.64	0.6	0.07
P	<0.001	<0.001	<0.001	<0.45	<0.797

Table 2. ANOVA scores for each of the behavioural tests, illustrating simple and main effects of response, chronic stress and interactions between the two.

Correlations between morphology and behaviour

Presented below is a series of correlations performed between the dendritic lengths of both control and stressed rats, of all the ROIs, against each rat's behavioural performance. Due to the exploratory nature of this thesis, the amount of comparisons being made dictated a family-wise error correction. As such a Bonferroni correction was performed to protect against committing Type-I errors, this correction changed the required alpha value for a significant correlation to $p<0.005$. Each battery of correlations will be grouped by behavioural test, note no correlations were found within reinstatement and so are instead presented within the appendix:

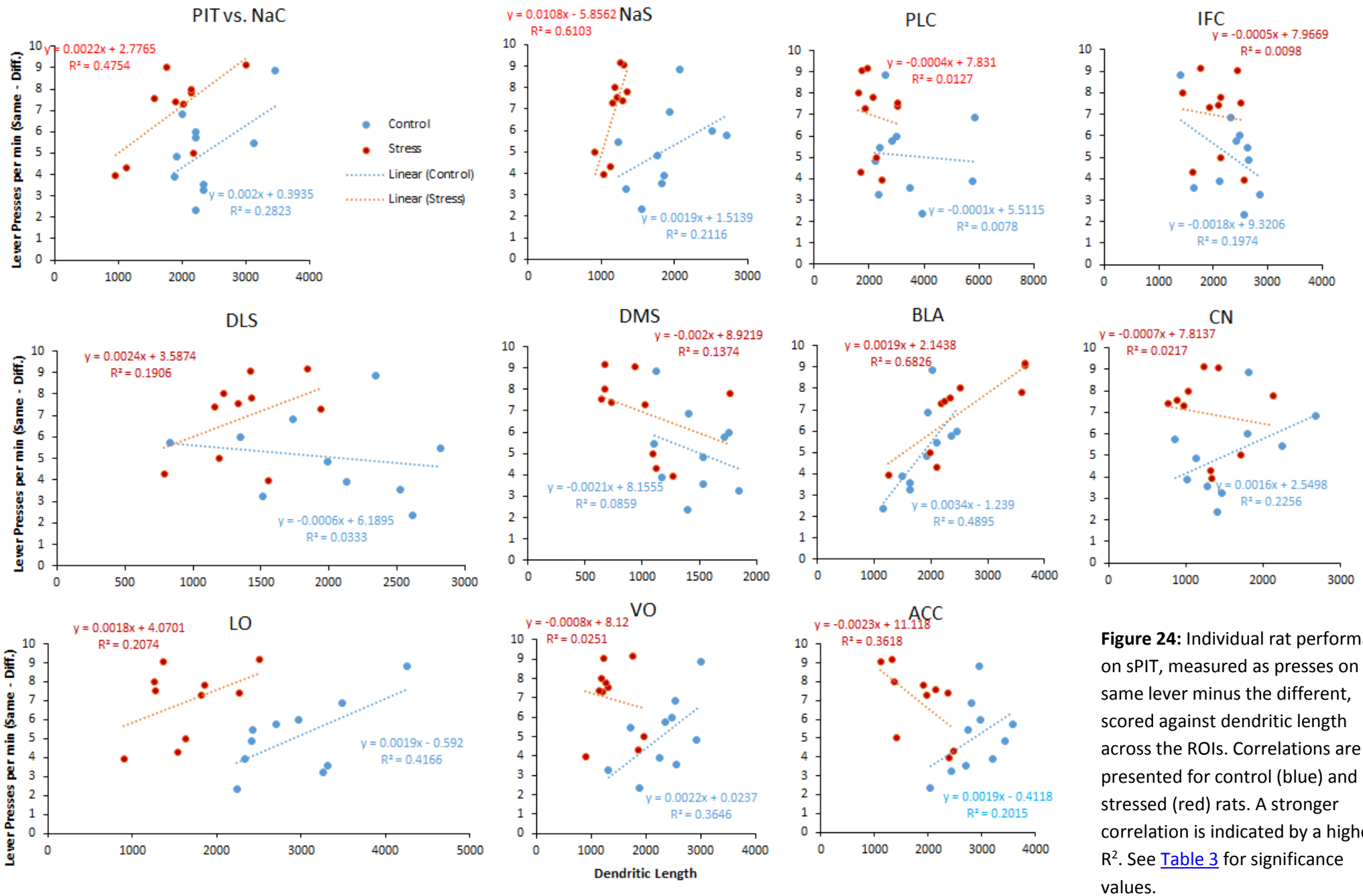

Figure 24: Individual rat performance on sPIT, measured as presses on the same lever minus the different, scored against dendritic length across the ROIs. Correlations are presented for control (blue) and stressed (red) rats. A stronger correlation is indicated by a higher R^2. See Table 3 for significance values.

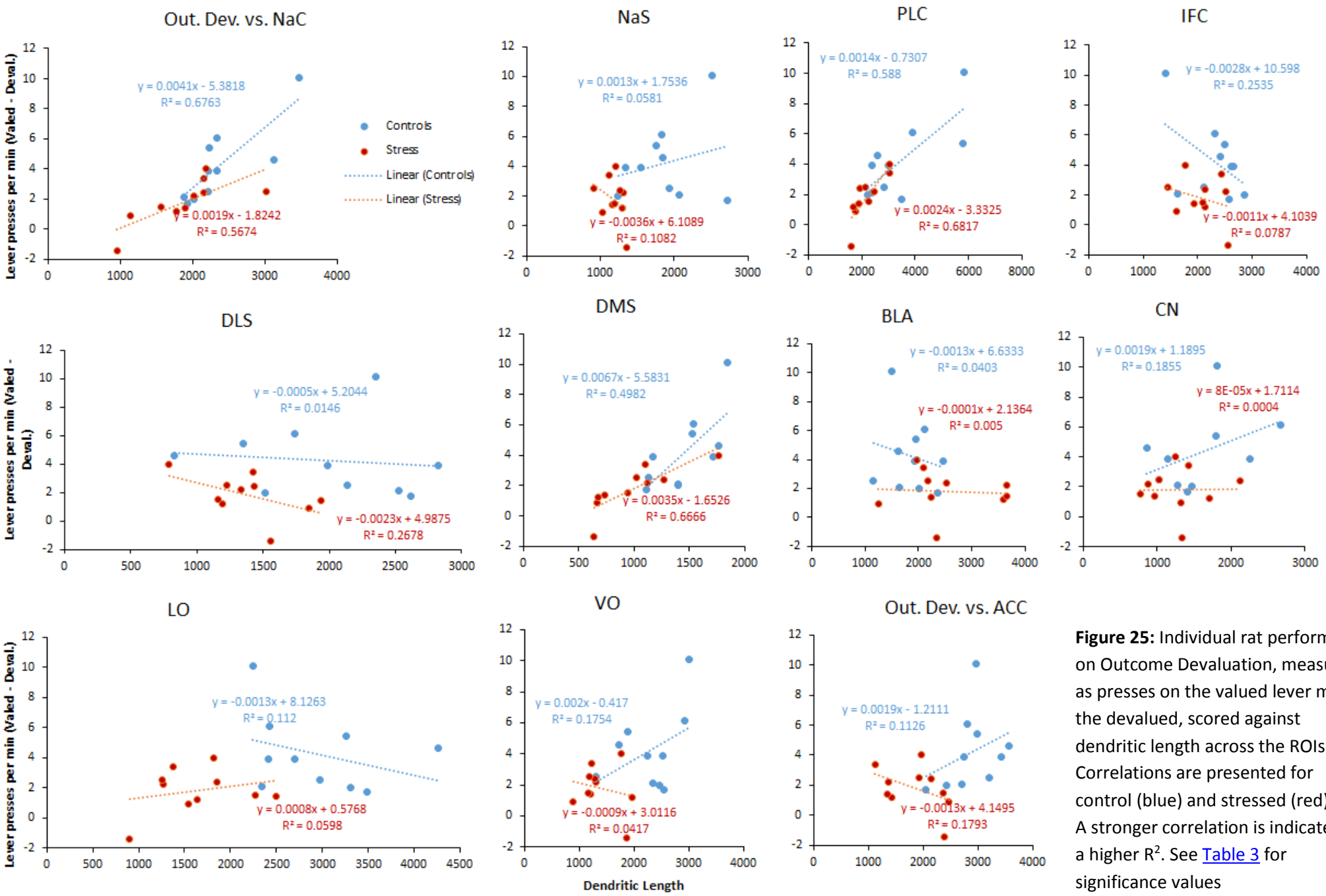

Figure 25: Individual rat performance on Outcome Devaluation, measured as presses on the valued lever minus the devalued, scored against dendritic length across the ROIs. Correlations are presented for control (blue) and stressed (red) rats. A stronger correlation is indicated by a higher R^2. See Table 3 for significance values

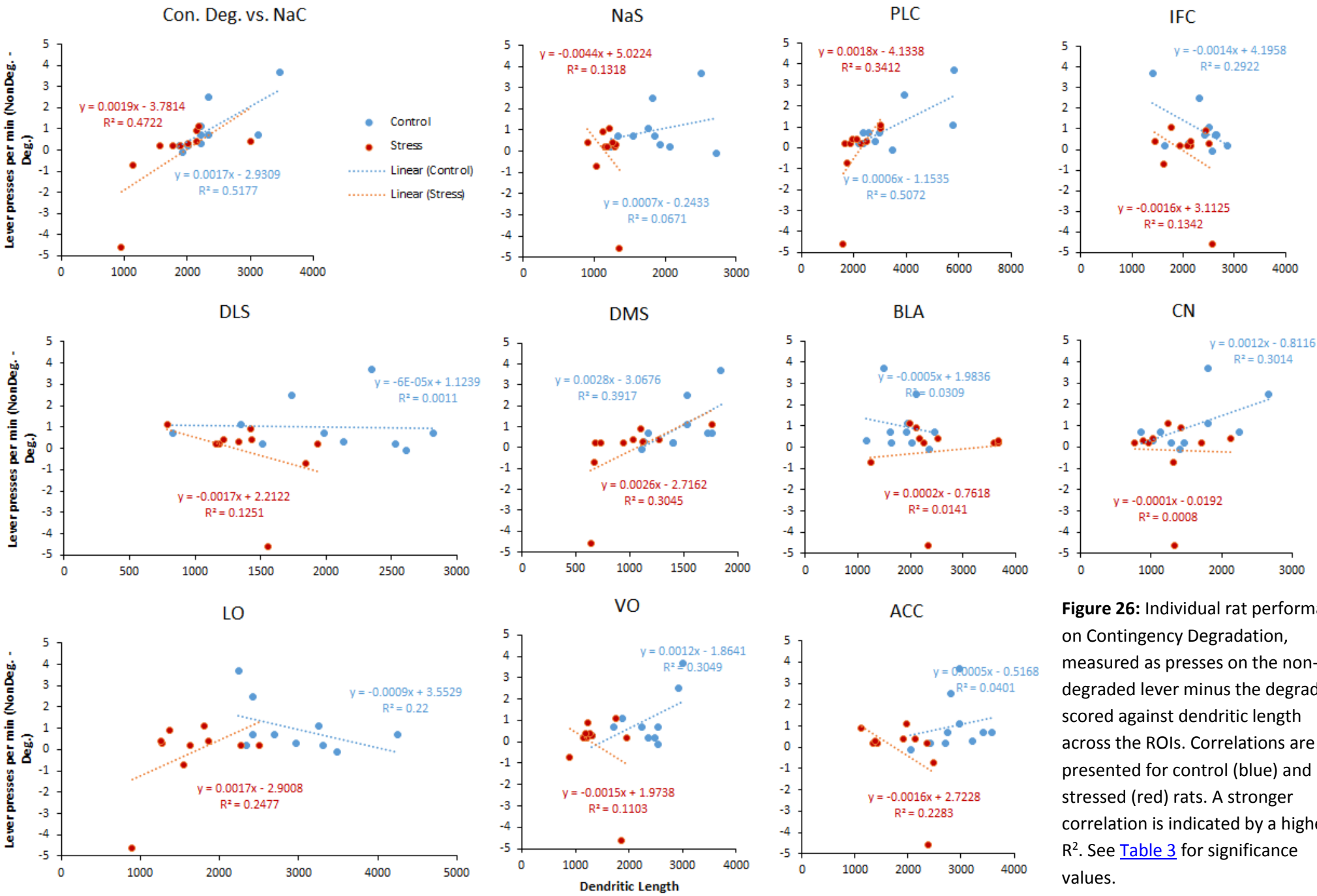

Figure 26: Individual rat performance on Contingency Degradation, measured as presses on the non-degraded lever minus the degraded, scored against dendritic length across the ROIs. Correlations are presented for control (blue) and stressed (red) rats. A stronger correlation is indicated by a higher R^2. See Table 3 for significance values.

The p values for each of the correlations is presented below, note that as specified, we have modified the traditional <0.05 significance cut-off via Bonferoni correction to <0.005, the below table represents both alpha values for speculative purposes to be discussed, regardless, only the scores highlighted in red and bold should be considered statistically significant.

CONTROL	NaC	NaS	PLC	IFC	DLS	DMS	BLA	CN	LO	VO	ACC	Bonferoni
PIT	0.114	0.181	0.808	0.198	0.614	0.411	**0.024**	0.165	**0.044**	0.065	0.193	**<0.005**
OUT.DEV	**0.003**	0.502	**0.01**	0.138	0.739	**0.023**	0.578	0.214	0.345	0.228	0.343	
CON.DEG	**0.019**	**0.047**	**0.021**	0.107	0.928	0.053	0.627	0.1	0.171	0.098	0.579	
REINST	0.59	0.722	0.827	0.735	1	0.836	0.63	0.961	0.522	**0.049**	0.811	

STRESS	NaC	NaS	PLC	IFC	DLS	DMS	BLA	CN	LO	VO	ACC	**Unmodified**
PIT	**0.027**	**0.008**	0.756	0.785	0.207	0.292	**0.003**	0.685	0.186	0.662	0.066	**<0.05**
OUT.DEV	**0.012**	0.353	**0.003**	0.432	0.126	**0.004**	0.847	0.954	0.496	0.571	0.223	
CON.DEG	**0.028**	0.302	0.076	0.298	0.316	0.098	0.744	0.94	0.143	0.348	0.163	
REINST	0.917	0.901	0.777	0.715	0.056	0.975	0.498	0.499	0.09	0.255	0.155	

Table 3*:* Significance scores for each of the correlations across all behavioural tests and ROI. Scores highlighted in red and bold are statistically significant correlations based on Bonferroni correction for amount of comparisons, those simply in bold are only significant to <0.05.

Experiment 3 – Quantifying Morphological Changes in Golgi Stained Neurons Using Novel Photoluminescence caused by Multi-Photon Excitation

Development

What can be seen throughout Experiment 3, is that two-photon imaging has a clear advantage over the reflection imagining done in Experiment 1, that being that synaptic spines are delineated and visualized far more clearly. Neuronal soma is visualisation is roughly the same, however the increased resolution of two-photon allows better 3D rendering, especially at greater magnification (Fig. 31 and 32). As with Experiment 1 and Fig. 9, Fig. 27 represents snaps taken from a 3D render, presented here for convenience, for a video see Sup Fig. 4.

The energy emitted by the mercury ions within the stained cyto-structure of the neuron was captured between 620-670 nm. This auto-florescence is triggered by energisation at 690nm. The result is a neuron that is visualised with greater control, the images in Figs. 29, 30, 31 and 32 were caught with two-photon microscopy, which makes use of the PSF offered by two-photon microscopy allowing background staining to be all but removed. As seen in the comparison of Fig. 29c and d, where the image caught via two-photo, has no background illumination, while the image caught via brightfield (Fig. 29d) presents with background stained neurons visualised. This allows for even greater isolation of neuronal morphology than that presented in Experiment 1. Two-photon illumination also allowed for greater detection of synaptic spines, shown explicitly in Fig. 31 and 32.

As seen in Fig. 30, the comparison between two-photon auto-florescent and brightfield visualisation seems almost identical, but for one difference. The dendrites in Fig.30a and c have illuminated synaptic spines, this allows for easier detection of synaptic spines in planes of view towards and away from the objective, i.e. perpendicular to the tissue, parallel to the objective. In brightfield imaging visualisation of spines in these planes is impossible, as seen in Fig. 31. This is the greatest strength of two photon Golgi imaging, is the increased resolution, and spine identification, best shown in Fig 32, a dendrite is shown at 100x; these snaps taken from the 3D render video Sup

Fig. 5. It can be seen that spines are protruding in all directions. The first of the three snaps in Fig. 32, is originally in the plan parallel to the objective, clearly there are spines that would normally not be visible via brightfield or reflections imaging.

It should also be noted that using power levers greater than 3mW caused irreparable damage to the tissue; as mentioned in the methods, imaging was optimised at 0.3mW at 690nm excitation, using power levels exceeding 3mW produced the tissue damage seen in Fig. 28. Lastly for completeness, Fig. 33 shows a series of shots taken from different excitations, intensities and collection points, illustrating the explorative nature, but also specificity of the methods described.

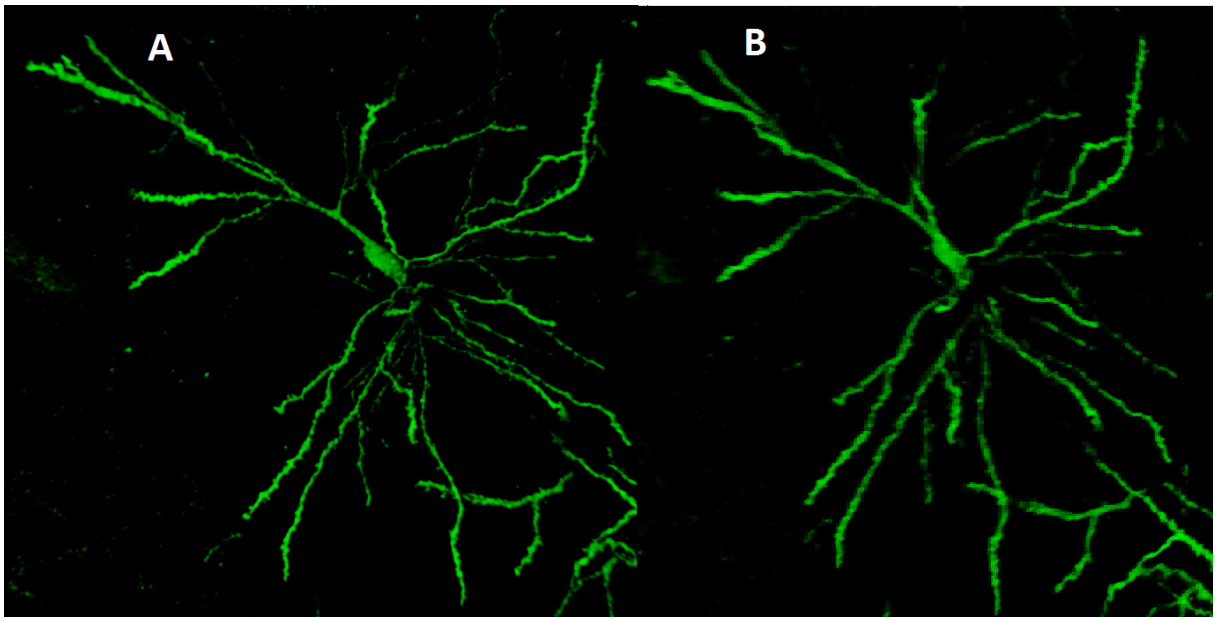

Figure 27: Snaps taken from a neuron stained via URG and visualised with 2-photon and rendered in 3D via Volocity software. **A** is a snap taken at from the direction parallel to the objective and **B** is a snap taken from the 3D render as it is rotated. These snaps are of reduced resolution and shown here for convenience, for a video see Sup. Fig. 4

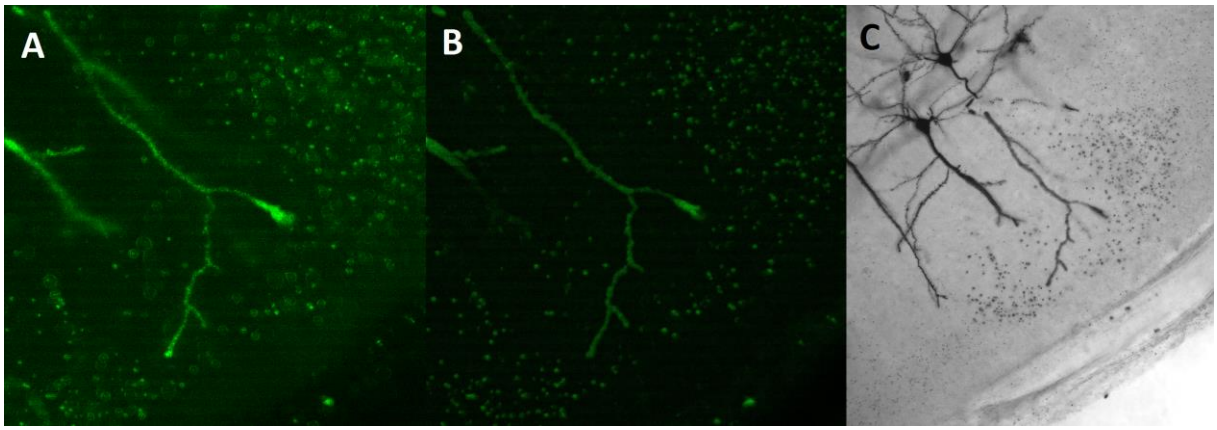

Figure 28: A series of snaps illustrating the damage power levels exceeding 3mW can do. A is a snap just as the burning process is beginning, the bubbles and dots appearing around the dendrite indicate that it is beginning to burn. B is after 10 minutes of burning, the fluorescence has decreased and morphology has begun to become indistinguishable. C is a shot taken from 20x objective shown in brightfield to see the scope of the burning and the distinct black bubbles/dots burning produces.

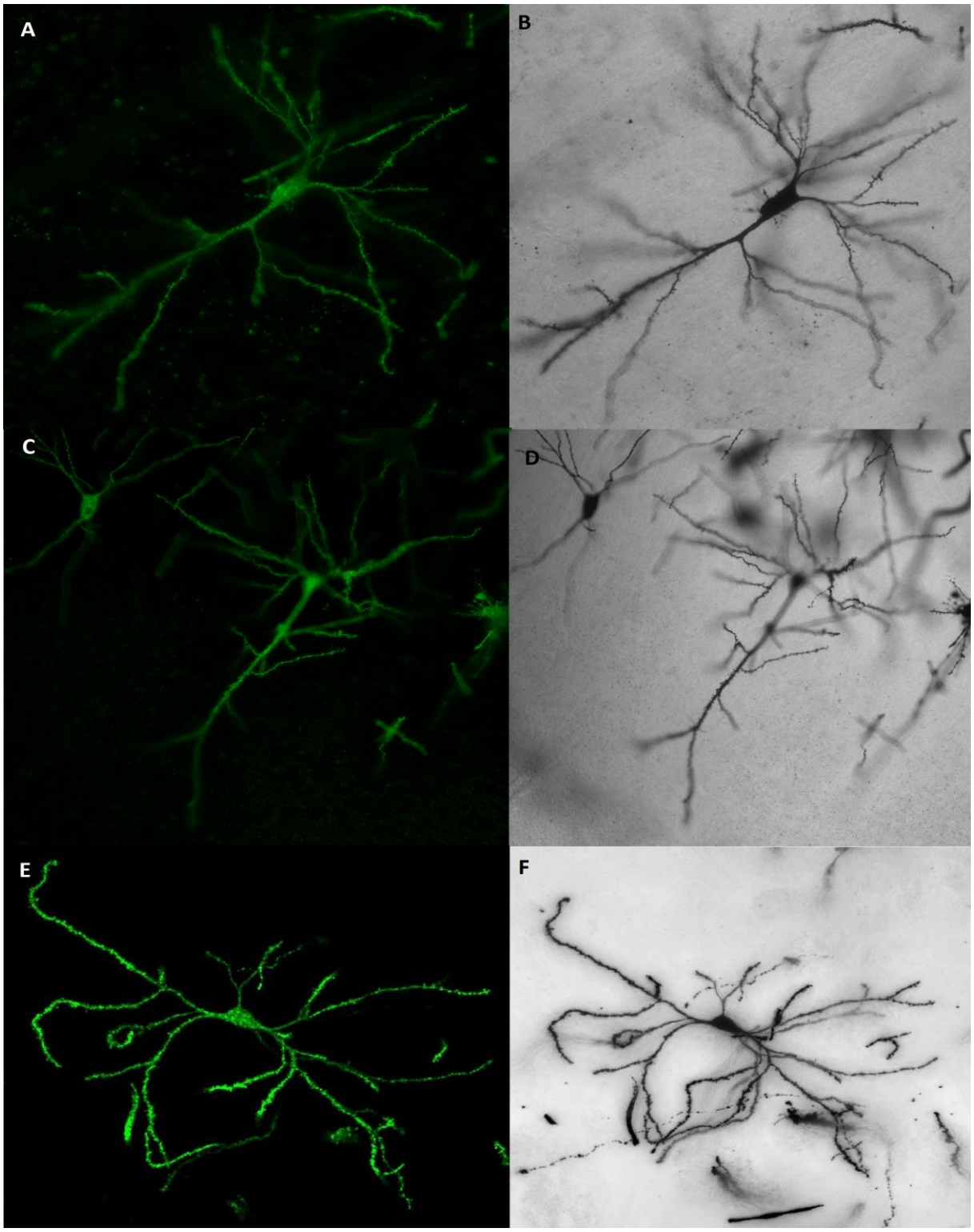

Figure 29: **A, C** and **E** Two-photon micrographs, caught at 20x, illustrating pyramidal and medium spiny neurons; soma, synaptic spines and dendrites are visible with very minimal background staining. Photoluminescence emitted at 620-670nm. For s detailed video see Sup. Fig. 4 and 5, for a 3D reconstruction see Sup. Fig. 6. **B, D** and **F** Bright-field micrographs shown for comparison.

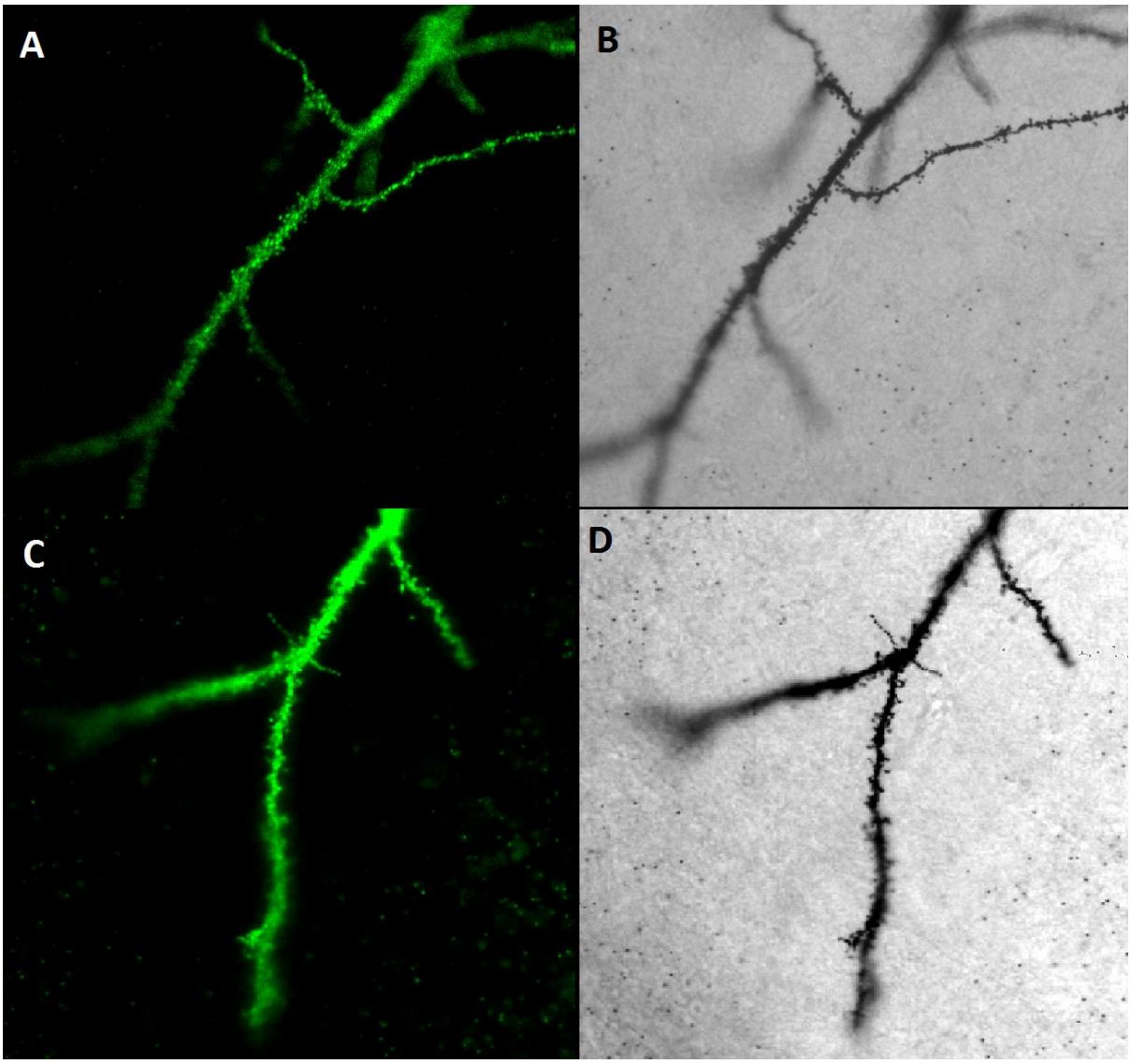

Figure 30: A and **C** Two-photon micrographs illustrating photoluminescent synaptic spines. Spines are clearly delineated and can be seen by eye in the typically non-visible perpendicular plane. The brighter spots of green light visible on the dendrite indicate synaptic spines oriented towards the camera. Photoluminescence emitted at 620-670nm. Compared to **B** and **D** (bright-field) where only spines protruding from the side are visible.

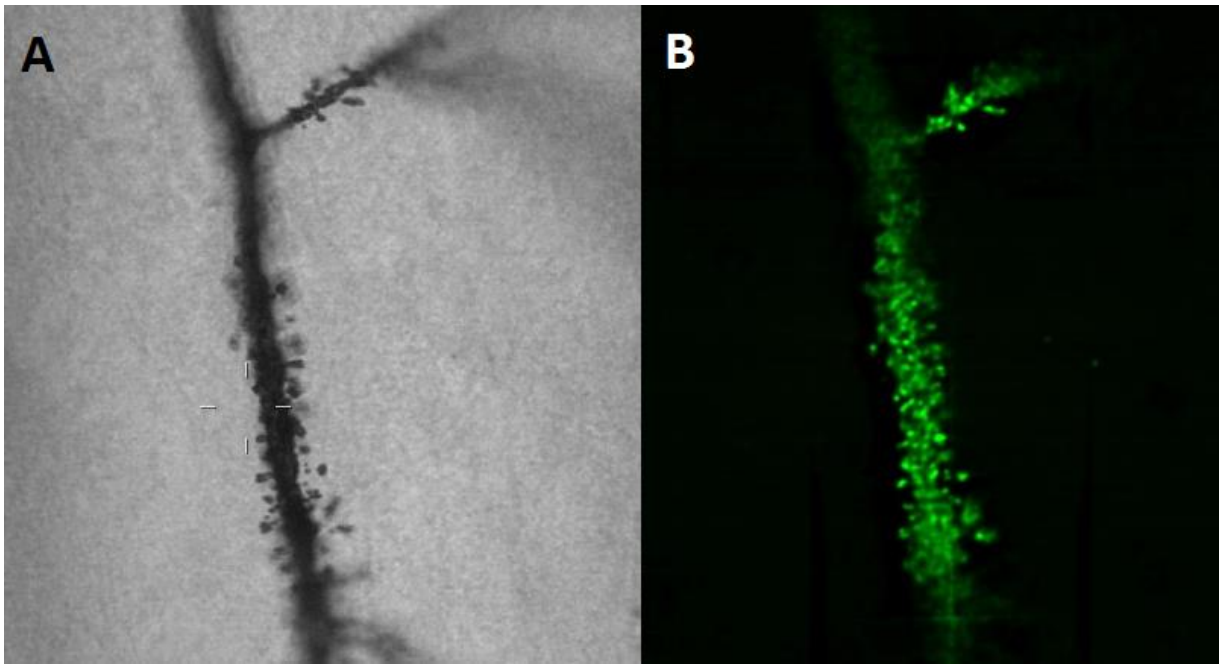

Figure 31: A pair of photomicrographs taken of a dendrite, stained via URG, at 100x objective and visualised via brightfield (A) and 2-photon (B). Comparing the shots, the brighter illumination dots in B correspond to synaptic spines, not visible via brightfield. See Fig. 32 for a 3D render.

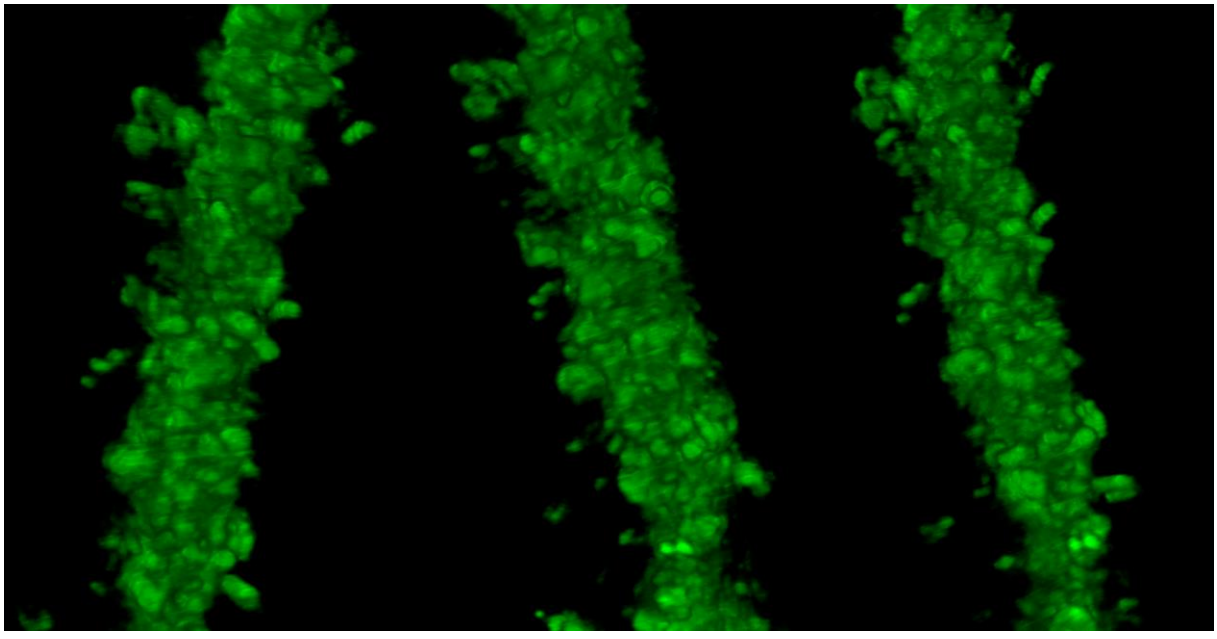

Figure 32: A 3D render of the images from Fig. 31. These snaps illustrate the amount of synaptic spines that are not being visualised via brightfield. The 2-photon resolution makes rendering these high magnification shots in 3D a possibility. For the video see Sup. Fig. 5.

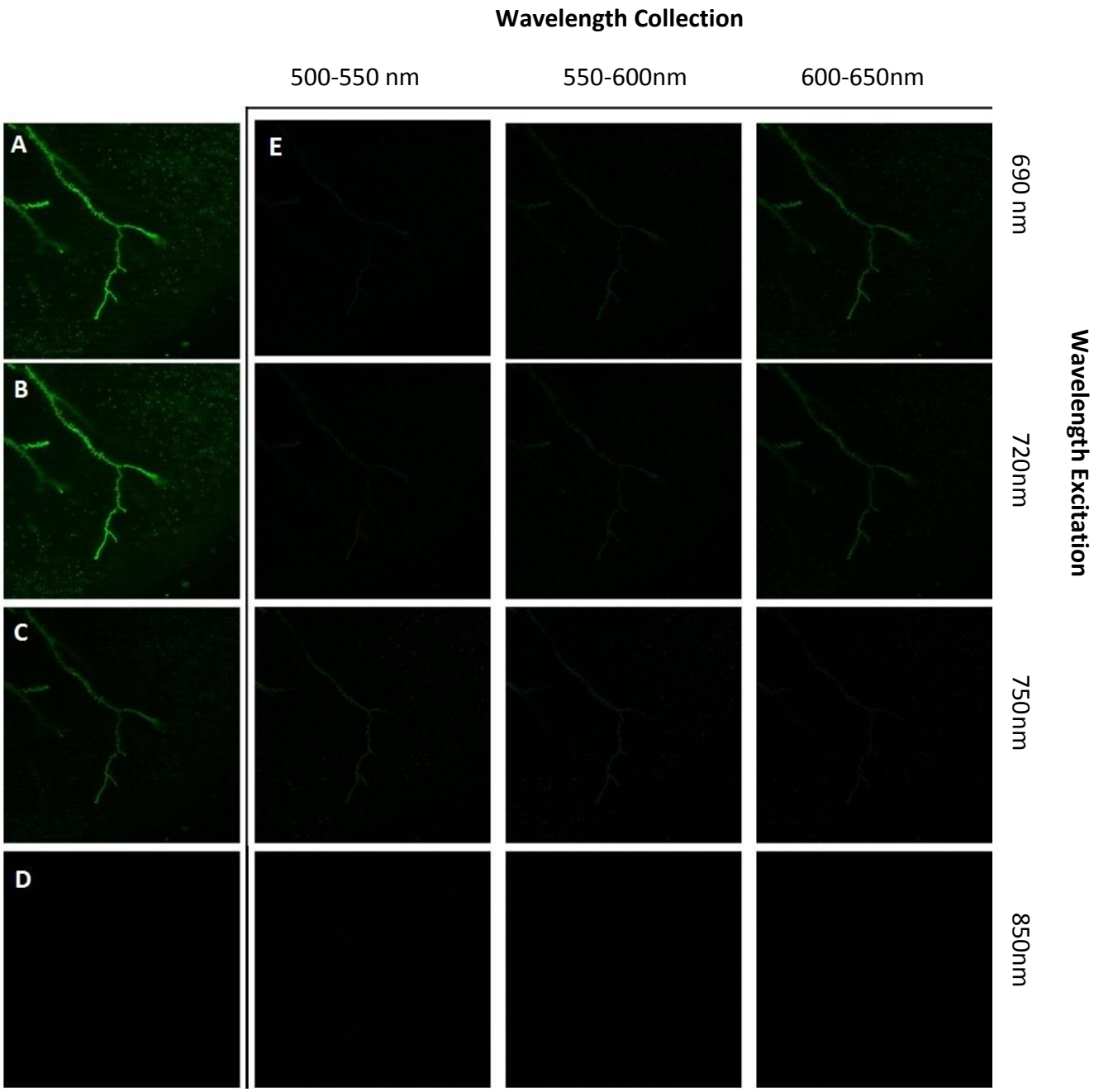

Figure 33: A series of photomicrographs taken from the same dendrite, illustrating the explorative nature and specificity of these methods. **A** shot taken at optimal settings, **B** at optimal setting but at 710-760 collection, **C** at optimal settings but excitation at 710 excitation with 6mW power, **D** optimal settings but 1000nm excitation, **E** a battery of shots showing progressively increased excitations and collections.

Experiment 4 – The effects of Predictable Chronic Stress on Neuronal Morphology and Decision-making

Changes in Body Weight Following Stress

The random stress treatment had a clear effect on body weight compared to controls, an effect that was also clearly ameliorated in the predictable stress group. The weights of the groups at the during and at the end of the stress treatment are illustrated in Fig. 34. ANOVA revealed that random CRS significantly decreased the body weight of rats. Main effect of group ($F(2, 45) = 44.17$; $p < 0.0001$) and time ($F(20, 900) = 27.81$; $p < 0.0001$), additionally there was a significant interaction of group x time ($F(40, 900) = 22.04$; $p < 0.0001$). Random stress rats had 75% body weight by day 21 compared to controls and 79% compared to predicted stress. Post hoc analysis revealed that the mean weight of the random stress group differed both from the predicted stress group ($p < 0.0001$) and from the controls ($p < 0.0001$) which did not differ significantly from each other ($p > 0.067$). Together these data indicate that random stress decreased weight and continued to do so as the paradigm continued. The observed weight loss within the random stress group provides evidence that the effect of stress was greater in this group consistent with previous studies (Sousa et al., 1998, Watanabe et al., 1992b, Coburn-Litvak et al., 2003, Conrad et al., 2004, Kassem et al., 2013) . Additionally, the finding that the predicted stress group showed little to no weight loss suggests that the effects of restraint stress where ameliorated in this group.

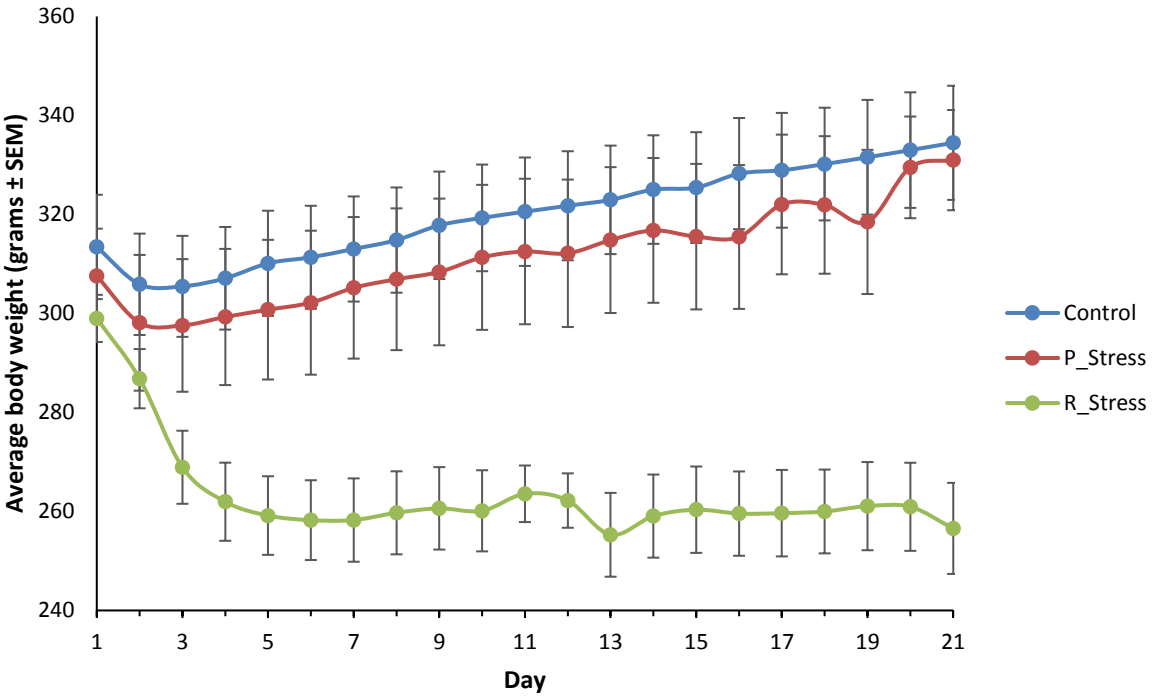

Figure 34: Illustration of the loss of body weight across the three groups of animals, controls, predicted stress rats (P_Stress) and random stress rats (R_Stress) across the paradigm. Controls and predicted stress rats maintain weight, random stress rats shown stress induced weight loss.

Histological controls

As described in the methods an additional 6 rats per group were not subject to any behavioural training or testing. Analysis of the histological data in these rats did not interact significantly with their specific treatment conditions compared to their behavioural counterparts (*p* values main effects and of the treatment (behavioural treatment x histological control) x stress group interactions across all ROI > 0.2). This finding that behavioural testing was not an additional variable that interacted with dendritic length or spine density suggested that it did not affect this measure in and of itself and, as such, the histological measurements of these rats were combined with those of their respective groups for analysis.

Changes in Dendritic Length

The cumulative length of the dendrites of individual neurons was measured for the battery of ROIs for control, predicted and random stress groups. Fig. 35 and 36 provide photomicrographs illustrating the group changes and Fig. 38 shows the summary data for each group on this measure across all ROI. Note that, whereas random stress produced a decrease in dendritic length across most regions, the NaC, BLA and InC showed an increase. Unlike in Experiment 2 the DLS did not trend towards a difference ($F(2, 45) = 1$; $p > 0.362$). Furthermore, as shown by Fig. 38b, there were changes of up to 45% in both directions. These changes indicate that chronic restraint stress caused decreases in all ROIs except the DLS and CN and caused increases in the BLA and NaC. Some changes were clearly more prominent between the two levels of stress and these differences were supported by ANOVA and by post hoc analyses (Tab. 4), with changes in many ROIs ameliorated in the predicted stress relative to the random stress group. Of particular note, we observed that the VO showed an increase in dendritic length in the predicted stress group but a decrease in the random stress group.

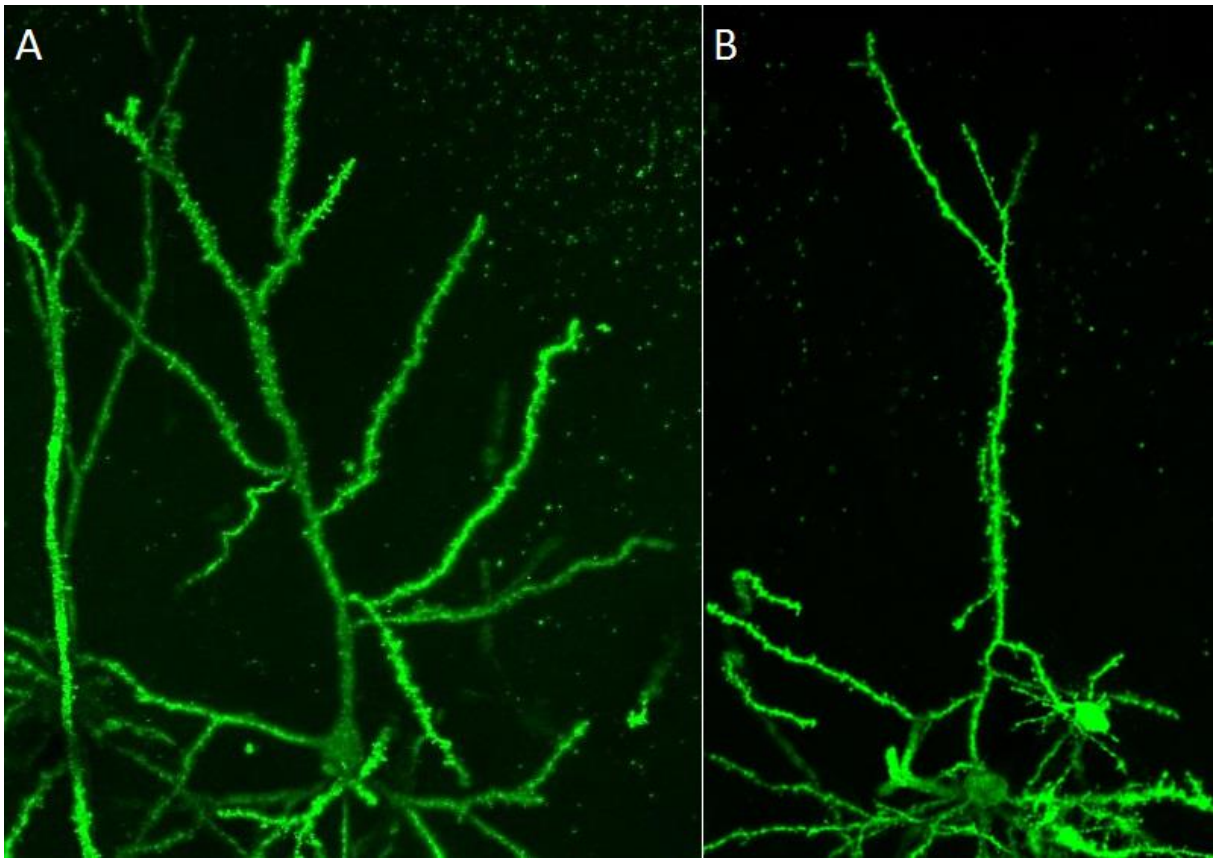

Figure 35: Photomicrographs depicting apical dendritic deterioration within pyramidal neurons in the ACC. Illustrating the decreases in dendritic length caused by chronic stress. **A** Healthy non-stressed neuron. **B** Chroncially stressed neuron

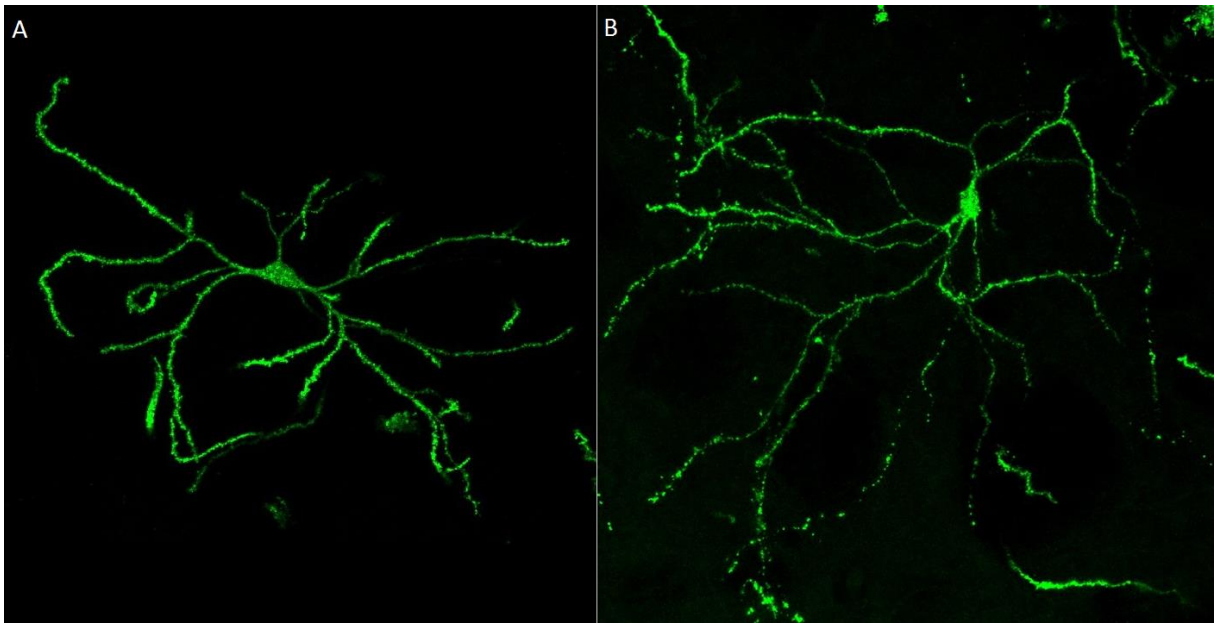

Figure 36: Photomicrographs depicting dendritic proliferation within medium spiny neurons in the NaC. Illustrating the increase in dendritic length caused by chronic stress. **A** Healthy non-stressed neuron. **B** Chroncially stressed neuron.

Changes in Synaptic Spine Density

As in Experiment 2, synaptic spine density was measured in each ROI and were defined and separated by dendritic order. Dendritic orders 2-5 were measured and the density changes illustrated in Fig 37 and shown Fig. 39. ANOVA revealed significant changes in all ROIs (Tab. 4). The changes in spine density in each ROI match the changes in dendritic length. Dendritic order 1 is not shown as no significant changes in any ROI were found (*p* values > 0.1). This is consistent with observations in Experiment 2 and in previous studies (Kassem et al. 2012, Padival et al. 2013) where spine density changes were rarely found on order 1 dendrites. The changes reported in Fig. 39 indicate that predicted and random chronic restraint stress causes decreases in synaptic spine density in all ROIs and increases in the BLA, NaC and InC. Some changes are more or less prominent between the two levels of stress, these are supported by ANOVA and post hoc tests (Tab. 4).

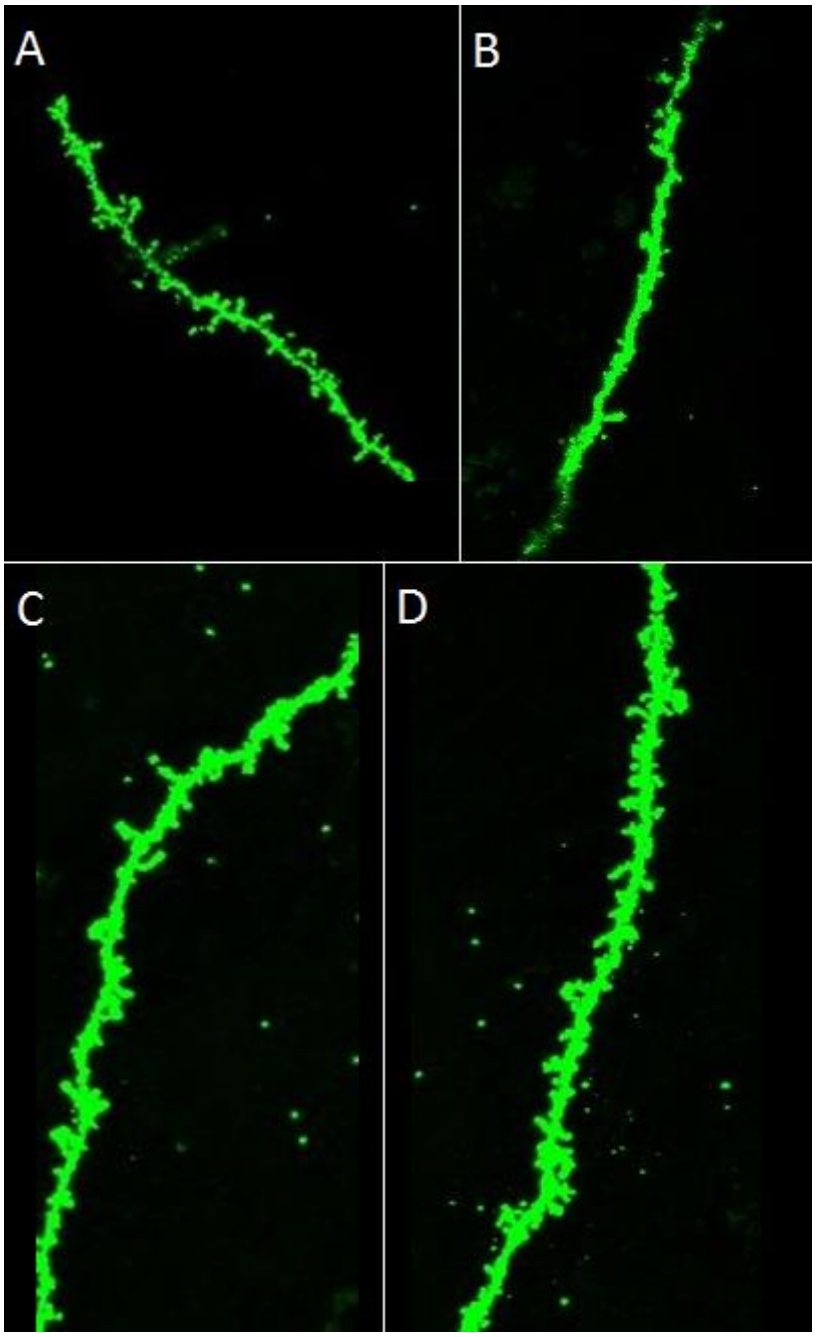

Figure 37: Photomicrographs depicting synaptic spine changes within chronically stressed rats. **A** & **B** illustrate the decrease in synaptic spines caused by chronic stress, depicted in the LO, **A** Healthy synapic spine density. **B** Spine density decreased in chronically stressed rat. **C** & **D** illustrate the increase in synapitc spine denisty caused by predicted chronic stress, depicted in the VO **C** Healthy synapic spine density. **D** Spine denisity proliferation in predicted stressed rats.

Behavioural Measures

Pavlovian training

To determine whether the animals acquired the association between the stimulus and the outcome, magazine entries were compared during the stimulus and during a prestimulus interval of equal length. As illustrated in Fig. 40a, all three groups of rats entered the magazine at significantly higher rates during the stimulus than the pre-stimulus period and to a similar degree. A statistical comparison of stimulus and outcome counterbalancing conditions showed no main effects or interactions with group and so responding was collapsed across these factors for the overall analysis (p values > 0.35). Three-way mixed ANOVA conducted with factors of stimulus, group and training sessions revealed no effect of group ($F(2, 27) = 1$; $p > 0.377$), but a main effect of training session ($F(7,189) = 115.89$; $p<0.0001$), of stimulus ($F(2,27) = 10.78$; $p<0.0001$) and a significant training x stimulus interaction ($F(14, 189) = 1.93$; $p < 0.026$). Post-hoc simple effects analyses revealed significant effects of stimulus for groups control $F(1, 18) = 73.44$; $p <0.0001$, predicted stress $F(1, 18) = 54.82$; $p <0.0001$, and random stress $F(1, 18) = 48.67$; $p <0.0001$), confirming that increase in responding during the stimulus relative to the prestimulus period on all three groups. This indicates that as training progressed responding increased, according to Fig. 40a, during the CS period relative to the pre-stimulus period and that the two stressed groups did not differ from the controls (unlike Experiment 2) or each other.

Instrumental training

The training data are presented in Figure 40b. It is clear from this figure that all group showed a general increase in performance as the ratio requirement was incremented and that this increase was similar in all three groups. This was supported by the statistical analysis. There was no effect of the counterbalancing factors (all p values > 0.3) and so the data were collapsed across these for the overall analysis. Two way ANOVA revealed that there was a main effect of training day ($F(7,$

189) = 79.561; p <0.0001), but no main effect of stress (F(1,18) = 1.434; p >0.256) and no interaction between these factors (F(14, 189) = 1.06; p > 0.92) confirming that the stress treatment did not affect instrumental training..

Pavlovian-Instrumental Transfer

As in Experiment 2, the data in the Pavlovian-instrumental transfer test in Experiment 4 were presented for each lever, separated by whether the lever delivered the same outcome as that predicted by the CS or a different outcome. These data are presented in Figure 41a. It appears, as was found in Experiment 2, that the random stress group showed if anything a larger specific PIT effect than the control group. More importantly, the size of this effect appeared to be further increased in the predicable stress group; i.e., that predictable stress appears to have generated a much larger specific PIT effect than in either the random or no stress control groups. For the analysis we first assessed the effects of the counterbalancing factors and, as these had no effect (all p >0.3) we collapsed across these factors for the overall analysis. The use of standard parametric statistics to assess this description of the data is complicated in this situation because ANOVA is relatively insensitive to detecting ordinal interaction effects in three group designs such as this one, traditional ANOVA revealed an effect of response (F(2,27)=38.52; p<0.001) and group (F(2,27)=3.48; p<0.045), but no interaction effect (F(4,27)=1.59; p>0.22). Bobko (1986) argues that in situations where the hypothesis predicts an ordinal interaction effect (in this case group x response) it can be lost to main effects and the loss of power to detect said interaction. An alternative approach involves testing planned comparisons, in this case a comparison between the size of the transfer effect in (**1**) the control group vs. the random stress group, (**2**) between both the control and random stress groups vs. the predictable stress group and (**3**) the control group vs. the random and predicted stress groups, may reveal interaction effects hidden by traditional ANOVA. After testing assumed homogeneity (p>0.323), planned comparisons performed reveal (**1**, p>0.18; **2**, p<0.05; **3**, p<0.04). These indicate that stress uniquely interacts with rat performance on the sPIT test, facilitating a

greater enhancement of lever press compared to controls, with predicted stress facilitating more than random stress.

Outcome Devaluation

The outcome devaluation test was conducted as described in Experiment 2. The data from this test are presented in Fig 41b, for each group separated into responding on the lever that in training delivered the valued vs. the now devalued outcome. Inspection of the figure suggests that, although there was a significant outcome devaluation effect in the control group, as observed in Experiment 2, random stress appeared to abolish this effect. Importantly, making restraint stress predictable appeared completely to restore the outcome devaluation effect in that group which looked similar to controls. This description of the data was supported by the statistical analysis. For the analysis we first assessed the effects of the counterbalancing factors and, as these had no effect (all $p > 0.5$) we collapsed across these factors for the overall analysis. Mixed model ANOVA revealed no effect of Group, $F(2,27)=2.06$, $p>0.05$, but a main effect of response (valued vs. devalued), $F(2,27)=18.04$, $p<0.001$, and, importantly, a significant interaction between these factors, $F(2.27)=3.54$, $p<0.043$. Simple effects analysis conducted to establish the source of the interaction revealed a significant devaluation effect in the control group, $F(2.27)=11.43$, $p<0.003$, in the predictable stress group, $F(2.27)=7.98$, $p<0.011$, but no effect in the random stressed group, $F(2,27)=0.08$, $p>0.77$. These results suggest that random stress reduced sensitivity to outcome devaluation and that the lever pressing in these rats was no a goal-directed action. In contrast, making the stress predictable appeared completely to mitigate this effect consistent with goal-directed control in the predictable stress group.

Reinstatement

The reinstatement test was conducted in similar manner to the test conducted in Experiment 2 with the same distinction between responding on the lever that previously delivered the same

outcome as the reinstating outcome relative to the other 'different' lever. The results from this test are presented in Figure 41d. Although the figure appears to indicate a diminution of the outcome-specific reinstatement effect in the predictable stress group relative to control, and further reduction in random stressed group, in fact none of these interaction effects achieved significance in the overall analysis. The only significant effect in the ANOVA was a main effect of response (same vs. different), $F(2,27)=6.575$, $p<0.016$, with no effect of group nor a significant group x response interaction, both $F<1$. Hence, as was found in Experiment 2, stress appears not to influence the ability of animals to use the outcome as a stimulus (S^O) to select the appropriate response (R) – a test of the rats' ability to encode S^O–R associations

Contingency Degradation

To confirm the conclusion that instrumental performance in the predictable stress group was goal-directed, we conducted a contingency degradation assessment as described in Experiment 2. Contingency degradation training produced a clear reduction in the performance of the degraded action relative to non-degraded action in the control group. When all training days were analysed via repeated measures ANOVA, a main effect of response ($F(2,27)=7.69$; $p<0.01$) and training day ($F(4,108=4.24$; $p<0.003$) were revealed, with no main effect of group ($F(2,27)=0.04$; $p>0.96$), furthermore, no interaction was found between group x response ($F(4,27)=0.43$; $p>0.95$), but one was found between response x training day ($F(8,108)=8.34$; $p<0.004$). Simple effects used to establish the source of these effects revealed that both control and predicted stress groups significantly pressed more for the non-degraded lever over the degraded lever (Control $F(1,9)=6.49$; $p<0.033$, Predicted $F(1,9)=5.31$; $p<0.048$) , while the random stress group did not ($F(1,9)=0.84$; $p>0.383$). These indicate that as training progressed control and predicated stress rats significantly pressed more for the non-degraded lever over the degraded lever, while random stress rats did not. The results of the contingency degradation test conducted at the end of training are presented in Figure 41c. In contrast, as was observed in Experiment 2, random stress appeared to abolish this effect and the

performance of this group of rats showed complete insensitivity to the degradation treatment. With regard to the statistical analyses, there were no effects of the counterbalancing factors (all $p>0.55$) and so we collapsed across these for the overall analysis. Two way, group x response, ANOVA revealed no effect of group, ($F(2,27)=1.06$, $p>0.36$) but a main effect of response (degraded vs. non-degraded, $F(2,27) =18.47$, $p<0.001$), and a significant interaction between these factors, ($F(2,27)=5.134$, $p<0.013$). Simple effect analyses conducted to establish the source of the interaction found a significant effect of response in both the control group, $F(2,27)=6.93$, $p<0.017$, and the predictable stress group, $F(2,27)=8.02$, $p<0.011$, but no effect of the degradation treatment in the random stress group, $F(2,27)=0.06$, $p>0.81$, suggesting, with reference to Fig. 41c, that randomly stress caused instrumental action to be insensitive to contingency degradation.

Delayed Discounting

The object of the delayed discounting test was to measure how well a rat could understand the value of their action-outcome contingences as well as action flexibility and impatience, early on in the test, one action outcome contingency offers a greater reward, however as the tests progress, that lever's time delay become greater, flexibility in behaviour is required to change decision making to preference the outcome that offers the best ration of delay to reward. Additionally, a preference for short delay would indicate impatience. i.e. LL offers a short delay and 1 pellet, RL a delay for 3 seconds and 5 pellets, as there is a ITI between each choice, the RL offers the best time to reward ratio, however by delay 30 seconds the short delay LL offers the best ratio. In Fig. 41e, a line graph illustrates the mean percentage preference for the delayed lever out of total lever presses. Random stress rats appear to learn the time delay ratios earlier on, with the predicted stress rats remaining patient and inflexible as the test continues. AVOVA revealed via simple and main effects of response that all groups learnt and performed delayed discounting, no main effect of group was found, but an interaction between group and response was (Tab. 5). This indicates that although post hoc

comparisons reveal no specific difference between groups, group impacts on response choice. This will be expanded on in discussion.

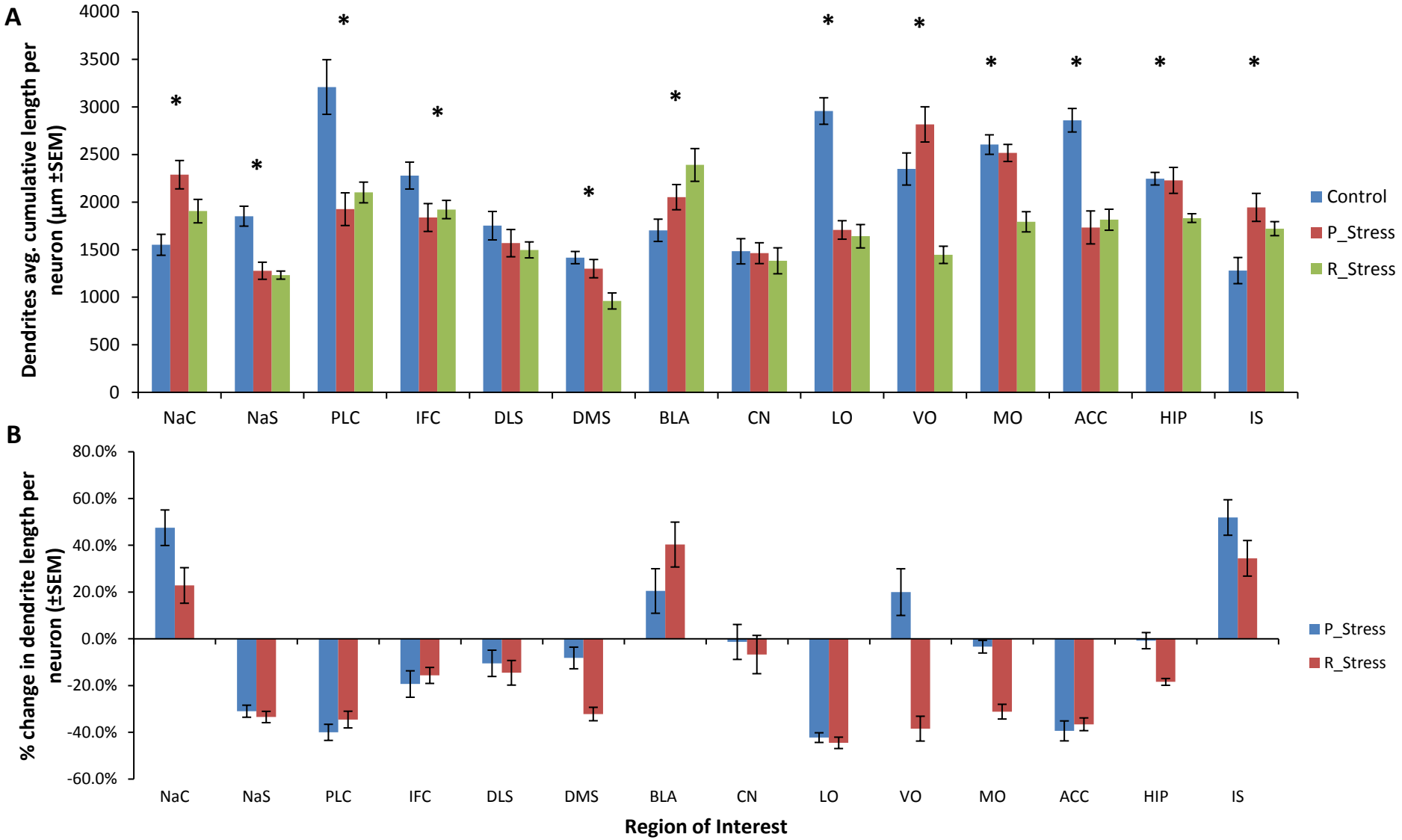

Figure 38: Changes in dendritic length due to chronic restraint stress, predicted or random. **A** Comparison between controls, predicted and randomly stressed rats within regions of interest. **B** Percentage changes within stressed animals compared to controls. P_Stress represents predicted stress, R_Stress represents random stress. * indicate significant differences see Tab. 4

Core		Post	Hoc		Shell		Post	Hoc		Pre		Post	Hoc		Inf		Post	Hoc	
F(2, 45)	*p*	CvP	CvR	PvR	F	*p*	CvP	CvR	PvR	F	*p*	CvP	CvR	PvR	F	*p*	CvP	CvR	PvR
Dendrite																			
8.22	<0.001	<0.001	0.14	0.10	17.16	<0.001	<0.001	<0.001	0.90	11.74	<0.001	<0.001	<0.001	0.80	3.24	<0.048	0.05	0.14	0.89
Order 2																			
6.70	<0.003	0.39	<0.001	0.07	0.78	0.47	0.94	0.67	0.45	4.23	<0.02	0.06	0.03	0.95	7.52	<0.002	0.01	<0.001	0.75
Order 3																			
30.76	<0.001	0.05	<0.001	<0.001	12.10	<0.001	<0.001	0.01	0.24	13.10	<0.001	<0.001	<0.001	0.87	23.44	<0.001	<0.001	<0.001	0.01
Order 4																			
9.50	<0.001	0.54	<0.001	0.01	2.47	0.10	0.10	0.31	0.97	20.90	<0.001	<0.001	<0.001	0.96	80.55	<0.001	<0.001	<0.001	<0.001
Order 5																			
0.99	0.39	0.95	0.81	0.35	12.00	<0.001	0.09	<0.001	<0.001	48.00	<0.001	<0.001	<0.001	1.00	62.54	<0.001	<0.001	<0.001	0.02
DLS		Post	Hoc		DMS		Post	Hoc		BLA		Post	Hoc		Cen		Post	Hoc	
F	*p*	CvP	CvR	PvR	F	*p*	CvP	CvR	PvR	F	*p*	CvP	CvR	PvR	F	*p*	CvP	CvR	PvR
Dendrite																			
1.00	0.36	0.57	0.35	0.92	8.20	<0.001	0.59	<0.001	0.02	5.81	<0.006	0.20	<0.001	0.22	0.17	0.84	0.99	0.89	0.89
Order 2																			
9.76	<0.001	0.04	<0.001	0.75	5.20	<0.009	0.93	0.01	0.03	19.23	<0.001	0.50	<0.001	<0.001	3.88	<0.028	0.76	0.03	0.13
Order 3																			
2.98	0.06	0.50	0.05	0.39	4.98	0.11	0.18	0.01	0.37	7.00	<0.002	1.00	0.06	0.06	11.95	<0.001	<0.001	<0.001	0.66
Order 4																			
11.86	<0.001	<0.001	<0.001	0.85	15.32	<0.001	0.14	<0.001	<0.001	10.92	<0.001	0.36	<0.001	0.01	14.59	<0.001	<0.001	<0.001	0.21
Order 5																			
23.95	<0.001	<0.001	<0.001	<0.001	595.00	<0.001	0.32	<0.001	<0.001	2.35	0.11	0.98	0.24	0.12	8.64	<0.001	<0.001	0.02	0.98
LO		Post	Hoc		VO		Post	Hoc		MO		Post	Hoc		ACC		Post	Hoc	
F	*p*	CvP	CvR	PvR	F	*p*	CvP	CvR	PvR	F	*p*	CvP	CvR	PvR	F	*p*	CvP	CvR	PvR
Dendrite																			
37.48	<0.001	<0.001	<0.001	0.90	20.57	<0.001	0.09	<0.001	<0.001	19.96	<0.001	0.81	<0.001	<0.001	20.63	<0.001	<0.001	<0.001	0.90
Order 2																			
8.73	<0.001	<0.001	0.10	0.11	10.69	<0.001	0.99	<0.001	<0.001	2.17	0.13	0.99	0.20	0.16	15.60	<0.001	<0.001	<0.001	0.59
Order 3																			
24.00	<0.001	<0.001	<0.001	0.76	17.63	<0.001	0.73	<0.001	<0.001	1.98	0.15	0.93	0.16	0.29	15.20	<0.001	<0.001	<0.001	0.14
Order 4																			
12.74	<0.001	<0.001	<0.001	0.90	35.78	<0.001	0.06	<0.001	<0.001	8.55	<0.001	0.77	0.01	<0.001	23.50	<0.001	<0.001	<0.001	0.02
Order 5																			
30.75	<0.001	<0.001	<0.001	0.14	43.37	<0.001	<0.001	<0.001	<0.001	7.10	<0.002	0.75	0.02	<0.001	41.90	<0.001	<0.001	<0.001	0.99
Hippo.		Post	Hoc		Insula		Post	Hoc											
F	*p*	CvP	CvR	PvR	F	*p*	CvP	CvR	PvR										
Dendrite																			
6.55	<0.003	0.99	0.01	0.01	7.45	<0.002	0.01	0.04	0.41										
Order 2																			
0.99	0.38	0.86	0.35	0.66	1.44	0.25	0.22	0.76	0.59										
Order 3																			
14.39	<0.001	<0.001	0.00	0.81	2.61	0.08	0.07	0.36	0.65										
Order 4																			
34.70	<0.001	<0.001	<0.001	1.00	12.13	<0.001	<0.001	0.03	0.06										
Order 5																			
25.22	<0.001	<0.001	<0.001	<0.001	8.04	<0.001	0.00	0.01	0.01										

Table 4. Quantitative changes between controls, predicted and randomly stressed rats, revealed by ANOVA in dendritic length in the above ROIs. Also shown are Post Hoc comparisons. CvP represents control compared to predicted group, CvR control compared to random group, and PvR predicted compared to random group.

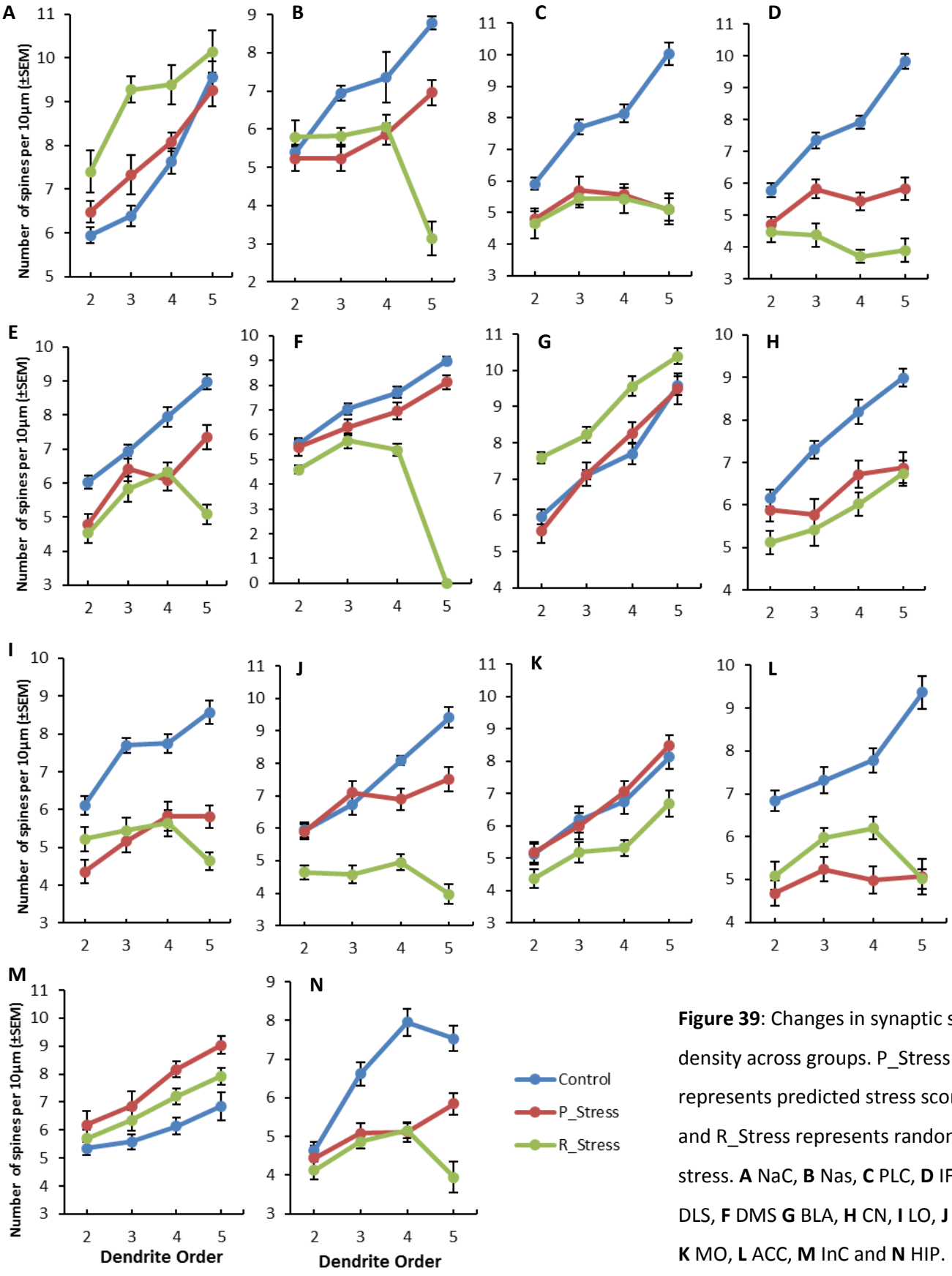

Figure 39: Changes in synaptic spine density across groups. P_Stress represents predicted stress scores and R_Stress represents random stress. **A** NaC, **B** Nas, **C** PLC, **D** IFC, **E** DLS, **F** DMS **G** BLA, **H** CN, **I** LO, **J** VO, **K** MO, **L** ACC, **M** InC and **N** HIP. Significant changes are seen at every dendritic order across all ROIs. Refer to Tab. 4 for values.

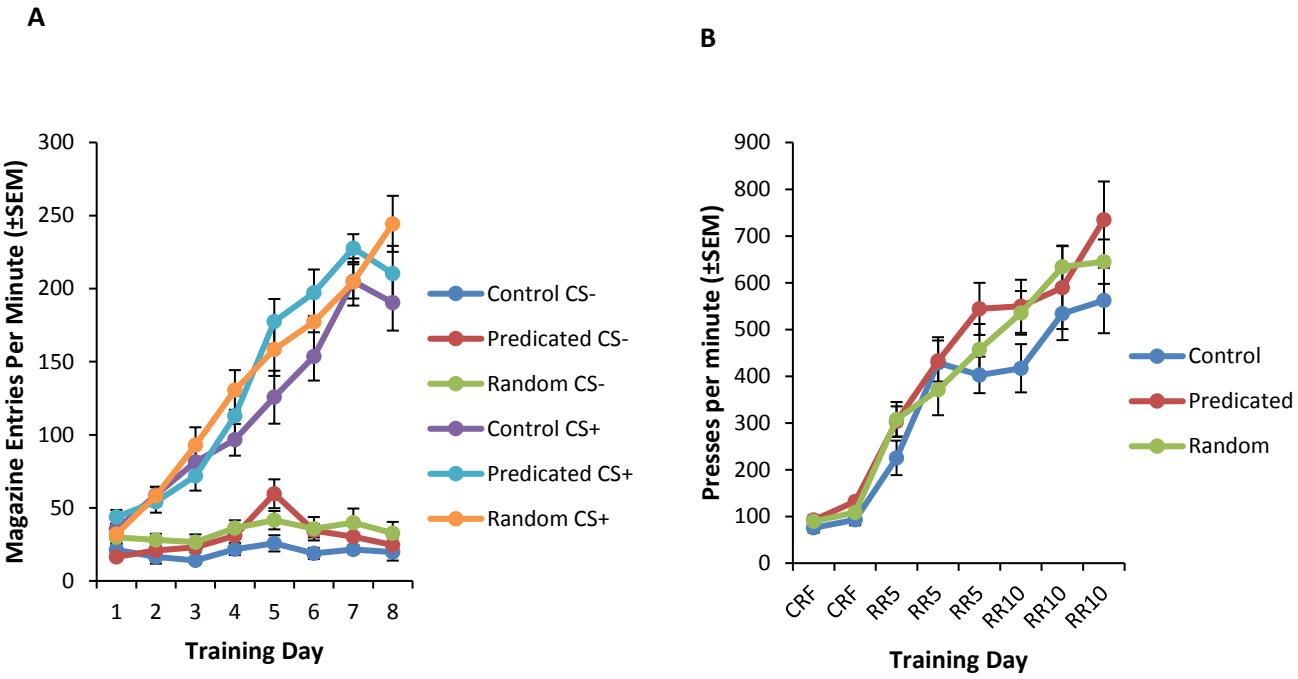

Figure 40. All groups learnt stimulus outcome and action outcome associations. **A** Pavlovian training, showing magazine entries during CS+ and CS-. **B** There was no difference between groups during instrumental training, all accelerated lever pressing as training increased. CS+ represents the period when the stimulus is presented and CS- during the prestimulus interval.

Correlations between morphology and behaviour

Presented below are a series of correlations performed between the dendritic lengths of control, predictable and random stress rats, of all the ROIs, against each rat's behavioural performance; for delayed discounting, correlations were made at both zero and 30s delay. Due to the exploratory nature of this thesis, the amount of comparisons being made dictated a family-wise error correction. As such a Bonferroni correction was performed to protect against committing Type-I errors, this correction changed the required alpha value for a significant correlation from $p<0.05$ to $p<0.004$, see Tab 6 for each of the resultant correlational significance scores. However, $p<0.05$ cut-off is still shown for speculative purposes to be elaborated in discussion. Each battery of correlations will be grouped by behavioural test, none or few correlations were found within reinstatement or delayed discounting and so are instead presented within the appendix.

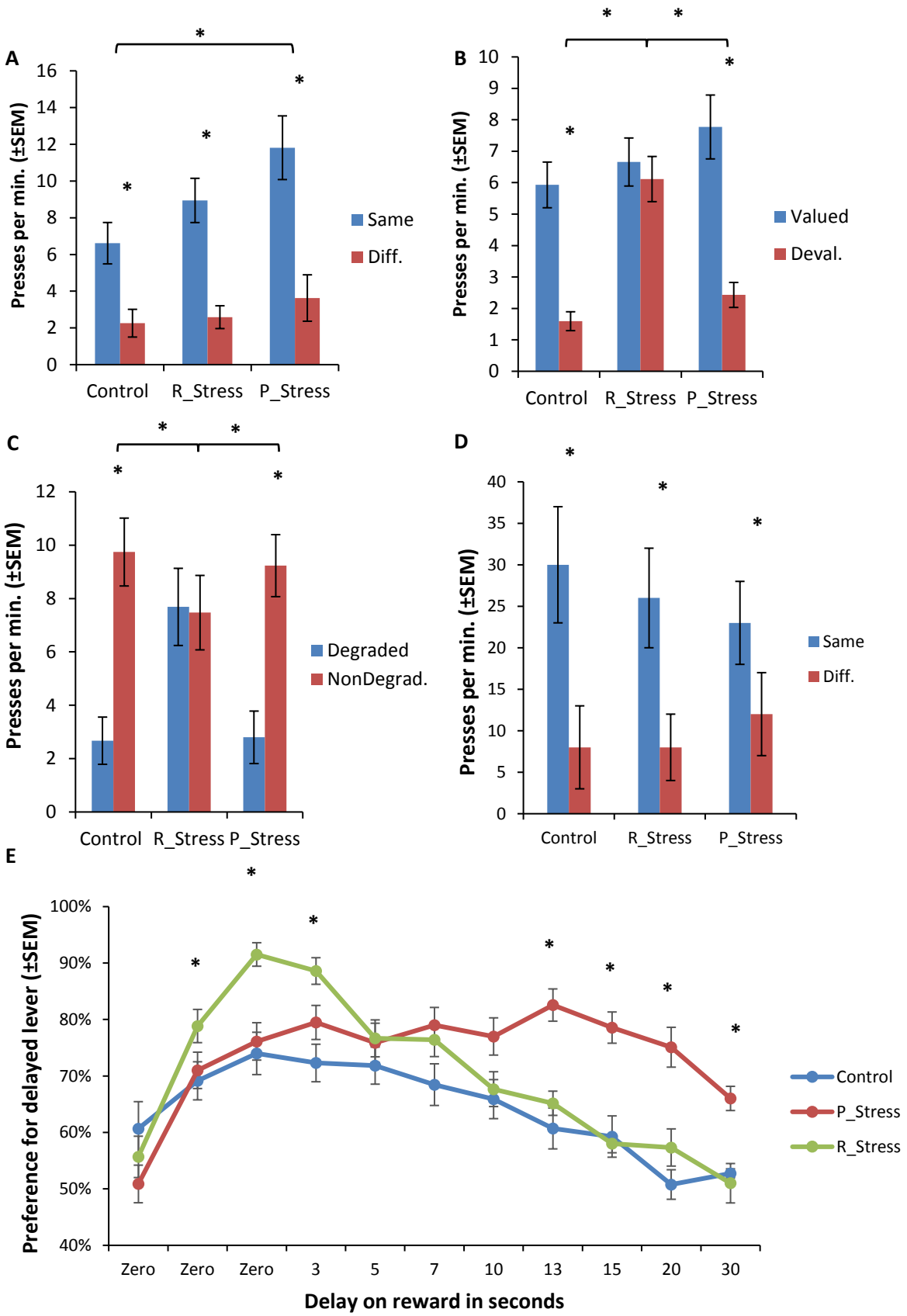

Figure 41. The five behavioural tests performed represented as histograms or a preference line graph. **A** PIT, **B** Outcome Devaluation, **C** Contingency degradation, **D** Reinstalment and **E** delayed discounting. Scores are means averaged across animals in each group and as lever presses per minute. * indicate a significant changes see Tab. 5 for further information. P_Stress represents predicted stress group, R_Stress represents random stress group.

PIT

	Simple Effects			Main Effects			Post Hoc		
	Control	P_Stress	R_Stress	Response	Group	Interaction	CvP	CvR	PvR
F (2, 27)	6.017	8.68	12.072	38.52	3.48	1.59	-	-	-
p	<0.025	<0.009	<0.003	<0.001	<0.045	<0.22	<0.036	<0.474	<0.33

Outcome Devaluation

	Simple Effects			Main Effects			Post Hoc		
	Control	P_Stress	R_Stress	Response	Group	Interaction	CvP	CvR	PvR
F (2, 27)	11.43	7.98	0.084	18.04	2.058	3.536	-	-	-
p	<0.003	<0.011	<0.77	<0.001	<0.147	<0.043	<0.641	<0.025	<0.026

Contingency Degradation

	Simple Effects			Main Effects			Post Hoc		
	Control	P_Stress	R_Stress	Response	Group	Interaction	CvP	CvR	PvR
F (2, 27)	6.93	8.02	0.06	18.47	1.062	5.134	-	-	-
p	<0.017	<0.011	<0.807	<0.001	<0.36	<0.013	<0.696	<0.011	<0.02

Reinstatement

	Simple Effects			Main Effects			Post Hoc		
	Control	P_Stress	R_Stress	Response	Group	Interaction	CvP	CvR	PvR
F (2, 27)	8.525	5.593	3.665	6.575	0.945	0.243	-	-	-
p	<0.009	<0.029	<0.039	<0.016	<0.945	<0.786	<0.947	<0.963	<0.998

Delayed Discounting

	Simple Effects			Main Effects			Post Hoc		
	Control	P_Stress	R_Stress	Response	Group	Interaction	CvP	CvR	PvR
F (2, 27)	25.85	72.99	19.25	8.407	1.298	2	-	-	-
p	<0.001	<0.001	<0.001	<0.001	<0.29	<0.008	<0.265	<0.59	<0.818

Table 5. ANOVA scores for each of the behavioural tests, illustrating simple and main effects of response, stress and interactions between the two. P_Stress represents predicted stress group, R_Stress represents random stress group. Post Hoc comparisons shown on the right, CvP represents control compared to predicted group, CvR control compared to random group, and PvR predicted compared to random group.

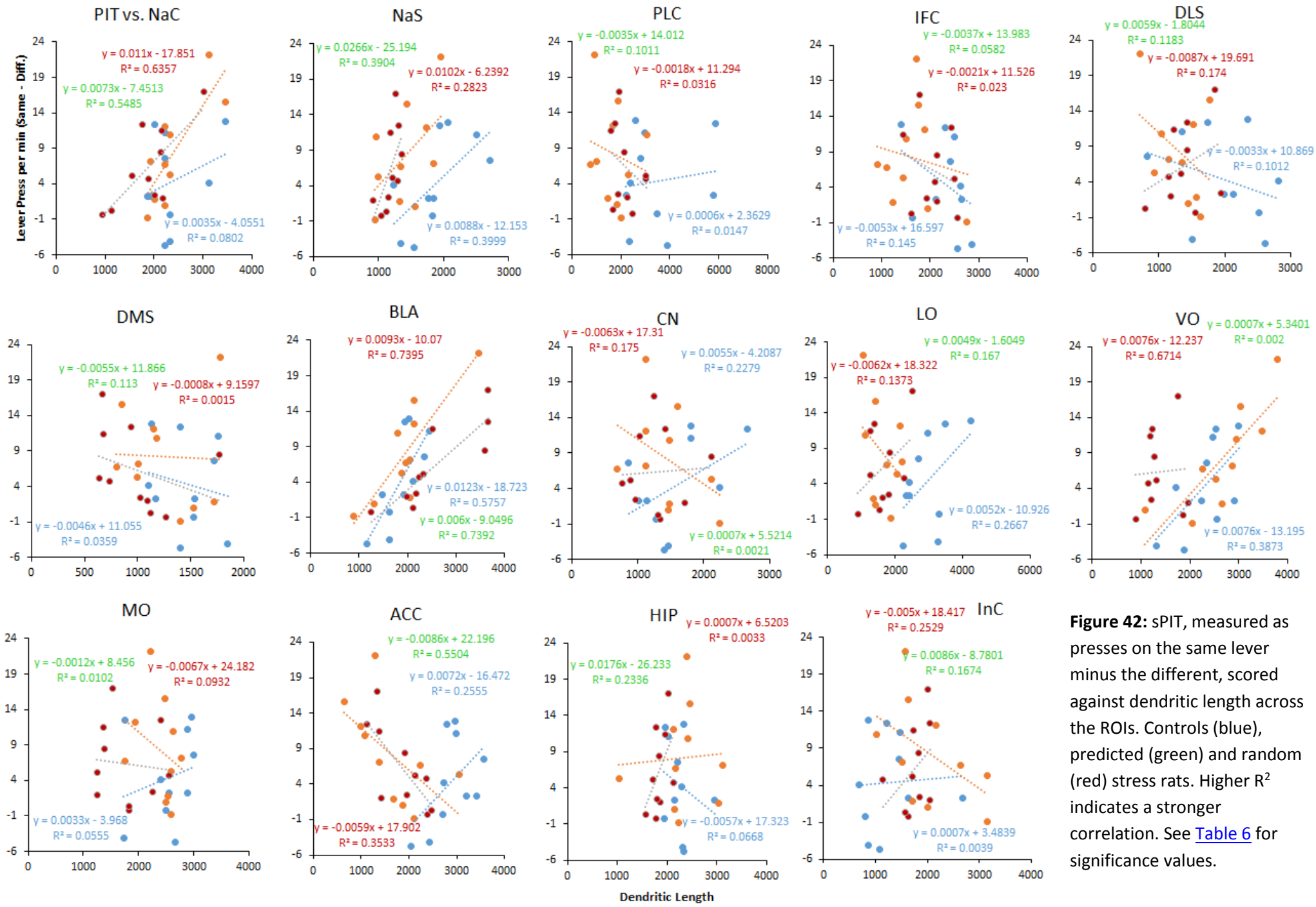

Figure 42: sPIT, measured as presses on the same lever minus the different, scored against dendritic length across the ROIs. Controls (blue), predicted (green) and random (red) stress rats. Higher R^2 indicates a stronger correlation. See Table 6 for significance values.

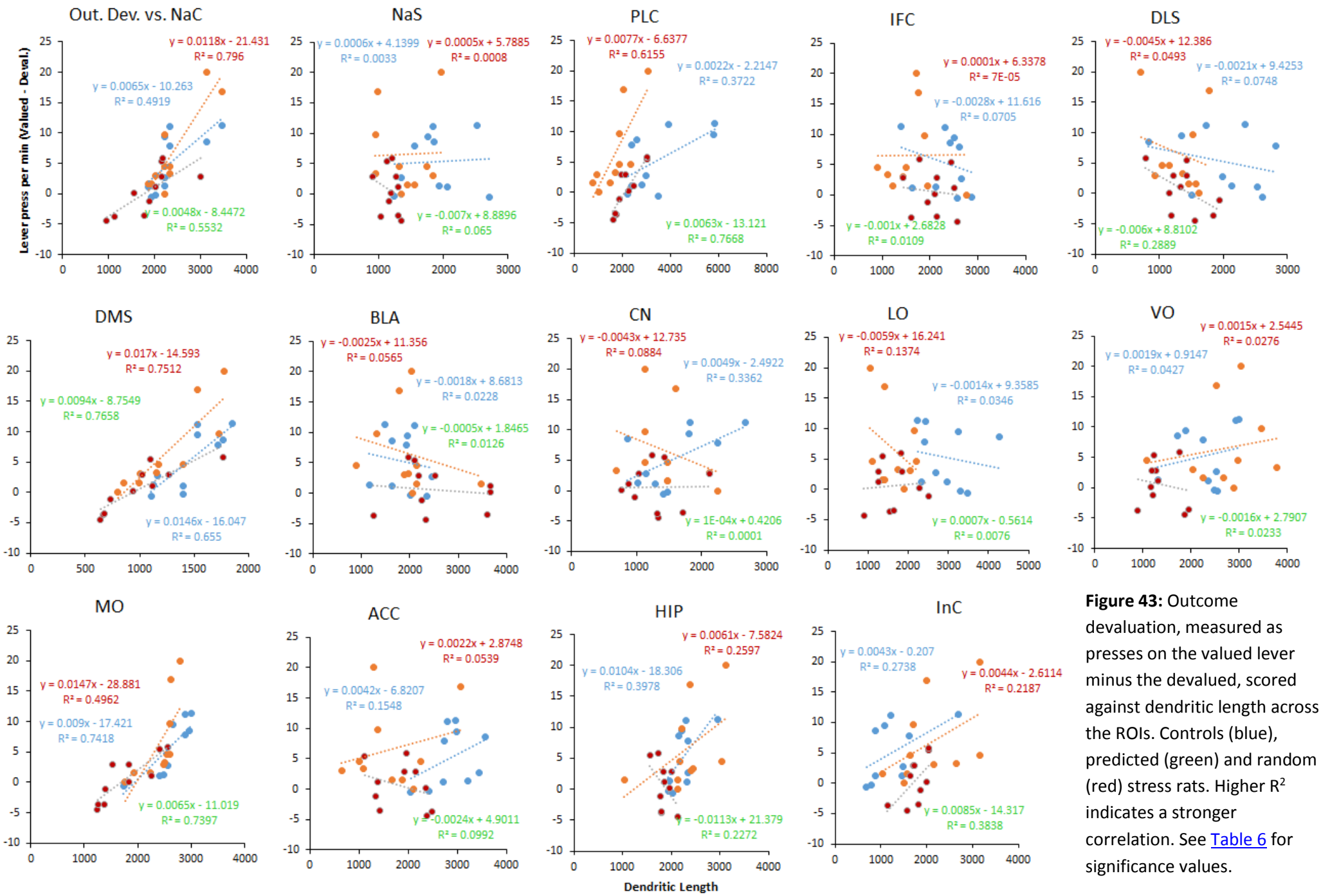

Figure 43: Outcome devaluation, measured as presses on the valued lever minus the devalued, scored against dendritic length across the ROIs. Controls (blue), predicted (green) and random (red) stress rats. Higher R^2 indicates a stronger correlation. See Table 6 for significance values.

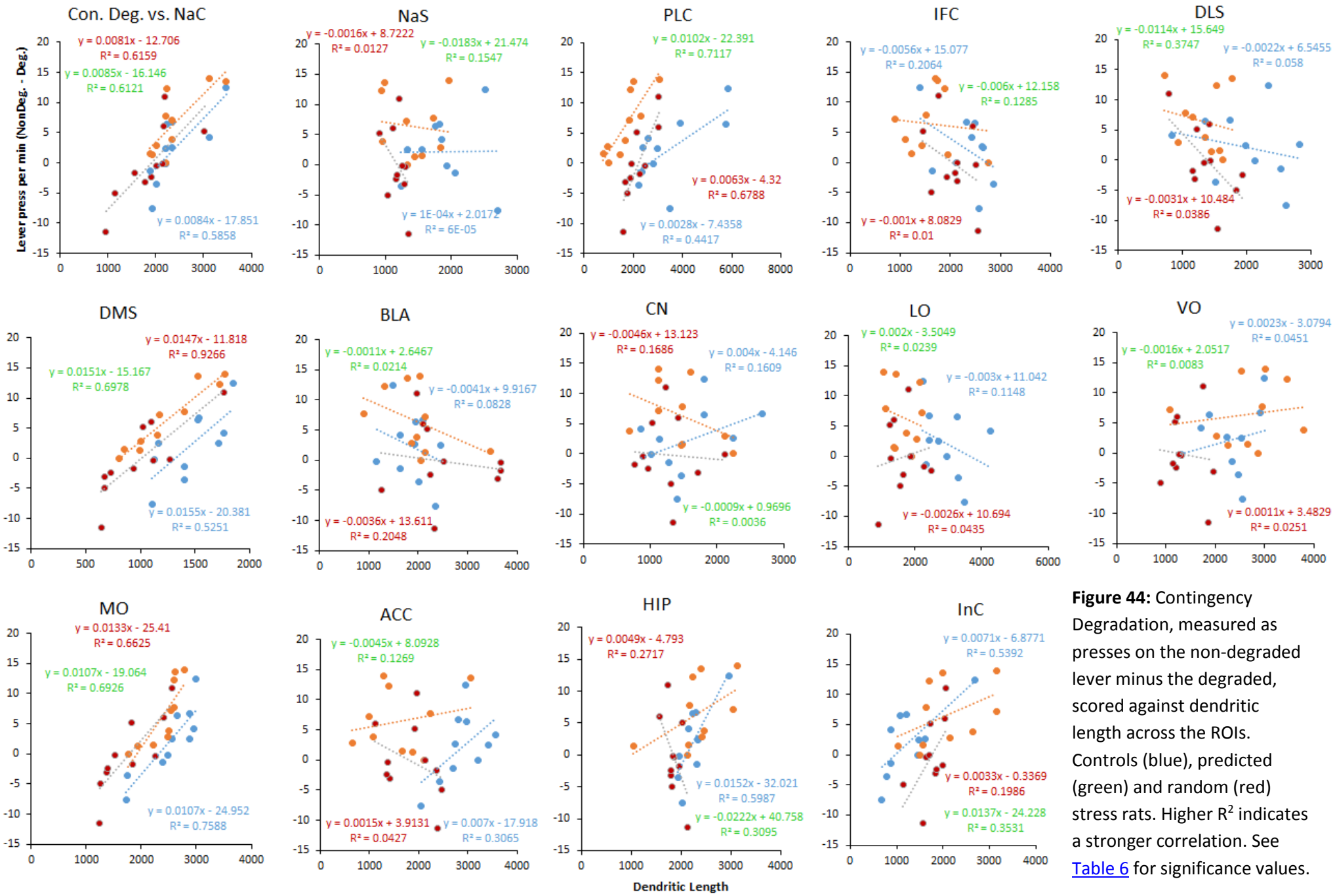

Figure 44: Contingency Degradation, measured as presses on the non-degraded lever minus the degraded, scored against dendritic length across the ROIs. Controls (blue), predicted (green) and random (red) stress rats. Higher R^2 indicates a stronger correlation. See Table 6 for significance values.

CONTROL	NaC	NaS	PLC	IFC	DLS	DMS	BLA	CN	LO	VO	MO	ACC	HIP	InC	Bonferoni
PIT	0.402	**0.043**	0.786	0.258	0.349	0.631	**0.009**	0.183	0.113	0.055	0.492	0.128	0.458	0.898	**<0.004**
OUT.DEV	**0.024**	0.874	0.061	0.458	0.445	**0.005**	0.676	0.079	0.607	0.567	**0.001**	0.261	0.051	0.12	
CON.DEG	**0.012**	0.995	**0.049**	0.171	0.547	**0.016**	0.383	0.225	0.297	0.577	**0.001**	0.096	**0.009**	**0.016**	
REINST	0.091	0.205	0.927	0.512	0.126	**0.001**	0.407	0.626	0.531	0.689	0.173	0.446	0.776	0.369	
DEL. DIS. Zero	0.698	0.564	0.209	0.112	0.216	0.083	0.274	0.095	**0.035**	0.999	0.89	0.957	0.075	0.363	
DEL. DIS. 30s	0.799	0.364	0.649	0.67	0.987	0.631	0.151	0.343	0.766	0.361	0.437	0.881	0.44	0.61	

PREDICTED	NaC	NaS	PLC	IFC	DLS	DMS	BLA	CN	LO	VO	MO	ACC	HIP	InC	**Unmodified**
PIT	**0.005**	0.119	0.623	0.664	0.253	0.896	**0.002**	0.219	0.274	**0.004**	0.382	0.064	0.85	0.13	**<0.05**
OUT.DEV	**0.001**	0.939	**0.007**	0.982	0.538	**0.001**	0.508	0.404	0.292	0.646	**0.023**	0.519	0.132	0.173	
CON.DEG	**0.006**	0.768	**0.004**	0.753	0.6	**0.0001**	0.207	0.25	0.526	0.649	**0.003**	0.535	0.113	0.195	
REINST	0.733	0.303	0.673	0.644	0.454	0.719	0.344	0.17	0.283	0.16	0.352	0.145	0.259	**0.003**	
DEL. DIS. Zero	0.941	**0.033**	0.653	0.486	**0.006**	0.549	0.834	0.829	0.149	0.413	0.075	0.515	0.466	0.989	
DEL. DIS. 30s	0.796	0.184	0.802	0.147	**0.003**	0.666	0.55	0.608	0.939	0.535	**0.003**	0.053	0.238	0.769	

RANDOM	NaC	NaS	PLC	IFC	DLS	DMS	BLA	CN	LO	VO	MO	ACC	HIP	InC
PIT	**0.014**	0.057	0.42	0.528	0.327	0.321	**0.002**	0.925	0.226	0.908	0.79	**0.016**	0.133	0.264
OUT.DEV	**0.014**	0.476	**0.001**	0.773	0.109	**0.001**	0.756	0.977	0.81	0.674	**0.001**	0.376	0.163	0.056
CON.DEG	**0.028**	0.957	**0.028**	0.143	0.569	**0.02**	0.339	0.149	0.233	0.505	**0.004**	0.203	**0.013**	**0.02**
REINST	0.531	0.816	0.911	0.946	0.36	0.449	0.661	0.371	0.529	0.918	0.414	0.815	0.188	0.878
DEL. DIS. Zero	0.962	**0.001**	**0.046**	0.104	0.831	0.498	**0.018**	0.793	**0.011**	0.193	0.924	0.967	0.702	0.888
DEL. DIS. 30s	0.172	0.42	0.354	0.805	0.86	0.565	0.1	0.265	0.923	0.053	0.137	0.119	0.471	**0.004**

Table 6: Significance scores for each of the correlations across all behavioural tests and ROI. Scores highlighted in red and bold are statistically significant correlations based on a Bonferroni correction for the amount of comparisons, those simply in bold are only significant to <0.05.

Chapter 4: Discussion

Through the research described in this thesis I developed the URG stain, a stain that works with modern tissue clearing techniques, and offers not only improved image quality with far less restrictions than its predecessor, but also hastens the clearing protocols (CLARITY & CUBIC) to work within a fraction of the time. I then used this new technique to measure and analyse entire neuronal morphology within a 3D environment, gathering the most informative morphological data. During Experiment 2 I used chronic stress as a treatment to manipulate neuronal morphology and decision-making. Morphological changes were seen across a number of regions as well as changes in animal decision making performance; stressed animals appeared to show, generally, a deterioration in morphology and to perform worse than controls on goal directed tasks such that their decisions appearing to be dependent on habits.

With further experimentation I was still not satisfied with the improvements of the URG, and I developed and optimized a novel method that used two -photon microscopy to image Golgi stained neurons, articulating the laser strength and intensity to cause florescence. This never before seen feature of the Golgi stain, offered comparatively increased resolution and 3D rendering power. Using this new method, we investigated neuronal morphology changes in chronically stressed animals in the context of controllability. We argued that predictability of a chronic stressor should protect the animal from the effects of stress in a similar way to the effects of controllability reported in particularly in the LH literature. Controllability had been shown to offer the animal a level of protection from uncontrollable stress, protecting the animal from the harmful effects of stress behaviourally. We went on to argue that the behavioural protection produced by predictability should be explicable by reference to protection from the changes in morphology normally induced by stress. Further, due to the limited literature, that these protective effects may even protect regions not previously reported as protected but reported as involved.

I found that rats exposed to predictable stress were protected against deficits in outcome devaluation and contingency degradation, that together provide the gold standard for assessing goal

directed action, and also protected against morphological changes specifically in the DMS, HIP and MO, with changes across many other regions, showing proliferation or degeneration. In this discussion chapter I will discuss the results in a similar order to the way they were introduced in Chapter 1, first addressing the new methods developed out of Experiments 1 and 3 and then the results of Experiments 2 and 4. During discussion of Experiments 2 and 4, I will look at morphological changes region by region followed by discussion of the behavioural results and their correlations. This thesis has a large battery of regions, and for the reader's convenience, the expected and resultant morphological changes are presented in the table below for reference.

Changes in Morphology Expected vs. Results

	Expected	Results	
ROI		Predicted	Random
NaC	Increased	Increased	Increased
NaS	Decreased	Decreased	Decreased
PLC	Decreased	Decreased	Decreased
IFC	Decreased	Decreased	**No Change**
DLS	Increased	**No Change**	**No Change**
DMS	Decreased	**No Change**	Decreased
BLA	Increased	Increased	Increased
CN	Decreased	**No Change**	**No Change**
LO	Decreased	Decreased	Decreased
VO	Decreased	**Increased**	Decreased
MO	Decreased	**No Change**	Decreased
ACC	Decreased	Decreased	Decreased
HIP	Decreased	**No Change**	Decreased
InC	Decreased	**Increased**	**Increased**

Table 7. Table comparing the expected direction of morphological change and the changes identified from Experiment 4. Changes which were not as expected, or where the predicted stress group differs from the random stress group are highlighted in bold.

4.1. CLARITY/CUBIC and Golgi

The URG method described here offers the advantage of highly detailed neuronal information within a 2-day period, instead of that offered by the standard rapid stain of 14 days (Gibb and Kolb, 1998, Spiga et al., 2011, Kassem et al., 2013, Levine et al., 2013). URG also has the advantage of delineating fewer neurons and fewer artefacts (Fig. 6), allowing for easier analysis and more precise measurement, which may otherwise be difficult due to overlapping or background staining of neuron or artefact present with the standard stain (see Fig. 4 and Fig 6). Further, URG has been shown to work well within PFA fixed tissue, which has reportedly been difficult to stain and image (Ranjan and Mallick, 2010, Ranjan and Mallick, 2012). Increasing the incubation temperature to 42^{o}C and increasing incubation time to 36 hours was found to facilitate metallic compound impregnation. Furthermore, as see in Fig. 8 these methods allow Golgi staining of neurons in tissue which has been pre-sliced to as thin as 100um; traditional Golgi stains are restricted to staining tissue before it has been sliced, otherwise resulting in an amount of artefact rendering the tissue useless (Levine et al., 2013). Additionally, the methods described here offer the opportunity to use the Golgi stain, which completely stains neurons capturing all their information from soma to synaptic spines in one clear image (Das et al., 2013) in conjunction with imaging techniques exclusive to or most effective in cleared tissue.

Variations on the URG Method - CLARITY

In the course of this experiment eight variations in the application of URG to CLARITY were reported, varied by (i) whether tissue was fixed or fresh, (ii) developed prior to or post clearing and (iii) whether the tissue was cleared via active of passive CLARITY. Noticeable differences between the various methodologies used were found, however, all were effective in delineating neuronal soma and general dendritic information. Nevertheless, as will be discussed, particular methods offered greater information. Staining and developing the tissue before performing a clearing technique

appeared to offer the greatest detail and delineation (Fig. 10 & 11). Images captured from CLARITY cleared tissue, where development was performed prior to clearing, offered the best quality images. Tissue that was developed following clearing, however, should be imaged via reflective imaging due to restricted visibility (Fig. 12c, d). This is due to the metal compound impregnation's development not being sufficient to stain the neuron to a level visible via brightfield, although the reflective properties of the metallic compounds have still impregnated within the neuron and are visible via reflective laser. When compared, passively cleared tissue offered greater detail and delineation while also requiring less resources and time (Fig. 10 & 11). Quality differences were not apparent between PFA fixed and fresh non-fixed tissue when observed after being cleared with CLARITY. A qualitative change between fresh and PFA tissue becomes apparent when the clearing technique is extended to CUBIC clearing.

Variations on Method - CUBIC

As seen in Fig. 13a, b, CUBIC cleared PFA fixed tissue offers the greatest level of detail compared to fresh. Fresh non-fixed tissue fails to delineate dendrites adequately, however still offers somatic and synaptic spine information. CUBIC cleared tissue was easier to clear as compared to the CLARITY methods requiring only submerging the tissue within reagent 1 to clear over two days. CUBIC cleared tissue poses an unforeseen problem, it became apparent that the URG stain within the CUBIC cleared tissue decays over time. Within 1 week the staining became faint and weak. By the end of a two-week period the stain had completely been removed. CLARITY cleared tissue does not pose this problem. However, this fading may be advantageous if there is a requirement to remove the stain from the tissue, dependent upon a researcher's requirements, if this is not desired, prompt imaging is necessary. Arguably, it may be the urea components of reagent 1 that lead to the deterioration of the stain; the protein denaturant effect of urea (Tanford, 1968) could cause a breakdown of the secondary and tertiary structure of the cell, potentially causing the de-impregnation of the metallic compounds.

Imaging

The use of both the URG and clearing techniques, allows the use of lightsheet imaging techniques to their full effectiveness. By using the same methods as a reflective laser, we use the lightsheet to visualise the neurons via reflecting the lightsheet off the metallic compounds of the URG stained neurons. Lightsheet offers the same detail as confocal imaging within a much larger image, allowing for 3D reconstruction with immense detail (see Fig. 13c & Sup. Fig. 2). Fig. 13c offers detail and neuronal delineation to the same level as the methods described to achieve images in Fig. 10 and 11. The use of lightsheet imaging to visualise neurons is still quite recent, and its use within cleared tissue is becoming more prevalent within the literature. With the advent of these new techniques we are now able to visualise entire populations of neurons with a morphological detail and accessibility previously unmatched.

One of the most fascinating aspects of the development of these methods was the finding that, when combined with the URG method, both CLARITY and CUBIC techniques required only 48 hours to clear the URG stained tissue. Further, due to this effect, active and passive clearing times did not ultimately differ. Chung et al. (2013) describes how several days are usually required for CLARITY to clear tissue, and CUBIC has been described as needing up to 14 days (Susaki et al., 2014, Susaki et al., 2015). It is conceivable that the ammonium hydroxide and sodium thiosulphate used during development may play roles as the ammonia has properties of urea (required for CUBIC) and sodium thiosulphate has the desired refractive index required for tissue clearing. If this were the case, however, then tissue developed after clearing would not clear as quickly because the ammonium hydroxide and sodium thiosulphate are only present during development, and the time required for clearing the tissue did not differ whether development was conducted prior to or following clearing. Alternatively, it may be the potassium chromate and dichromate present within the Golgi stain. These chromates bind to cells during a Golgi-Cox stain (Das et al., 2013), and the URG stain is no different. These chromates likely produce protein vulnerability when they bind to the cells; protein vulnerability is a pre-step that potentially weakens the protein bonds to more readily allow protein

denaturation and lipid removal when clearing techniques are applied. If the chromates are in fact damaging the proteins as described the stain itself would act as a pre-step and hasten the clearing process. This aspect of the URG stain needs to be further investigated as its potential to improve clearing techniques may be of great future use.

URG allows researchers to stain fresh and PFA fixed tissue by increasing incubation temperature to 37°C or 42°C respectively, offering detailed visualisation of neurons. Further the URG stain allows facilitation of current tissue clearing techniques, in addition to staining entire neurons within cleared tissue, improving investigative power, 3D reconstructions and a clearer understanding of neuronal morphology. From all the methods described herein, for the best results independent of fixation procedure, the recommendation would be to stain and then develop the tissue followed by clearing using CLARITY via the passive clearing method.

4.2. Two-Photon and Golgi

I described in Experiment 3 a novel means if visualising URG stained neurons using a two-photon laser, capturing two-photon laser excited photoluminescence from mercuric sulphide crystals impregnated within the cell. This ability to visualise neurons via photoluminescence through the use of a two-photon microscope offers the user the advantage of increased depth, detail, point spread function, and resolution, while reducing background staining visibility (see Fig. 29 and 30). Further, the 3D reconstructions created from these clearer higher resolution images offer greater insight into neuronal morphology than typically achieved via conventional microscopy, particularly when attempting to visualise synaptic spines (see Fig. 27 and 32, and Sup. Fig. 4 and 5). Additionally, the ability to see synaptic spines by eye in planes of view normally hidden through bright-field imagining offers more precise measurement of spine density (see Fig. 30 and 31), potentially allowing the user to see changes in spine density or morphology that would traditionally be missed. Some care must be taken when utilizing this method however, as severe tissue damage is a possibility with higher laser

intensity levels. We utilised the two-photon laser at 690nm at power levels optimally less than intensity 0.5mW, as higher power levels (3mW>) were found to photo-damage and burn the tissue (Fig. 28). Through the use of these new methods we believe we were able to get more accurate morphological information about the neuron, its dendrites and synaptic spines.

Use with Tissue Clearing Techniques

Like Experiment 1's URG stain, the methods described for Experiment 3 can be applied to tissue clearing techniques in the same way except that imaging is conducted via the methods required for two-photon. Use of this method with tissue clearing techniques allows even greater depth of view into tissue. CLARITY, among other modern clearing techniques, has shown to completely clear tissue (Chung and Deisseroth, 2013, Chung et al., 2013, Susaki et al., 2014, Susaki et al., 2015), allowing two-photon lasers to penetrate greater than 5mm into tissue while still retaining very high resolution (Richardson and Lichtman, 2015). Applying this clearing method will allow researchers to use more intact tissue, requiring reduced or even no sectioning. Further to this, although not applied in Experiment 3, like in Experiment 1, lightsheet imagining could take this new method even further. As discussed previously, lightsheet is a technique that allows for larger visual fields to be imaged at once (Dodt et al., 2007). Through the use of planar excitation as opposed to laser excitation (Eberle et al., 2015, Richardson and Lichtman, 2015), light-sheet imaging illuminates an entire plane at once, making imaging of large tissue samples exceedingly fast, as compared to confocal and two-photon imaging techniques (Becker et al., 2008, Becker et al., 2013). Light-sheet imaging allows for structural and whole tissue 3D imaging, illustrating regional changes and morphology (Dodt et al., 2007, Becker et al., 2008, Becker et al., 2013, Eberle et al., 2015). With the advent of two-photon lightsheet imaging (Planchon et al., 2011), whole cleared brains could be visualised via photoluminescence while still having the high detail offered by Golgi stained neurons. The combination of these techniques would allow users to visualise entire tissue samples and analyse

regional information in a 3D environment at a much higher resolution due to a better point spread function.

Future Applications

As discussed in more detail in subchapter 1.3, the use of photoexcitation on metals is not inherently novel, however, the application of photoexcitable metals in a histological context is. The use of other metal compounds when coupled with these methods could potentially allow for many different histological applications. A traditional silver stain, predecessor to the modern Golgi-Cox stain, would be expected to look similar to the more modern Golgi stain, however, the luminescent properties of the metallic compounds will be different. The infancy of this concept allows for broad ideas for new histological techniques to be proposed. One such idea would be the application of gold nano-clusters. The unique molecular behaviour of gold nano-clusters could be used to penetrate cells, or specific features of the cells, then when excited by 330 nm wavelength light, the particles will emit at 476 nm (Matulionyte et al., 2015). Matulionyte et al. (2015) goes on to explain that the low toxicity level of the gold nano-clusters makes it appropriate for biological material, should it ever see use within biological labs, this quality makes gold a prime example of a metal compound that may yet be used in future histology research. Further, the application of any other photosensitive metal compounds may reveal completely novel cellular staining and binding, which, when photoexcited, would luminate at a different wavelength to mercury, allowing for double staining; gold and mercury could, for example, be visualised at different wavelengths within the same sample, at 476 nm and 620-670 nm respectively. Additionally, unlike traditional fluorescent stains, the photoluminescent properties of the Golgi stained neurons will not fade over time nor will they photo-bleach, a common disadvantage of fluorescent histology which makes conservation of the stain difficult (Wilson and Bacic, 2012, Longin et al., 1993, Gill, 1979, Amor et al., 2016, Bolognesi et al., 2016, Jacobson et al., 1976).

The Golgi stain continues to reveal novel imaging applications 100 years after its invention. The superior delineation of neuronal morphology offered by the Golgi stain is still desirable and, with the use of two-photon imaging, the superior depth and image clarity allows for novel and exquisite photoluminescent neuronal imaging. The use of the described methods with modern clearing techniques and lightsheet imaging will allow high morphological resolution deep within tissue.

4.3. Morphological Effects of Stress

In this section I will discuss the physical effect of chronic stress on rats found in Experiments 2 and 4. Beginning with the effect of stress on weight loss and on morphological changes in the HIP and ACC, I will proceed through the remaining ROIs, discussing the effect of chronic stress on dendritic and synaptic spine morphology and, if any, on differences within each ROI between the random and predictable stress treatments with reference to Tab. 7 and the expected changes to morphology.

Primary Indicators: Weight loss and HIP and ACC

As presented in the results, chronically stressed rats in Experiment 2 and random chronic stress rats within Experiment 4 both showed significant weight loss by the end of the CRS paradigm, indicating that this stress treatment was effective relative to the controls and, in the case of Experiment. 4, the predictable stress group. Weight loss has been used as an indicator of chronic stress since the beginning of chronic stress study research and the weight loss presented here is congruent with the weight loss reported in the literature (Sousa et al., 1998, Watanabe et al., 1992b, Coburn-Litvak et al., 2003, Conrad et al., 2004, Kassem et al., 2013). However, so as to increase reliability but also ensure a wider range of measures with which to differentiate the random and predictable stress groups of Experiment 4, we also used morphological measurements of neurons in the HIP and ACC because these regions have been consistently reported to degenerate following

chronic stress (Alfarez et al., 2003, Alfarez et al., 2008, Brunson et al., 2001, Conrad et al., 2004, Conrad et al., 1999, Donohue et al., 2006). Consistent with this, in Experiment 2, stressed rats showed a decrease in HIP and ACC dendritic length and synaptic spine density. Similarly, in Experiment 4, random stress rats showed this same effect compared to control and predictable stress. Interestingly however, the HIP was protected against degeneration within the predictable stress group, and I will elaborate on this below. The decreases in the ACC and HIP across both experiments in stressed animals are indicative of successful treatment, as similar changes have been observed in other studies (Chen et al., 2009, Cook and Wellman, 2004, Kassem et al., 2013, Perez-Cruz et al., 2007, Radley et al., 2005, Radley et al., 2008, Shansky et al., 2009a). It should also be noted, as discussed in subchapter 1.7, that Yang et al. (2015) found in his study of controllability, that IS rats showed reduced HIP spine density compared to ES and control rats. Similarly, significantly decreased HIP spine density was found in our random chronic stress group (arguably analogous to IS rats) compared to controls and the predictable stress group (analogous to ES).

As mentioned, there are changes within ROIs that the predictable stress group shares with controls and others that it shares with the random stress group. What this emphasises is that the predictable stress group differs from both the controls and random stress rats in particular ways. Predictable stress produced no degeneration within the HIP, indicative of a protective effect, more of which are presented and elaborated below. When we look at the HIP region individually, predictable stress rats present morphologically as controls, however, there are other regions for which making stress predictable is not protective; i.e., the predictable stress rats present as random stress rats. We can argue from this that, because the predictably stressed rats are showing degeneration of the ACC, that the treatment was effective and the rats were adequately stressed, but, due to the protection from weight loss and change in HIP morphology, that predictably stressed rats are significantly different from their random stress counterparts. This is supported by, the protection that making stress predictable provides to changes in decision-making, which will be discussed subchapter 4.4. We will now proceed to discuss the morphological changes in the other ROIs.

Prelimbic Cortex

In prelimbic cortex chronic stress decreased dendritic length and synaptic spine density regardless of its predictability across both Experiments 2 and 4 (See Figs. 20 & 38). The predictably and randomly stressed rats did not differ in dendritic length and spine density (See Tab. 4) and both showed reductions relative to the unstressed control group. The decreases in the PLC observed in this study are mirrored in previous research (Radley et al., 2004, Radley et al., 2006, Cerqueira et al., 2007b, Dias-Ferreira et al., 2009), showing similar dendritic decreases of approximately 40%. And the results are also somewhat mirrored in the only similar analogous morphological study of controllability, where a decrease in synaptic spine density was found in IS (analogous to random stress rats) compared to ES (analogous to predicted stress) and controls in the PLC (Yang et al. 2015). Although both random and predicated stressed groups showed similar levels of degenerated in Experiment 4, a larger decrease in the random stress group was still evident, and at least does not conflict with the idea that decreases in the PLC exist following uncontrolled stress. The failure of the predictable stress group to replicate the effect of ES in the Yang et al. (2015) paper may be due to the different paradigm, or that predictability was not sufficient to protect the PLC from the effects of 21 days of stress.

Infralimbic Cortex

Chronic stress treatment significantly reduced dendritic length in all stressed groups in Experiments 2 and 4. (See Figs. 20 & 38) and reduced synaptic spine density in both groups relative to controls (See Figs. 21 & 39). Although random stress in Experiment 4 did not produce significant decreases in dendritic lengths compared to controls, the spine measurements for this group indicate degeneration and the dendritic lengths had a downward trend, although did not differ from controls or predictable stress groups. We must remember that morphological measures in the PLC and IFC were expected to decrease, as years of chronic stress work have indicated decreases in the PFC as a whole (Bennett, 2011a, Bennett, 2011b, Brown et al., 2005, Cerqueira et al., 2007a, Cook and

Wellman, 2004, Radley et al., 2005, Radley et al., 2006, Radley et al., 2008, Radley et al., 2004, Shansky et al., 2009a), and specifically the IFC (Cerqueira et al., 2007b, Shansky et al., 2009b). Nevertheless, we must report that, although there is trending dendritic lengths and blatant degeneration of synaptic spines in support of this, our morphological measures within the random stressed group do no confer with previous literature. Out of the numerous possibilities that could have caused this unlikely shift from the literature, I would argue that it is an artefact of statistical power.

Orbitofrontal Cortex

In Experiment 2, stressed rats exhibited decreased dendritic length and synaptic spine density in the LO and VO compared to controls. In Experiment 4 we found a similar result in the random stress group, a decrease in dendritic length and synaptic spine density, but now also inclusive of the MO. This is as predicted, we expected the regions of the OFC to decrease as indicated by the literature, and divided the regions into it various sub-regions for explorative work, as little to no research has looked at the effects of chronic stress on OFC subregions. The novel morphological results reported here, are supported by the many investigations into the degenerative effect of chronic stress on neuronal morphology within the OFC (Cerqueira et al., 2007a, Goldwater et al., 2009, Perez-Cruz et al., 2007, Radley et al., 2006, Radley et al., 2008, Radley et al., 2004, Shansky et al., 2009a). The results are congruent with the one study which did delineate subregions (Liston et al., 2006), which showed decreases in the OFC, which upon inspection, were regionalised to the LO. As no other study has attempted to delineate OFC subregions for morphological study within stressed animals, the results presented here offer a novel set of results.

The predictable stress group showed similar degeneration to dendrites and spines in the LO as the random stress group, but was protected from degenerative changes in the MO, and actually showed an increase in these morphological measures in the VO relative to controls. This range of change within subregions is both interesting and completely novel. It reinforces the heterogeneous

nature of the OFC, as well as the complexity of the effects of stress on neurons within the OFC. Only via the interaction of prediction and chronic stress can these divergent morphological changes within sub-regions be observed. It was expected that predictability would mitigate the morphological changes caused by chronic stress. And as will be discussed, there are other regions that have also been protected from these changes. Interestingly we found that the MO in predictably stressed rats is morphologically similar to the controls, and as will be discussed, these rats performed similarly to controls on measures of decision-making. The degeneration of morphology in the LO, is congruent with that seen in the random stress group and indicates that this subregion is likely not sensitive to the predictability of stress. More interestingly still, the VO showed an increase in morphological measures when stress was predictable, indicating that prediction and chronic stress interacted in a unique way within this subregion. This increase was significantly different from control, and the random stress group, which showed a decrease.

Dorsomedial Striatum

Dendritic length and spine density decreases were found in the DMS following chronic stress in Experiment 2 and in Experiment 4's random stress group (Figs. 20, 21, 38 and 39). These decreases in morphology were as expected. Importantly, however, these decreases were ameliorated in the predictable stress group; in Experiment 4 only the random stress group showed reduced dendritic length and synaptic spine density. This change in the DMS is contradictory to the increase found by Taylor et al. (2014), but in line with the downward trend observed by Dias-Ferreria et al. (2009). In addition, it correlates with the translational work conducted by Blix et al. (2013), which showed that people suffering from chronic work related stress had reduced grey matter volumes of the caudate and putamen, which is indicative of dendritic atrophy (Kassem et al., 2013).

This is another region that is protected against the detrimental effects of chronic stress on morphology. It would appear the protective effect of controllability may translate to predictability; the effects observed in Experiment 4 are consistent with those reported by Amat et al. (2014), and

their argument that the DMS is crucial to the protective effects of controllability observed in ES rats. We observed that the DMS in the predictable stress group was healthy and not significantly different from controls (Tab. 4). We also observed that the protective effect of predictability in this region extended to goal directed performance in the behavioural tasks; the DMS is crucial for outcome devaluation and contingency degradation, and on both of these assessments the predictable stress group performed similarly to controls. This is the first study to report morphological changes associated with control/predictability over a stressor, and the protective effects appear to occur across several regions.

Dorsolateral Striatum

We observed no significant change in dendritic length within the DLS across any group in both Experiments 2 and 4. There were synaptic spine decreases in both Experiments 2 and 4 observed sporadically within both predicted and random stress groups and this degeneration of synaptic spines may have been indicative of the beginnings of dendritic atrophy (Bennett, 2008, Bennett, 2009, Bennett, 2010, Bennett et al., 2010, Kassem et al., 2013). But these changes did not reach significance; as seen in Fig. 20, there is a visual trend in dendritic length, but it is small. The receptivity to degeneration within the DLS may be restricted to synaptic spines, or an even more severe stress paradigm may be required to see dendritic change. With morphological change restricted to the synaptic spines, and those changes not consistent within the predicted or random stress groups, it may be that the DLS is not as sensistive to chronic stress as previously thought. This would be in contradition to the increases in the DLS dendritic length reported by Dias-Ferreira et al., (2009); however, they failed to observe any synaptic spine changes, which would normally indicate a shift in dendritic morphology, and which were, ironically, reversed in our study; i.e., we saw sysnaptic spine changes and no dendiritc changes. Further these results do not clearly translate to human studies (Blix et al., 2013), where a decrease in grey matter might be expected.

Basolateral Amygdala

As discussed in subchapter 1.4, stress has been reported to induce increases in dendritic length and synaptic spine density in the BLA. We expected to see similar proliferation of neuronal morphology and this is what we observed (Figs. 20, 21, 38 and 39). This is in line with what was hypothesised and the literature (Mitra et al., 2005, Vyas et al., 2006). Specifically, Padival et al. (2013) showed that chronic stress proliferated synaptic spines within the BLA, most prominently along dendrite orders 2-4, and although we found a constant increase in synaptic spine proliferation as the orders ascended, spine proliferation did not begin until order 2. The increased proliferation in the BLA in the random stress group relative to the predictable stress group is, again, best interpreted in terms of the protective effects of making the chronic stressor predictable. It is, however, open to question how predictability has this effect, this will be discussed in subchapter 4.6.

Central Nucleus of the Amygdala

As morphological literature is lacking on dendritic and synaptic spine measures within the CN following chronic stress, these results are novel. However, from previous literature a degeneration of morphology within the CN was expected, as the literature indicates increased CRH and GC concentrations within the CN following chronic stress. Nevertheless, in contrast to this prediction, both Experiments 2 and 4 revealed no change in morphology within the CN following stress (Figs. 20 & 38). This failure to observe an effect of stress does not entirely conflict with previous literature because there are comparable reports of changes in the CN following chronic stress and the way CRH and GC interact within the CN may be different to that of other regions where it would be expected to cause degeneration. Further, the failure to see an overall change in morphology may have been due to the fact we sampled from the whole CN and it is possible that subregions within the CN are differentially affected by stress, this will be expanded on in subchapter 4.6.

Nucleus Accumbens Core

As discussed in subchapter 1.4, morphological measures within the nucleus accumbens are conflicting and do not use consistent stress paradigms; the literature indicates that chronic stress can cause increases, decreases and no effect on morphology but these findings have emerged from, differing stress paradigms to the one used here. We argued previously that Bessa et al's (2013) preparation (CMS) is the most similar of those used in the literature the one used here (CRS) and, as they reported a proliferation in NaC morphology, we expected the same. As seen in Figs. 20, 21, 38 and 39, we did in fact observe such an increase in dendritic length and synaptic spine density in chronically stressed rats in Experiment 2. And, regardless of group, in Experiment 4 both the predictable and random stress groups showed increased measures of morphology compared to controls; although the random stress group only trended towards an increase in dendritic length ($p<0.1$), the spine densities were significantly increased. Interestingly, across both experiments and in both the predicted and random stress groups, morphological proliferation following chronic stress appeared to specifically be targeting the medial dendrites (Orders 2-4), where the most significant increases in spine density were observed. This conceptually mirrors the proliferation observed by Padival et al. (2013), as described above, where they found that increases in synaptic spine density within the BLA were most prominent on order 2-4. Similarly, the increases in spine density reported here are across orders 2-4. It may be, unlike degeneration which targets the most distal orders first, and works its way towards the soma, that proliferation targets medial dendrites first, with the proliferation of synaptic spines and dendritic length radiating distally and proximally from there.

Nucleus Accumbens Shell

In contrast to the NaC, NaS morphology was found to decrease following chronic stress in Experiment 2 and, in Experiment 4, across both predictable and random stress groups. This degeneration of morphology is loosely contradictory to what we expected, and the observations reported by the Bessa et al. (2013) paper. We accepted that the CMS paradigm used by Bessa et al.

(2013) was the closest analogy to our CRS paradigm and, as such, tentatively expected a proliferation of NaS. Bessa et al. (2013) didn't find any increases in spine density, only dendritic length. It is possible that the CMS paradigm used by Bessa et al. (2013) differed sufficiently from the CRS one used in these experiments and that the latter is more intense

Regarding the limited literature relating controllability to changes in morphological changes, the changes, as already discussed, in the Yang et al. (2015) paper demonstrate a proliferation in nucleus accumbens in IS rats compared to ES rats and controls. Neverthless, as Yang et al. (2015) did not differentiate between the NaC and NaS, this change is difficult to interpret. Comparing spine density within the NaS, the most distal dendrites of predicable stress rats appear marginally more protected against degeneration than in the random stress rats, as we observed degeneration to the extent that order 5 dendrites disappeared whereas predictably stressed rats at least maintained order 5 dendrites (Fig. 39). Alternatively, it is possible that Yang et al. (2015) sampled more heavily from border regions. We reported proliferation in the NaC and degeneration in the NaS and it is possible that the proliferation in Yang et al (2015) was related more to the NaC than the NaS.

Insula Cortex

Experiment 4 revealed that chronic stress, whether random or predictable, caused proliferation in InC morphology and increased both dendritic length and synaptic spine density. Nevertheless, on closer inspection of the spine morphology, it can be seen that the predictable stress group had a stronger proliferation effect, illustrated by the significantly increased synaptic spine density across dendritic orders 3-5. This is now another region where chronic stress causes a proliferation in neuronal morphology (the others being the BLA, NaC). As previously discussed, rodent research is completely lacking within the realm of morphological changes in the InC in relation to chronic stress, or even acute stress for that matter. From human studies, sufferers of PTSD and major depressive disorder show reduced grey matter volumes in the InC (Chen et al. 2006, Horn et al. 2010, Herringa et al. 2012), which contradicts the results seen here. This may be the result of

different stressors, different degrees of prior experience with stress, or a failure for this change to adequately translate from humans to rodents. Further research is required into the morphological changes induced by chronic stress in the InC so as to validate these results.

Summary

Chronic stress, as has been commonly observed in the literature and as extended in the novel results presented here, changes neuronal morphology in many structures across the brain. With one exception out of the battery of regions analysed (the CN), chronic stress typically either caused degeneration of dendritic length and synaptic spine density or proliferation in these features in the BLA, NaC and InC. Firstly, as expected, chronically stressed animals showed degeneration of the HIP, ACC, PLC and IFC, although noting that little literature differentiates the PLC and IFC from the PFC. The morphological measures taken in the InC are completely novel, with no previous research investigating such changes in chronically stressed rats. Furthermore, pioneering morphological data was presented for the LO, MO and VO subregions of the OFC, illustrating a heterogeneous effect of chronic stress treatment in the OFC not previously observed.

These results were largely confined to randomly stressed rats. In the predictable stress group, the rats that could predict the stressor were largely protected from the changes in morphology caused by chronic stress. This was particularly true in the HIP, MO and DMS of the predictable stress group which appeared largely unaffected by the stress treatment and were morphologically similar to controls. In other cases, particularly the BLA and the VO, the effect at least differed from the random stress rats. Predicted stress rats were not completely protected however. In many structures the effects of chronic stress were morphologically the same as random stress, particularly in the NaS, PLC, IFC, ACC and LO. So although there was protection, and decision-making appeared to be protected too (see below), there was not complete protection.

There is still room for further explorative study however, of particular interest would be morphological study of the ventral tegmentum area (VTA), dorsal raphe nucleus (DRN) and thalamus,

which are involved in decision making and serotonergic and dopaminergic connections to the aforementioned ROIs (Balleine et al., 2007, Bradfield et al., 2013b, Chandler et al., 2014, Corbit et al., 2007, Feduccia et al., 2012, Haber and Knutson, 2010, Sirigu and Duhamel, 2016, Maier and Seligman, 2016). Assessment of these areas may reveal unique morphological changes following chronic stress that further correlate with changes in decision making and/or morphology. The large amount of morphological information presented here indicates that chronic stress changes morphology, and as we move to the behavioural discussion I would like to reiterate the philosophy of this thesis, all maladaptation's to behaviour can be explained by changes within neuronal morphology.

4.4. Effects on Decision Making

In this section I will discuss the changes in decision making exhibited by chronically stressed animals. I will divide this section by the various tests used to examine different aspects of decision making. I will begin with outcome devaluation and contingency degradation, which are tests of decision making that measure how well an animal performs on decisions that require goal directed action. We will then discuss sPIT, outcome specific reinstatement and delayed discounting, which measure how well an animal can extract predictive information from the environment to guide future actions and how flexible that guidance is.

Outcome Devaluation and Contingency Degradation

Experiments 2 and 4 assessed the effects of chronic stress on outcome devaluation and contingency degradation. In Experiment 2, stressed rats were insensitive to outcome devaluation (Fig. 23), i.e. they continued to press for the outcome which had just previously had free access, unlike controls that remained, as expected, sensitive to outcome devaluation and reduced pressing on the lever that, in training, delivered the devalued outcome. This result mirrors the work by Dias-

Ferreira et al. (2009) who showed that chronically stressed rats were insensitive to outcome devaluation. Further to this, in Experiment 4, controls again were sensitive to outcome devaluation, whereas randomly stressed rats were not, presenting the same as the chronically stressed rats in Experiment 2 (Fig. 41). Most interestingly, however, we found that the predictable stress group of rats, who we have argued might be expected to have developed a level of controllability over the stressor, were sensitive to outcome devaluation, pressing less for the devalued outcome and looked similar to the control group in this regard. It would appear that the making the chronic stress predictable protected against the negative effects of chronic stress in a similar manner to the way that ES rats, in an ES vs. IS experiment, are protected against the negative behavioural effects of an acute stressor. This protective effect also extended to contingency degradation.

Contingency degradation, like outcome devaluation, measures the animal's capacity for goal directed action; however, instead of manipulating outcome value it involves manipulating the contingency between actions and their outcomes. We found, in Experiment 2, that the control rats were sensitive to the change in contingency and adjusted their decisions appropriately, reducing pressing on the degraded lever. The stressed animals, however, continued to press on the degraded and non-degraded levers equally, indicative of an insensitivity to changes in the action-outcome contingency and a dependence on habits to guide behaviour. Again this effect mirrors the only other study which has looked at these measures of goal directed actions within chronically stressed rats; the Dias-Ferreira et al., (2009) study also showed that chronically stressed animals were insensitive to contingency degradation. Further to this, in Experiment 4, just as with outcome devaluation, controls reduced pressing on the degraded lever whereas random stress rats were insensitive to degradation. Furthermore, as observed with outcome devaluation, the predicted stress group performed in a similar manner to controls and were protected against the negative effects of the chronic stress, remaining sensitive to the degradation to the action-outcome contingency. This correlates with the hypothesis that the ability to predict the occurrence of a chronic stressor

provides the animal with a level of protection against the negative effects of that chronic stress has on behaviour.

Pavlovian-Instrumental-Transfer

Across all groups and both Experiment 2 and 4, all rats successfully exhibited a sPIT effect; i.e., were able to extract predictive information from the environment to guide future action. What we did see was that stress rats, regardless of group, had a stronger sPIT effect, the chronic stress seemly facilitating performance. The results from Fig. 23 & 41 indicate that control animals, as predicted, exhibited a reliable sPIT effect. In Experiment 4 both stress groups showed a greater facilitation of sPIT compared to controls, with the predicted stress group having an even greater facilitation than random stress. This result, repeated twice, is in contradiction to the Morgado et al. (2012) investigation which showed CMS lead to a transient decrease in sPIT performance. As discussed, ours is the only other experiment that has specifically investigated CRS effects on sPIT. and, as such, the difference in these results could easily be explained by the different stress paradigms; Morgado et al. (2012) results were found using a CMS paradigm, where the rats are less stressed than the rats in this study. As indicated by McLaughlin et al. (2007), CMS is not as robust as CRS nor does it provide the same reliability and consistency in dendritic changes as CRS. It is likely that lighter stress treatment leads to less impactful changes within morphology, leading to less impactful changes on behaviour.

As explained, stressed rats exhibited a facilitation of the sPIT effect compared to controls; however, on experimental repetition is was revealed that rats which suffered predicted stress showed an even greater facilitation compared to their random stress counterparts, this can be explained by investigation into the changes within the key ROIs the BLA, NaS and OFC. We observed that the random stress group showed greater dendritic proliferation in the BLA than predicted stress. It would naturally be assumed based on the evidence (Corbit and Balleine, 2005, Corbit et al., 2007, Corbit and Balleine, 2011) that a proliferation of dendrites within the BLA, would lead to a facilitation

of the sPIT effect as disconnection and lesion studies involving the BLA produced a deficit in the sPIT effect. Hence, if the random stress group has an even greater proliferation of BLA morphology then we would expect that this group would have an even greater facilitation of the sPIT effect. The problem with this claim is that the predictable stress rats had reduced proliferation of the BLA compared to random stress rats. Alternatively, Balleine et al. (2011) showed that when the VO is lesioned with the LO the sPIT effect is removed, if the LO is lesioned by itself no effect is seen. What we see from ROI analysis is that the LO of both stress groups decreases, but only the random stress group sees a concomitant decrease in the VO, the predicted group actually sees a proliferation in the VO. It may be that the protection/proliferation of the VO is driving the increased performance on the sPIT test, higher than that of the random stressed group, as the OFC involvement is no longer morphologically hindered as in the random stress group. Later in subchapter 4.6, I discuss a more speculative explanation

Reinstatement

To investigate whether sPIT performance was guided by action→outcome or outcome→action associations we performed an outcome-specific reinstatement test. This test assesses the ability of the rats to use the outcome itself, rather a stimulus paired with the outcome, to drive action selection. Across both Experiment 2 and 4 we see that there is no effect of chronic stress on reinstatement (Tab. 5). Both experiments showed that there was no effect of any factor except response (same vs. different), no interaction with stress or group. Although there is a trend towards a diminishing reinstatement effect in the predicted stress group, this does not reach significance. This indicates that chronic stress does not facilitate outcome-specific reinstatement. It is likely, as discussed in the introduction (p.57) that the facilitation in reinstatement seen by Ball et al. (2016) following chronic stress is in fact a measure of general reinstatement and not one outcome specific, if we recall from the subchapter 1.6 in the introduction, there is a difference between general and outcome specific cued action selection. In respect to drug and palatable food seeking

literature, it may be that the facilitation of reinstatement associated with chronic stress is specific to outcomes of extremely high incentive value, i.e. drugs and high palatable foods, but if investigated within an outcome-specific context with two outcome of relatively equal and incentive value, this facilitation is not present.

Delayed Discounting

In addition to the tests conducted in Experiment 2, in Experiment 4 we added a delayed discounting task in order to examine the effect of both random and predictable chronic stress on the flexibility of the animals' decision making. Interestingly, we found that it was the predicable stress group that was most impaired on this task. Both control and randomly stressed rats performed healthily, that is the rats preferred the delayed lever while the delay was short, and then changed to the non-delayed lever as the delay ascended. The predicted stress rats did not perform this way, instead their choices appeared inflexible, rigid, continuing to press for the delayed lever significantly more than non-delayed lever for significantly longer than control or random stress rats. To discuss this, we will review and expand on the literature introduced in subchapter 1.5.

Recall that Schwager et al., (2014) showed that rats administered a pharmacological stressor, yohimbine, prior to delayed discounting, preferred whichever lever they began the task pressing, whether it be the delayed or non-delayed lever, indicating an inflexibility in the animal's decision making. In Experiment 4 we found that controls performed as expected, initially preferring the delayed lever before returning to the non-delayed lever. Interestingly, random stress rats followed a similar pattern of preference to control rats, however they preferred the delayed lever significantly more and earlier on than controls, before returning and responding on the non-delayed lever at the same rate as controls. This is seen in Fig. 41, where the randomly stressed rats had a significantly higher preference scores on trials 2, 3 & 4, but then proceeded to show a similar preference to the controls. It was actually the predicted stress rats that performed similarly to the rats seen in the Schwager et al., (2014) study, initially preferring the delayed lever and then continuing to prefer it for

far longer than either the random stress rats or controls indicative of an inflexibility in the predicted stress group. As there was no literature that has investigated chronic stress effects on delayed discounting, let alone predictable stress, our early inferences led me to expect that randomly stressed rats would be similar to the stressed rats in Schwager et al. (2014), and the predicted stress rats to have increased flexibility with their decisions. The opposite was in fact observed. What this highlights first, is that acute stress and chronic stress are different, and secondly that controllability, although protecting animals against morphological changes and changes in outcome devaluation and contingency degradation, it may not protect the animal against all harmful effects. If we look into the efferent and afferents of the OFC, I speculate why this might be the case (see subchapter 4.6).

Summary

It is clear across these two experiments that random stress causes insensitivity to outcome devaluation and contingency degradation, indicating that rats which suffered chronic stress become dependent on habitual decision making over goal directed. We see that prediction gives the rat control over the stressor, offering a lever of protection that prevents the rat from needing to depend on habitual behaviours following stress, illustrated by their comparable performance on outcome devaluation and contingency degradation to that of control animals. Additionally, across both experiments we see that animals which underwent chronic stress treatment, regardless of controllability, successfully performed sPIT. Moreover, stressed animals displayed a facilitated PIT effect, pressingly for the "same" lever significantly more than controls, with predicted stress animal pressing even more so than random stress. Further, across both experiments, we determined that this facilitation in sPIT effect was not influenced by outcome→action associations, both experiments revealed, across all groups and treatments, that chronic stress did not impact in outcome-specific reinstatement, with all rats performing the same as controls. And lastly, in Experiment 4, interestingly it was predicted stress rats, and not random stress rats which suffered at the delayed discounting task, indicating that although it appeared that prediction of a stressor behaviourally

protected rats, this protection is not all encompassing. To create a better picture of the factors involved here we now look into correlations between the morphology and behaviour.

4.5. Correlations between Morphology and Behaviour

One of the aims to this thesis was to causally link changes in morphology to those in behaviour. We see in Experiments 2 and 4 that chronic stress treatment changed morphology across ROIs via degeneration, proliferation and in the case of the CN no change at all, additionally we saw that chronic stress deteriorated goal directed action and facilitated sPIT performance. Furthermore, if the animal could predict the stressor, it offered a level of protection to the animal ameliorated many of the detrimental effects of treatment, so much so that regions were entirely protected and animals presented as controls in outcome devaluation and contingency degradation. As was shown in the in the results sections (Fig. 42, 43, 44), in a few of the regions analysed, animal behaviour correlated with change in morphology; this was also evident, although less so, within control animals, indicating I believe, what is assumed but rarely tested, that morphology and behaviour are intimately linked.

Morphological Correlates of the Devaluation and Degradation Effects

Firstly, looking at the correlations in Fig. 42, 43, 44 and Tab. 6 we see that within all three groups, the MO has one of the strongest correlations with outcome devaluation and contingency degradation performance, so much so that control scores even come out as correlated with morphology. This reinforces the literature on the importance of the MO to outcome devaluation and contingency degradation. Three other regions particularly correlated with these tests were the DMS, NaC and PLC, and although only statically significant within stress groups at the corrected p value ($p<0.004$), under a traditional p value of ($p<0.05$) these correlations come out within control animals as well. Conceivably a less explorative study would not push these control correlations out of

significance. Similarly, to the MO, these correlations reinforce the literature on the importance of the DMS, NaC and PLC to goal directed action.

Secondly, comparing our results to those of the Dias-Ferreira et al. (2009) paper, it is evident that chronic stress damages an animal's ability to perform goal directed tasks which, as argued by Dias-Ferreira et al. (2009), appears largely to be due to the decreases and increases in dendritic morphology in the DMS and DLS, respectively. In line with their results, we observed degeneration of the DMS, among other structures responsible for goal directed action. However, in direct contrast to their results we observed, only a downward trend in the DLS. It should be remembered that Dias-Ferreira et al. (2009) did not report changes in spine morphology, but only changes in dendritic length. In fact, we observed the opposite; i.e., a decrease in spine density without a significant decline in dendritic morphology. This is across the stressed animals in Experiment 2 and the randomly stressed animals in Experiment 4. Although, we have already argued that a decline in synaptic spine density would be indicative of a future decrease in dendritic morphology within the DLS, the severity of the stress may need to be quite extreme, so much so that its relevance may be open to question. It may be, as already mentioned, that the DLS is more resistant to change, only seeing precursory changes to overall morphology. What we believe contrary to the Dias-Ferreira et al. (2009) study, is that there is only a decline in the regions required for goal directed action and that this isn't accompanied by an increase in the regions responsible for habits. What our findings generate by way of conclusion is, therefore, different from Dias-Ferreira et al.; as the animal's habit systems remained intact and similar therefore, to controls, it is the degeneration of the DMS, among other regions, that leads to a loss of goal directed control and a consequent dependency on habits.

Pavlovian-Instrumental-Transfer

Due to the Bonferroni correction, correlations were only found within stressed animals. Both random and predicted stress rats show a strong positive correlation in sPIT performance with BLA morphology, controls also correlate however it would only significant under a less exploratory study.

This correlation with morphology supports the literature's view on the BLA's crucial involvement in sPIT. Uniquely, within predicted stress rats, the VO also strongly correlated with sPIT performance, which may give more credence to the argument that it may be the VO that is specifically driving sPIT performance to be different from random stress rats. Additionally, it appears that, although failing to come out as significant (P_Stress $p<0.064$; R_Stress $p<0.016$), stress negatively correlates the ACC with sPIT performance, while positively doing so within controls. Inherently this doesn't mean anything, however when taking in the context of an argument made on BLA proliferation within stressed animals it does. Bennett (2009), argued, as the only argument at present explaining the proliferation of BLA morphology within chronic stress, that deterioration of the ACC leads to proliferation of the BLA. This is expanded on in speculation (subchapter 4.6), however, this change in correlation direction may place support for this argument and also indicate that stress treatment changes the ACC so as to influence behaviour differently. Nevertheless, it appears that sPIT performance appears to correlate with the morphology of the BLA across all groups and NaC specifically within stress groups.

Reinstatement & Delayed Discounting

As for outcome specific reinstatement, no morphology correlated with performance across any group across either the Bonferroni corrected p-value or even a traditional $p<0.05$, except for the InC within predicted stress rats. This correlation may be indicative of behaviour, and may be unique to predicted stress animals, in a similar way to how VO morphology within predicted stress rats correlated with contingency degradation. This isn't necessarily unlikely as predicted stress morphology is greater than controls and more proliferated than random stress. If this correlation were to be indicative, further lesion studies on predicted stress animals would reveal impairment in reinstatement. Similarly, delayed discounting also had very few correlations. Correlations were made at both trial 3 (zero delay) and 11 (30 sec delay), correlations at zero delay were found within random stress rats within the NaS, and if we were to extend the p-value range to $p<0.05$, correlations

are also found within the BLA, PLC and LO, as well as within the NaS and DLS within predicted stress rats, as it stands however, only the NaS could be considered indicative of performance. At the 30 second delay we see correlations within the DLS and MO in predicted stress rats, and within the InC for random stress rats. These correlations are more in line with literature, as Balleine and Dickinson (2000) and Bradfield et al. (2015) have argued that the InC and MO are required for retrieval of information used to guide choice, and in a delayed discounting task, such retrieval is needed to use time and value information to guide choice.

Predictability

So how do these correlations relate to predictability and control? Recall that the Amat et al. (2014) paper argues that, for the animal to show the protective effects of controllability over a stressor, the DMS must be intact and active. It is encouraging to see that the behavioural results in the predictable vs. random stress group not only showed protected from stress but also the degenerative effects of stress on neuron morphology observed in the DMS were also ameliorated, further to this as seen in Fig. 43 and 44 behavioural performance within outcome devaluation and contingency degradation positively correlated strongly with DMS morphology. The protection of the DMS in our predicable stress group indicates, as we argued and expected, that prediction of a stressor offers similar protection against the effects of stress as action controllability. In fact, it may be reasonable to regard stress prediction a form of controllability; the prediction of a stressor may well allow an animal to control its environment so as to ameliorate the effects of the stressor. The prediction of a stressor offers not only protection of behaviour, as indicated in Amat et al. (2014) and Baratta et al. (2015), but also cellular protection. The predictable stress rats performed behaviourally the same as controls in almost all measures of goal directed behaviour, and were shown to have dendritic morphology distinct from random stress rats, the same as controls in the DMS, MO and HIP, regions showing the clearest example of protection of morphology, with the DMS and MO having some of the strongest correlations between behaviour and morphology. The DMS and MO are crucial

for the encoding and retrieval of goal directed action and their protection explains the performance on the outcome devaluation and contingency degradation tasks. If we recall from subchapter 1.5, the DMS is required to generate and maintain goal directed action, and the MO is required to retrieve information about the value of an outcome. With both of these structures intact within predicted stress rats, we argue that this preservation of morphology, allowed the predicted stress rats to perform the same as controls on these tasks, where the random stress rat could not, due to a degeneration of the DMS and MO.

There may be limits to the relationship between predictability and controllability, however, particularly in the context of chronic stress. For example, another region thought to be involved in the protective effects of controllability is the IFC. Baratta et al. (2015) showed that control over a stressor in the same ES vs. IS shock paradigm used by Amat et al. (2014), accelerated the rate of extinction of drug seeking behaviour. This acceleration was abated when the IFC was inhibited. In the current study we observed degeneration of the IFC in stressed group in Experiment 2 and, importantly, also in the predictable stress group of Experiment 4. In this case the relationship between IFC degeneration and prediction from stress was not observed; something that could easily be as much due to the differences in the chronicity of the stress treatment as the differences of predictability and controllability, a speculative explanation is however given (see subchapter 4.6). Nevertheless, other regions may have been involved in protective effects of predictability. The MO, VO and HIP were all altered in predictable stress rats compared to random stress rats or controls. The MO and HIP were observed to be as healthy as controls following CRS and, most interestingly, the VO showed a proliferation in dendritic morphology. The intimate connection between the HIP and the OFC (Verschure et al., 2014, Wikenheiser and Schoenbaum, 2016, Takahashi et al., 2008) and the DMS (Delcasso et al., 2014, Kalivas and Kalivas, 2016, Verschure et al., 2014), may also place the HIP in a crucial position to mediate the protective qualities of prediction, and this protective effect may be as a result of interplay between these regions and circuits. Further research aimed at

elucidating this circuit may help to develop an explanation for the protective qualities of predictable stressor.

Summary

It is clear that there is a correlation between morphology and behaviour. Animals which performed worse on outcome devaluation and contingency degradation had reduced morphology within the MO. This correlation is sensitive enough to correlate less severe changes in morphology to less severe changes in behaviour as evidenced by correlations within control animals. But correlations were also observed between NaC and PLC and outcome devaluation and contingency degradation. With sPIT we saw an extremely strong correlation between performance and the BLA across all groups, as well as unique correlations within stressed groups; a correlation with NaC and an inverse correlation between control rats and stressed rats was observed within the ACC. Only one correlation was found with reinstatement, that being within the InC in predicted stress rats. And lastly delayed discounting saw correlations specifically within random stress for the NaS and InC, while predicted stress rats saw correlations with DLS and MO, no correlations were found within control animals. This is the first evidence for a direct correlation between morphological change, that is not a lesion, to goal directed behaviour.

4.6. Speculation

Within this final subchapter I collect a range of highly speculative arguments aimed to explain some of the unexpected results, and the unique morphological and behavioural changes seen within the groups of this thesis. Due to gaps in the literature some of the points are made out of conjecture, however throughout the arguments I point out where future research could be taken so as to provide validation.

BLA morphology Predicted vs. Random stress

At present there are only limited explanations in the literature for the proliferation observed in the BLA after chronic stress (Bennett, 2009). Bennett, argued that the proliferating effect seen in the BLA following chronic stress was due to a decrease in serotonin transporter promoting a decrease specifically in 5-HT_{1A} receptor activity in the ACC which it was argued had an inhibitory influence on NMDA receptor efficacy in the BLA via efferent innervation. Bennett further argued that reduced NMDA efficacy produced the decrease in synaptic spine density leading to a decrease in dendritic length (Bennett, 2009). So this arguably accounts for the BLA proliferation seen in chronically stressed animals in Experiment 2, as they have a degenerated ACC and a concomitant BLA proliferation. But how does this account suggest that predictability blocks this effect? i.e. the reduced BLA proliferation? It must do so by proposing that predictability blocks the change in ACC 5-HT_{1A} receptor activity. However, as we observed that the predictable stress group showed comparable decreases in morphology within the ACC to the random stress group, is this argument false? Perhaps not, as research indicates LH, the symptomology exhibited by IS rats, which are analogous to our random stress rats, are dependent on serotonergic innervation from the DRN to the BLA (Graeff et al., 1996), and it has been reported that other uncontrollable stressors, such as social defeat, also lead to this DRN serotonergic efferent activation (Amat et al., 2010), assumedly such activations would lead to the BLA. If we assume social defeat, just like IS, is analogous to CRS, then it would be fair to assume that within the rats present in this study that the DRN would be innervating BLA potentiation. Hence, in Experiment 4, predictable stress rats have more control over the stressor and so would suffer from less DRN→BLA innervation, leading to less BLA activation compared to random stress rats, and ultimately less morphology proliferation. Together with the work by Bennett (2009), it may be that a large portion of BLA proliferation is resultant from ACC degeneration, and the difference between predicated and random stress rats emerges from different activation of the DRN. Furthermore, in an acute stress preparation Rozeske et al. (2011), demonstrated that uncontrollable stress (IS), and not, controllable stress (ES), impaired specifically 5-HT_{1A} receptor

mediated inhibition of the DRN, and so it remains possible that similar changes in 5-HT function emerged in BLA; or that changes in DRN can also produce changes in NMDA function in the BLA and so explain increased proliferation in the random stress compared to predicted stress. If we continue to accept that the random and predicted stress groups are analogous to uncontrollable (IS) and controllable (ES) stress, then from the Rozeske et al., (2011) paper, we would expect that the random stress group would have a greater reduction in 5-HT_{1A} receptor activity than the predicated stress group, and so a more pronounced BLA morphological proliferation. This account, while highly speculative, is testable and worth developing in future research.

Morphological camouflage within the CN? Proliferation within the NaC?

This failure to see an overall change in morphology within the CN may have been due to the fact we sampled from the whole CN and it is possible that subregions within the CN are differentially affected by stress; e.g. the central medial amygdala (CeM) and the central lateral amygdala (CeL) have been argued to be involved in behaviourally distinct processes (Badrinarayan et al., 2012, Ciocchi et al., 2010, Duvarci et al., 2011) and may have divergent neuronal morphology following chronic stress; e.g., if the CeM degenerates but the CeL proliferates, sampling across subregions would camouflage any measurable effect. Although this is a speculative possibility, the internal division of the CN is worth assessing in future studies.

There are at present no arguments which explain why it is that the NaC proliferates following chronic stress, I propose a simple and testable explanation. NaC proliferation may be explained as an effect of the increased efferents from the InC and BLA, as both connect to the NaC (Cho et al., 2013, Loonen and Ivanova, 2015, Mannella et al., 2013) and both showed morphological proliferation. If the InC and BLA proliferated prior to the NaC, their resultant increased activity would encourage increased synaptic growth and hence concomitant dendritic growth in the NaC. This hypothesized order of influence makes specific testable predictions about what should happen were this influence

curtailed in some way; e.g. if the insular or BLA were lesioned in one hemisphere prior to the chronic stress treatment, a treatment that should block NaC proliferation.

The greater facilitation of sPIT in prediction

How is it that the predicted stress rats have greater facilitation of sPIT than random stress rats when they have a less proliferated BLA? Looking again at the changes within the OFC, as discussed in Balleine et al. (2011), there is a BLA→OFC→NaS circuit that is involved in sPIT, with greater proliferation of the BLA in random stress compared to predicated stress and the protection of the OFC in predicated stress rats, we see a twofold effect, where the BLA would send increased inhibitory connections to the OFC, which would then send less excitatory connections to the NaS, driving down its activity, resulting is poorer sPIT performance. Where as in the predicted group, the less proliferated BLA would send less inhibitory connections to the OFC, which with it being also protected against dendritic degeneration, would send comparatively increased excitatory connections to the NaS driving enhanced sPIT performance. This speculation is testable via Immunohistochemistry labelling of activity markers, or electrophysiology measurements of morphologically damaged tissue.

Furthermore, investigation into identifying and analysing any differences which may occur between the D1 and D2 dopamine receptors present within the striatum, may reveal that the sPIT effect is camouflaged within the striatum. The D1 receptor promotes striatal excitability and the D2 receptor promotes inhibition (Shiflett and Balleine, 2011a, Shiflett and Balleine, 2011b). Stress specific changes within these receptor subtypes would be of great interest, with recent studies indicating that chronic stress decreases D1 receptor transmission (Ball et al., 2015, Mizoguchi et al., 2000), and as D1 receptor activity is intimately involved with sPIT performance, there may be a relation. Such research may even reveal that chronic stress impacts on cholinergic interneurons (CIN) and their delta-opioid receptors, which are reported to influence D1 receptor expressing neurons which in turn modulate sPIT performance. A study by Laurent et al. (2014) revealed that not only is

the D1 receptor crucial for sPIT performance, but that CIN activity and delta-opioid receptor activity promoted sPIT performance. It may be that chronic stress, like in the BLA and NaC, promotes a proliferation of CIN morphology or even more specifically the delta-opioid receptor. If such proliferation were to occur, it would promote sPIT performance via modulation of D1 receptor expressing neurons (Bertran-Gonzalez et al., 2013, Laurent et al., 2014). This proliferation would be camouflaged during traditional morphological analysis, as interneurons are not measured as they are typographically different from neurons. It may be then that CIN or delta-opioid receptor proliferation has occurred within the NaS, which would promote increased sPIT performance and in part explain the facilitated sPIT effect seen in our chronically stress rats. There is no literature to date that has looked into CIN and any effects if any, chronic stress has in them, a caveat to be revealed with further research.

Delayed Discounting Predicted Stress Choice Rigidity

Mar et al. (2011), demonstrated in a lesion study that rats with their LO removed prefer immediate reward, and rats with the MO removed prefer delayed reward. In the current study both the predictable and random stress groups showed degeneration of the LO but only the random stress group showed degeneration of the MO. From the Mar et al. (2011) study this should lead us to predict that both groups should prefer immediate reward, as the 40% degeneration in morphological measures of the LO should arguably be congruent to a lesion, whereas the random stress group should show more of a preference for the delayed lever compared to the predictable stress group, due to the degeneration in MO morphology. However, this was not observed. The NaC and BLA both have heavily interconnections to the OFC (Carmichael and Price, 1995, Morecraft et al., 1992, Schilman et al., 2008), and the changes in the LO and MO, plus any concomitant changes within the NaC and BLA, must be taken into consideration before we can properly infer behavioural effects from these morphological changes.

Both the predictable and random stress groups showed a similar degree of degeneration in dendritic length and spine density in the LO but differed with respect to proliferation of the NaC and BLA. The predictable stress group had more proliferation in the NaC than the random stress, whereas the random stress group had greater proliferation in the BLA. These changes complicate the simple effects found in Mar et al. (2011). The greater proliferated BLA may allow the random stress rat to more readily encode the relationship between the stimulus and/or response and the outcomes causing the rats to prefer the delayed lever sooner than controls and predictably stressed rats, but then trailing off at the same rate as controls as the preference for the delayed lever decays. Proliferation in the NaC in the predictable stress rats suggests that there should be increased inhibitory transmission to the LO and MO, via the pallidal and thalamic relays, compared to the random stress rats (Carmichael and Price, 1995, Morecraft et al., 1992, Schilman et al., 2008). This increased inhibitory activity implies an increased preference for both the delayed and non-delayed levers because, if increased inhibition of the LO and MO is comparable to a lesion, the inhibition of the LO would increase preference of the non-delayed lever, and inhibition of the MO would increase preference for the delayed lever. In partial confirmation of this prediction, the predictably stressed rats with the greater NaC proliferation had an increased preference for the delayed lever only; their preference for the non-delay lever was unaffected. The specificity of this behavioural change may indicate a specific MO afferent is particularly proliferated or active. And it is this specifically increased inhibitory input onto the MO that would be inhibiting the ability of the MO to properly integrate choices. With the MO inhibited, the animal may become more dependent on a previously established action-outcome association, i.e. the initial preference of the delayed lever, as the animal is no longer able to update the value of its choices.

Controllability: Is it the mPFC the IFC or PLC

Regarding controllability, Amat et al. (2006), as already discussed, showed that the mPFC, the PFC and IFC, were required for the protective effects of controllability. This contradicts the findings

herein, both the PLC and IFC had degenerated morphology following chronic stress, regardless of how predictive it was. Now although the DMS maintained healthy morphology following specifically predictable stress, supporting the work by Amat et al. (2014), it still needs to be addressed how it is that the mPFC was deteriorated yet predicted stress rats present as though they are protected via controllability. It may be that specifically, within the PLC and IFC that predictability and behavioural controllability are different, however I feel that unlikely, I propose that it is instead specifically the IFC that is required. Baratta et al. (2015) showed that it was specifically the IFC that was required for the protective effect of controllability to accelerate cocaine extinction. Additionally, if we look more closely at the degeneration within the mPFC within Experiment 4, the IFC is less reduced than the PFC in measures of dendritic length (20% vs. 40% respectively) and spine density (40% vs, 60% respectively). It may be that the IFC is the region specifically required, and that its involvement prevented it from becoming as degenerated as the PLC, and/or, that the region is still able to contribute to controllability until a certain degeneration threshold is met. To test this, simple lesions studies would reveal whether the PLC, IFC or both are required for the protective effect of predictability.

Chronic stress switches appetitive to aversive: the CHR-Dopamine Switch

Within this speculation, I argue that conditioned stimuli traditionally considered appetitive, i.e. within pellet and sucrose training, instead influence an animal's decision making process from an aversive perspective, following chronical stress treatment. Firstly, as mentioned through the introduction and discussion, Lemos et al. (2012) found that CRH locally administered to the nucleus accumbens induced an appetite response, and when rats were tested on place preference they preferred the room associated with CRH injections. This effect was shown to be associated with dopamine release, when examined ex vivo, CRH application to nucleus accumbens tissue increased dopamine release compared to vehicle. Further to this they examined chronic stress effects on CRH-dopamine interactions, showing after chronic forced swim stress, and examined under the same

conditioned place preference test, stressed rats saw a greater aversion to the CRH paired room compared to vehicle and controls animals. The authors argued that chronic stress dysregulates CRH modulation of dopamine release, and this changes the appetite quality of CRH interaction within the nucleus accumbens to aversive. Note that this dysregulation is specific to CRH, as when the authors used a k-opiod agonist to reduce dopamine release it was unaffected by the chronic stress. If this is extended to other behaviours, such as sPIT, this switch may have impacted on the stressed rats performance. If CRH interaction within the nucleus accumbens is now promoting an aversive instead of appetitive response, it is conceivable that, that appetitive S-O associations, albeit, likely still learned as appetitive, are, when called upon to extract information from the stimulus to infer decision, inferred as an aversive stimulus; the stressed animals are already primed to aversive stimuli, that being the stress itself, and combined with their proliferated BLA, may be promoting an increased receptiveness to aversive stimuli, or making aversive stimuli more salient, and driving performance in an sPIT task as such. This argument is testable via modifications to training, and monitoring for anxious behaviour. Furthermore, one study revealed that all structures within the striatum that were receptive to dopamine, had higher dopamine intake following CRS (Copeland et al., 2005). With the reduced output and higher intake of dopamine following CRS, it is conceivable that dopamine dependant or associated efferents would become less effective. The bed nucleus of the stria terminalis (BNST) and ventral tegmentum area (VTA) (Johansen, 2013, Silberman and Winder, 2013), both heavily involved in stimulus outcome association, in particular, the BNST sends GABAergic and glutamatergic efferents to the VTA which are used in the association of aversive and appetitive stimuli respectively. The glutamatergic appetitive connection is dependent on dopamine release (Silberman and Winder, 2013). If dopamine is less available, as discussed, activity would then bias towards the aversive GABAergic connection. This argument is testable via morphological and pharmacological analysis and intervention within said structures, and with literature indicating that the BNST and VTA have the highest non-neocortex CRH receptor binding sites, (De Souza et al., 1985,

Makino et al., 1995), parallel CRH release and effects (Makino et al., 1994, Makino et al., 1995), and being anatomically associated, this I believe is a far assumption.

An Intracellular Explanation for Concomitant Change in Morphology and Behaviour following Stress

We understand that regions are protected via predictability, however understanding how this protection mediates damage to morphology and the behaviour, is the next step. So far, we have given some explanation for how the predicted stress group has different morphology, i.e. within the BLA, the reduced proliferation within predicted stress groups is likely due to change in serotonin within the ACC and BLA. This is specific to the BLA, and a broader explanation may be possible. Explanations for the cellular protection in prediction and control are limited, Amat et al. (2006, 2014) showed that only the ES rats showed increased *c*Fos activity within the mPFC and DMS, i.e. only the rats with control over the stressor showed increased mPFC and DMS activity compared to rats who did not have control over the stressor or control rats. This increased activity could be driving increased activity and synaptic growth, helping maintain the region closer to or at healthy morphology. But I believe another explanation comes from intracellular processes. I posit below that changes within GC and CRH, calcium and calmodulin-dependent kinase 2 (CaMKII), extracellular signal regulated kinase (ERK) and Dopamine, can together explain the morphological and concomitant behavioural changes between random and predictable stress rats seen herein.

To begin, the process of synaptic regression is dependent on CaMKII. Bennett (2008) showed that CaMKII regulates adenosine 5'-triphosphate (ATP) and F-Actin, which are necessary for synaptic spine stability, and should CaMKII become downregulated or disrupted synaptic spine collapse occurs. We recall that GC and CRH receptor activation interacts with NMDA receptors within synaptic spines, specifically causing a decreases in efficacy of NMDA receptors, and a downstream dysregulation of CaMKII, leading to synaptic regression and dendritic atrophy. Next, it has been reported that animals with controllability over a stressor have reduced GC and CRH concentrations (Lawler et al., 1993, Pitman et al., 1995) which may, among other explanations, directly explain the

reduced morphological impact of predicable stress. However, dopamine must also be considered in this speculation, as it is involved in synaptic modulation of learning behaviour and is uniquely affected by chronic stress. Dopamine changes radically in chronic stress, Lemos et al. (2012), showed that CRH no longer upregulates dopamine release in chronic stress, as it does is acute stress, and many studies have shown that chronic stress leads to a decreases in dopamine (Charmchi et al., 2016, Fadda and Liguori, 1981, Faramarzi et al., 2016, Holly and Miczek, 2016, Ida et al., 1982, Karkhanis et al., 2016, Kasanova et al., 2016). Predicted stress rats may conceivably suffer from less intense losses of dopamine, in a similar way to how predicted stress rats have reduced GC and CRH concentrations. Should this be the case, based on work by Shiflett and Balleine (2011a, b), the learning required intracellular compound ERK would be maintained more so in predicted rather than random stress rats. In their reviews of the literature, the gluta- and dopaminergic dependent compound ERK was heavily involved in action-outcome learning, stimulus response learning, and reward association, going on to discuss that for animals to adequately learn, intracellular changes must occur; glutamate and dopamine innervation must promote ERK, which allows the neurons to generate learning-related plasticity; and bringing the argument full circle, these glutamate and dopamine interactions within the synaptic spine are dependent on CaMKII. If chronic stress causes a decrease in CaMKII, it may not only be affecting neuronal morphology, but also indirectly affecting behaviour, and as a result, impacting on the intracellular processes needed to develop adequate action-outcome learning.

Summarising (see Fig. 45), predicted stress rats appear both morphologically and behaviourally to be resilient to the effects of chronic stress, not as healthy as controls but far healthier than random stress rats. If their resilience to morphological change is represented not only by increased dopamine, but also reduced GC and CRH concentrations, comparatively to random stress rats, this would lead to less synaptic spine and dendritic atrophy, which is what is observed. This would mean more CaMKII is present within the synaptic spine. In fact, there must be more CaMKII present within the synaptic spine, as there is less atrophy. So the predicted stress rats have

higher CaMKII within the synaptic spine, which would allow greater gluta- and dopaminergic innervation compared to random stress rats. This would lead to more ERK activity, and a greater resilience to changes, not only to morphology but also decision making. Understandably, this theory is highly speculative, but can be validated via manipulations within a chronic stress treatment to any of the key factor described. Based on the limited literature and the conceivable correlation between GC, CRH, dopamine and CaMKII, I believe that this may explain a unified argument of protected morphology across the regions and the outcome devaluation and contingency degradation results seen in our stress rats.

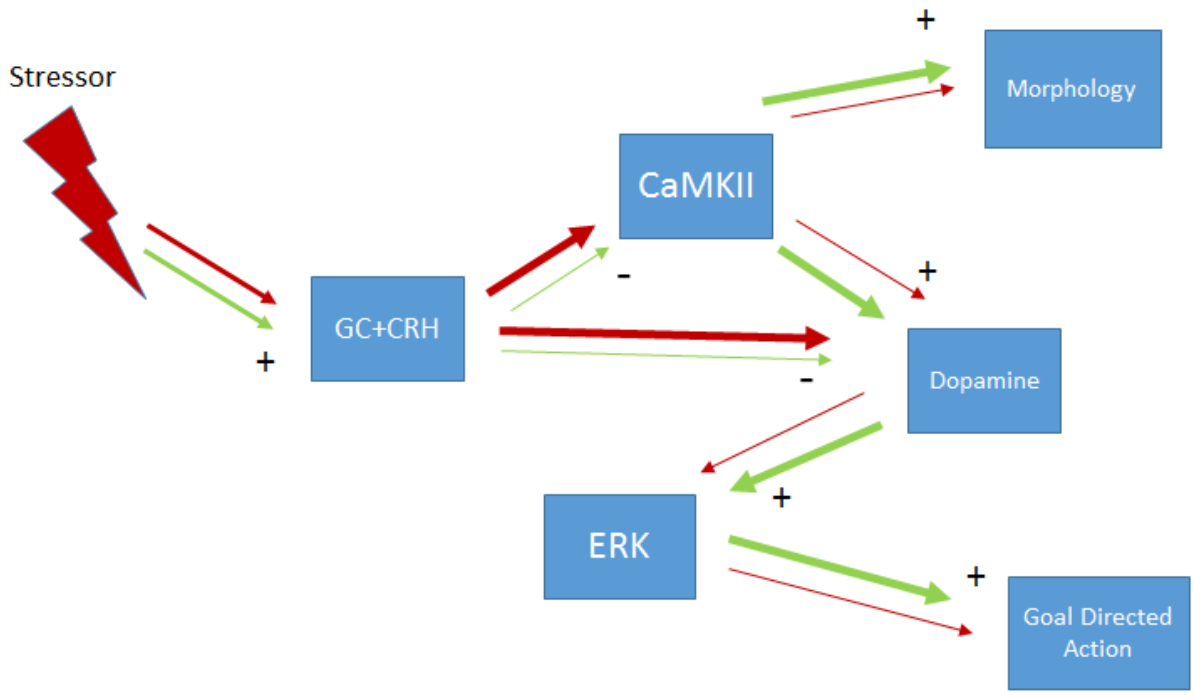

Figure 45: An illustration of the intra-cellular argument, where predictable stress rats (green) maintain morphology and goal directed action via reduced GC and CRH release, leading to less downregulation of CaMKII, resulting in increased dopamine and ERK. Random stress rats (red) suffer from greater GC and CRH concentration, downregulating CaMKII more so than predicted rats, leading to less dopamine and ERK, and hence, degenerated morphology and deteriorated goal directed action. (+) indicates a promoting and (-) a decreasing impact from the connection, and the larger the bar the greater the impact, e.g. GC +CRH cause a decrease in CaMKII with the red bar indicating a larger decrease and the green a smaller.

Summary

Clearly further studies into the differences between controlled and non-controlled stressors are required to further understand the mechanism behind the protective quality prediction has and how this impacts morphology and behaviour. I speculate that: the increased proliferation within the BLA between predicted and random stress is due to a combination of decreased efferents from the ACC and DRN; that morphological change following chronic stress within the CN may be camouflaged by discerning populations of neurons within the CeM and CeL, and an initial hypothesis that NaC proliferation is due to highly active efferents from the InC and BLA; that greater sPIT performance in predicted stress rats may be due to less inhibitory signals within the BLA→OFC→NaS circuit, as well as, that this increased facilitation may be camouflaged within dopamine subpopulations or CINs; I go on to say; predicted stress rats have increased choice rigidity within the delayed discounting task due to increased NaC inhibitory connections to the OFC; that it is in fact the IFC, and not the PLC, within the mPFC that is in part responsible for the protective effects of controllability; that switching from appetitive to aversive stimuli association, and a reduction in dopamine, following chronic stress, influences choice by aversive associations; And lastly I describe how the concomitant downregulation of CaMKII, dopamine, and ERK following chronic stress lead to the degeneration of morphology and desensitisation to change in outcome value and contingency, with this downregulation less so in predicted stress rats, explaining their improved morphology and sensitivity. As originally stated these are all highly speculative, but highly testable, and I believe that such direction would reveal fruitful observations.

4.7. Conclusions

We now reach the end of this thesis, and across the four experiments performed herein there are multiple novel findings found from an exhaustively explorative study, within the realm of stress, learning, decision-making and histological science. It is observed that chronic stress changes

neuronal morphology in many regions that spread across the neo- and sub-cortex. From these morphological changes stressed rats change their decision making processes to bias habits, performing significantly worse on measures of goal directed action. As I have argued, I believe that these changes in decision making are the result of degeneration and proliferation to the various ROIs investigated, which is supported by correlational data. Further to this if a rat can predict the chronic stressor, they are offered a degree of controllability over the stress, and this controllability offers morphological protection of many ROIs and mediation to others. This protection to key regions, then explains the protected goal directed action seen in predicted stress rats. Rats that could predict the chronic stressor performed the same as controls on outcome devaluation and contingency degradation. This protective effect of prediction does not extend to all regions nor to all measures of decision making. Predicted stress rats still saw degeneration of many ROIs and an inflexibility in decision making, evidenced by their impaired performance on the delayed discounting task. In addition to this, stressed rats performed better on sPIT tasks, indicating that they are better able to extract information about a stimulus to guide decision making. Again, both the inflexibility and facilitated sPIT effect are argued to be due to the changes in morphology within the stressed animals. From these arguments we go on to explain how prediction and controllability over a stressor protect morphology. We raise explanations based in speculation, as further explorative research is required; among the many explanations, we covered topics from serotonin innovation, to a switch in how dopamine is innervating neurons, to more intracellular explanations involving the complex interaction between CaMKII, dopamine, GC, CRH, and ERK. What this makes clear, is that the research presented within this thesis offers not only new avenues of research aimed at explaining the effects observed in these predicted stress rats, but also new directions to study the effects of stress and by contrast disorders we use stress as a model for.

If we refer back to the beginning of this thesis, the philosophy proposed by Nissl and shared by this paper, that all maladaptations in behaviour can be explained by changes in morphology, then I believe that this thesis has exemplified this idea. Chronically stressed animals have degenerated

PLC, NaS, DMS, LO, VO, MO, ASS and HIP, with proliferated NaC, BLA and InC. These changes in morphology were then correlated with, and argued to explain the degeneration in goal directed behaviour; randomly stressed, but not predictably stressed, animals being insensitive to changes in outcome value and action-outcome contingencies, but facilitated in sPIT performance. Hence, changes in morphology appear to cause and to explain the changes in behaviour seen in our rats within this thesis. Further, reference to history sees that our development of the novel predicted stress model and histological methodologies presented herein were only achievable through the study of the work by Maier and colleagues, and Golgi and Cajal. Maier and Seligmans's original work on LH and the work that followed, allowed the inference of the predicted stress model as a congruent of controllability. And were it not for me taking Golgi and Cajal's original methods, and as many have done, attempting to develop them further, I would not be at the point where I was able to visualise neurons in their entirety within hours, within transparent tissue, with an unparalleled resolution, and a photoluminescent feature previously unrecorded. This work allowed the development of novel morphological measures to study regions previously never measured in chronically stressed rats (InC and CN) or regions which have not had their sub-regions investigated at all or only briefly (LO, VO, MO, NaS, NaC, DMS, DLS, PLC and IFC). This new and validating morphological work can only add to the reservoir of neuroscience knowledge. I expect that from this work, research would reveal further methods of stress treatment, both morphological and decision making manipulations, a greater understanding of the mechanisms of stress and controllability, and open up further novel histology and visualisation, as well as potentially bringing literature closer to an understanding of how and why the Golgi stain works, a question that has plagued researchers for over a century.

Postface

It is astonishing to find that we can manipulate the situational context and more or less completely ameliorate most of the effects of a robust chronic stressor. This protective effect does not change the stressor itself it changes how the animals experiences it. This change to the experience of the stressor, by increasing its salience and predictive strength, allows the animal to accommodate this stressor in a way that does not seemingly harm homeostasis, or at the least, affects homeostasis less than a random stressor. If we look at ourselves now, we know that work and many other factors in our life are causing high levels of stress (Medibank-Private, 2008, Sherry, 2013), but for many of us, these stressors are predictable, i.e. work, family, etc. It is likely that very early on in our development as a species, when early societies began, we understood that predicting a stressor is important to our health at least compared to a stressor that could not be predicted. But this defensive mechanism now reveals that it is mostly protective from neurological harm. Nevertheless, as shown here, there is still morphological change going on; change that is not present in control animals. This generates a very tangible notion, I believe, that although we have accommodated stressors into our society, it may be that these stressors are insidiously still causing neurological harm, and perhaps, we have engineered a society which has camouflaged any behavioural changes that would be the result of this morphological change. In addition to my suggestions for further scientific study, perhaps philosophical study may also benefit from this research, study into societal differences between the ways we accommodate stressors may reveal differences in behaviour and also morphology. However, I also feel that, from this work, we may also see new treatment options for suffers of anxiety and depression for which we use chronic stress as a model, taking benefit from the development of methods to use prediction, in a way, as a possible treatment to abate the morphological and behavioural effects associated with these disorders.

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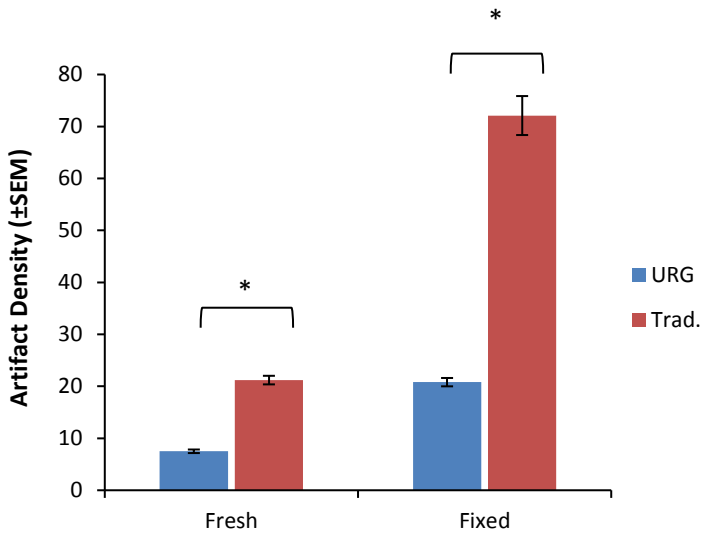
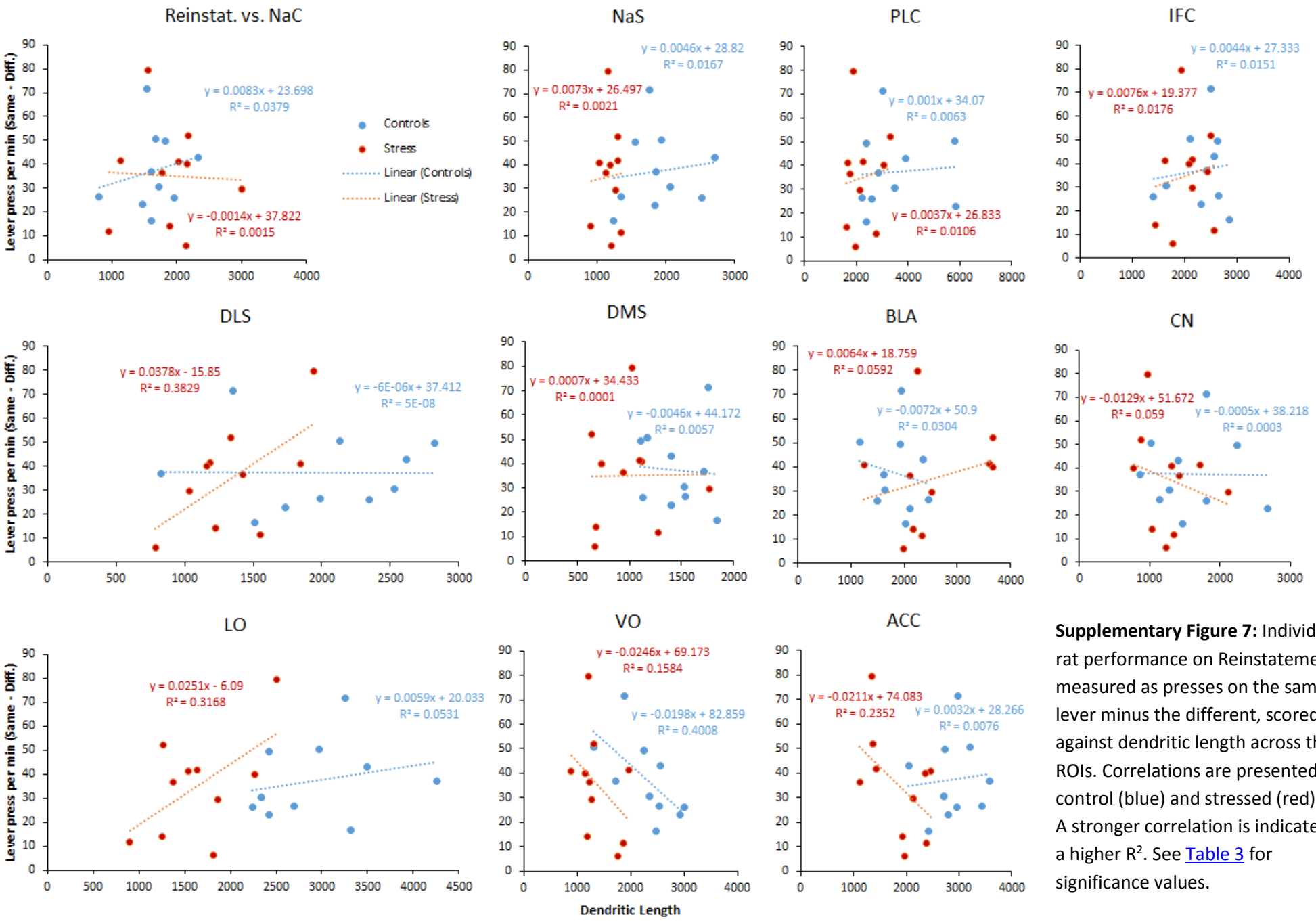
Appendix

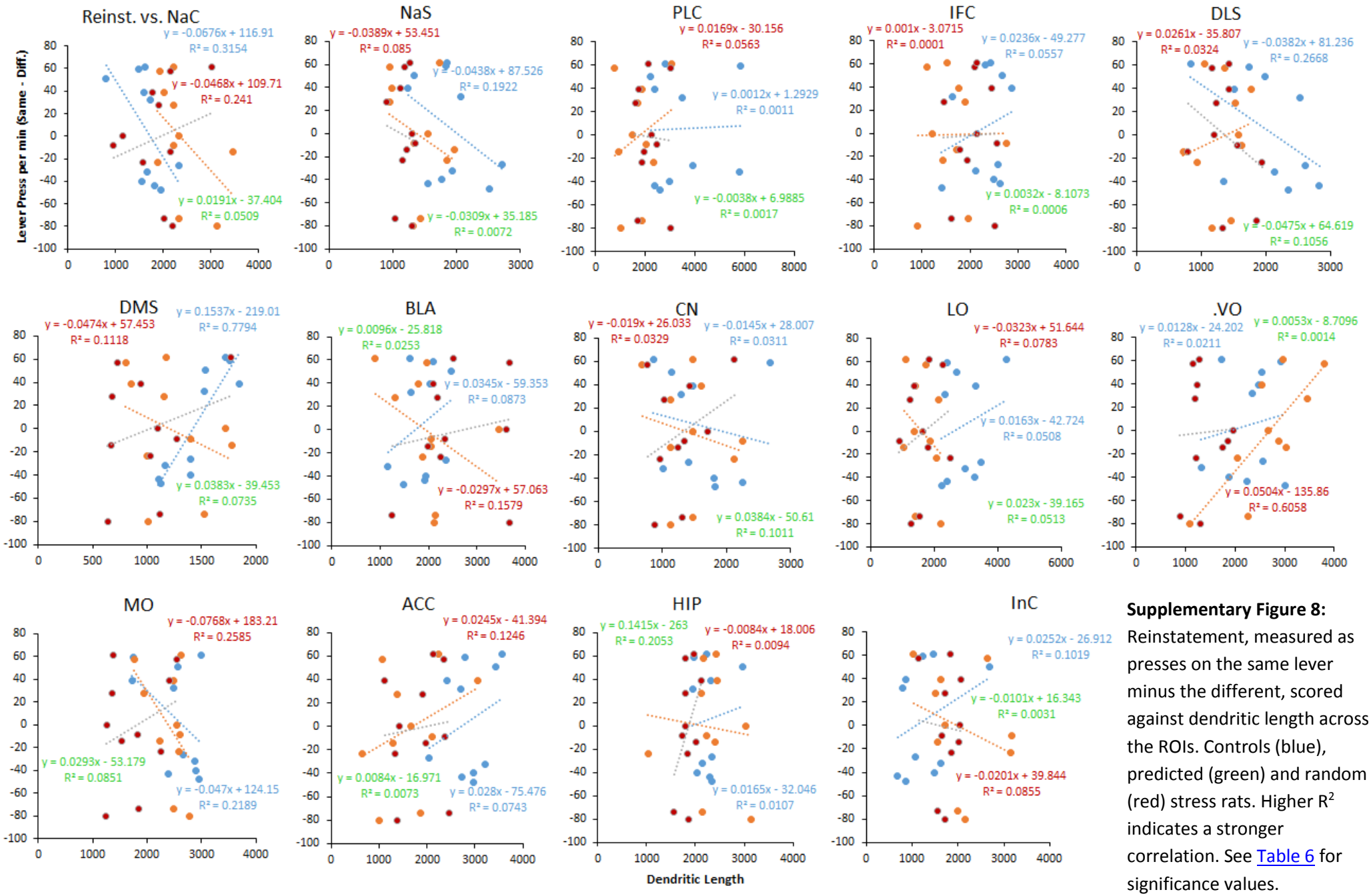
Supplementary Figures 1-5: Videos see attached files.



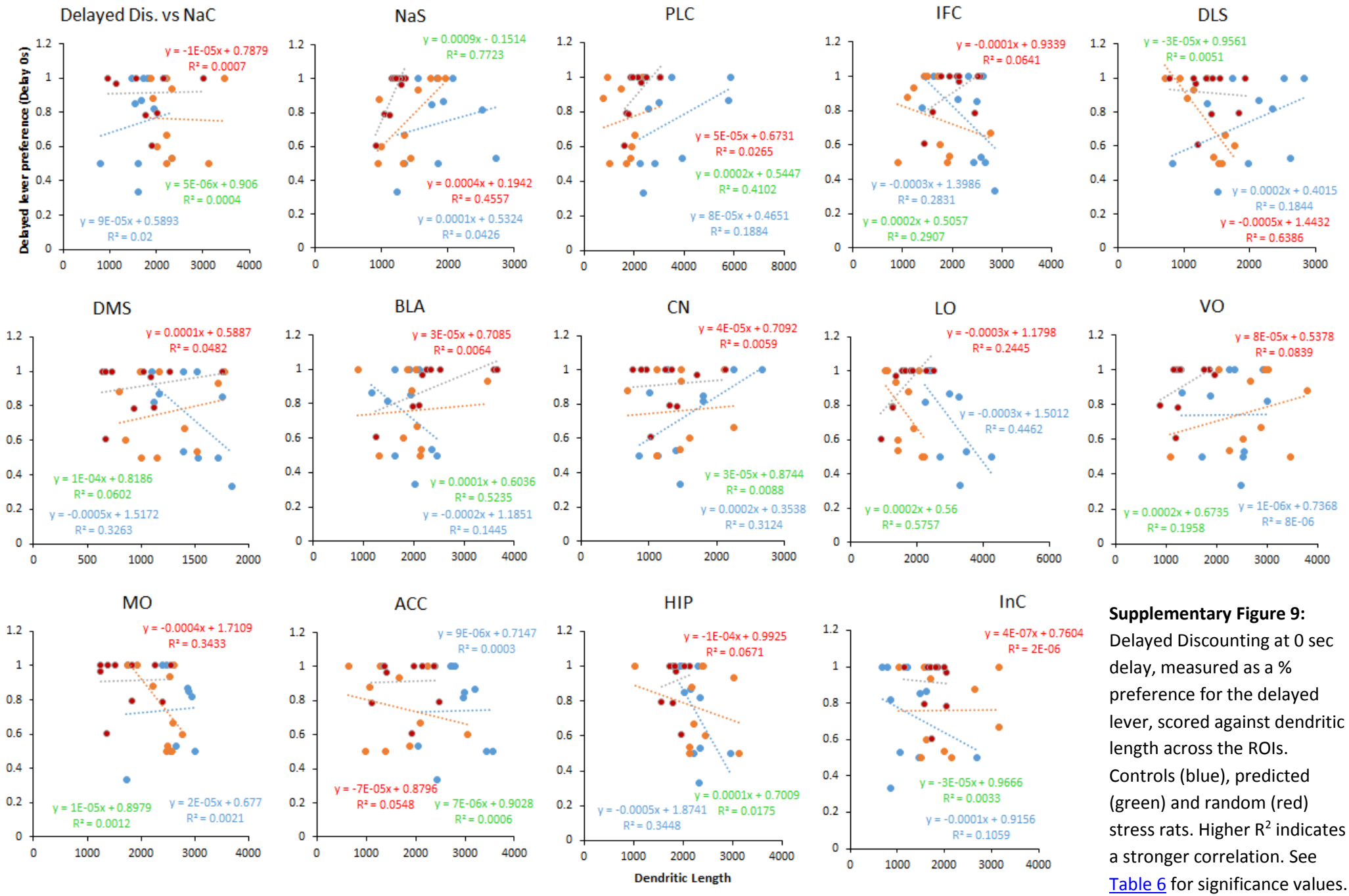
Supplementary Figure 6: Histogram illustrating the densities of artefact found between traditional Golgi stains (red) and the URG stain (blue). In both fresh and fixed tissue the URG has significantly less artefact. Further, the amount of artefact present within URG fixed tissue is comparable to the amount found within traditional stained fresh tissue.



Supplementary Figure 7: Individual rat performance on Reinstatement, measured as presses on the same lever minus the different, scored against dendritic length across the ROIs. Correlations are presented for control (blue) and stressed (red) rats. A stronger correlation is indicated by a higher R^2. See Table 3 for significance values.



Supplementary Figure 8: Reinstatement, measured as presses on the same lever minus the different, scored against dendritic length across the ROIs. Controls (blue), predicted (green) and random (red) stress rats. Higher R^2 indicates a stronger correlation. See Table 6 for significance values.



Supplementary Figure 9: Delayed Discounting at 0 sec delay, measured as a % preference for the delayed lever, scored against dendritic length across the ROIs. Controls (blue), predicted (green) and random (red) stress rats. Higher R^2 indicates a stronger correlation. See Table 6 for significance values.

Supplementary Figure 10: Delayed Discounting, at 30s delay, measured as a % preference for the delayed lever, scored against dendritic length across the ROIs. Controls (blue), predicted (green) and random (red) stress rats. Higher R^2 indicates a stronger correlation. See Table 6 for significance values.