Cortical and Brainstem Circuits Responsible for Pain Modulatory Responses in Healthy Humans

By **Lewis Crawford**

Faculty of Medicine and Health

The University of Sydney

March 2023

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Supervisor: Professor Luke A. Henderson

Table of Contents

Acknowledgements

I was once told completing a PhD is like digging into an iceberg with a teaspoon – each day goes by, and you think nothing really changes, and all of a sudden you look back and you've created a cavern. One pandemic, two years of industry engagement, and three manuscripts later and I can now say my digging is done. This thesis represents not just my work but is a testament to the guidance and support I've received along the way. To my mum Susan, my dad Robert, and their partners David and Louisa – thank you for always listening to my brain-based rants and instilling in me the qualities required to see this thesis through.

To my people I've worked with along the way – particularly the Neural Imaging Lab team: Rebecca, Fernando, Ash, Angela, and Emily – You were the first people I saw when I walked in Monday mornings and the last I saw on Friday evenings – our chats and coffee walks made even the longest days fun.

To Damien Boorman and Noemi Meylakh – the both of you quickly became mentors to me, guided my interest in the contents of this thesis and taught me the value of taking the time to understand before doing. Damien, as my placebro for life and Canada / USA compadre – I am consistently astounded by what you know and how you communicate it, and I am certain that one day I'll see your name up in lights in some beyond impressive position.

Professor Luke Henderson – the man who took a 3rd year university student on as a student in a neural imaging lab who had done next to no neuroanatomy – I'm not sure what you saw in me, but I am so incredibly grateful you gave me the opportunity to see the joys of research. I am beholden to your generosity and willingness to devote time and effort into my strange ideas – if I'm anything like you in 20 years I'll know I've done alright. I hope we can continue to work together for years to come.

Finally, to Emma Killalea, my best friend and partner – thank you for always putting up with me and putting a smile on my face – I honestly couldn't have done this without you.

Statement of Originality

I, *Lewis Sebastian Crawford*, certify that to the best of my knowledge, the content of this thesis constitutes 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.

Lewis Sebastian Crawford

Date: 21st of April, 2023

Authorship Attribution Statement

To the best of my knowledge, this thesis contains no material previously published or written by another person, except when referenced within the text.

Chapter 1 (introduction to placebo, nocebo, and neural imaging) includes sections that have been reproduced or adapted from the introduction of my Honours thesis, submitted to the University of Sydney in November 2019. Relevant sections are marked next to each of relevant section or subsection headings: * - reproduced, or # - adapted. Additionally, *Figure 1.6* and its corresponding legend has been reproduced from the peer-reviewed publication "The pain conductor: brainstem modulation in acute and chronic pain", which can also be found in full in Appendix B.

I am the primary author and Luke Henderson is the corresponding author for all publications included in this thesis. Permission to include the published material has been granted by the corresponding author.

Lewis Sebastian Crawford, 21 April 2023.

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are accurate.

Luke Anthony Henderson, 21 April 2023.

Publications contributing to this thesis

- Chapter 2 contains the research publication: **Crawford LS**, Mills EP, Hanson T, Macey PM, Glarin R, Macefield VG, Keay KA, & Henderson LA. (2021). *Brainstem mechanisms of pain modulation: a within-subjects 7T fMRI study of placebo analgesic and nocebo hyperalgesic responses*. Journal of Neuroscience, 41(47), pp.9794-9806. doi: 10.1523/JNEUROSCI.0806-21.2021

  CRediT: Conceptualization - LSC, EPM, KAK, LAH; Investigation - LSC, EPM, KAK, LAH; Resources - PMM, RG; Data analysis - LSC, TH, PMM; Writing - original draft - LSC

- Appendix A contains the research publication: **Crawford LS**, Mills EP, Meylakh N, Macey PM, Macefield VG, & Henderson LA. (2022). *Brain activity changes associated with pain perception variability*. Cerebral Cortex. doi: 10.1093/cercor/bhac332

  CRediT: Conceptualization - LSC, EPM, LAH; Investigation - LSC, NM, LAH; Resources - PMM, VGM; Data analysis - LSC, NM, PMM; Writing - original draft – LSC

- Appendix B contains the research publication: **Crawford LS**, Boorman DC, Keay KA, & Henderson LA. (2022). The pain conductor: brainstem modulation in acute and chronic pain. Curr Opin Support Palliat Care, 16(2), 71-77. doi: 10.1097/spc.0000000000000598

  CRediT: Conceptualization - LAH, KAK; Investigation - DB, LSC; Writing - original draft - LSC, DB

Other publications during candidature

The following manuscripts, whilst largely unrelated to the topics discussed in this thesis were published with my academic contribution throughout the course of my candidature:

- Doyen S, Taylor H, Nicholas P, Crawford LS, Young I, & Sughrue, ME. (2021). *Hollow-tree super: A directional and scalable approach for feature importance in boosted tree models.* PloS one, 16(10), e0258658. doi: 10.1371/journal.pone.0258658

- Doyen S, Nicholas P, Poologaindran A, Crawford LS., Young IM., Romero-Garcia R, & Sughrue ME. (2022). *Connectivity-based parcellation of normal and anatomically distorted human cerebral cortex.* Human Brain Mapping, 43(4), 1358-1369. doi: 10.1002/hbm.25728

- Robertson RV, Crawford LS, Meylakh N, Macey PM, Macefield VG, Keay KA, & Henderson LA. (2022). *Regional hypothalamic, amygdala, and midbrain periaqueductal gray matter recruitment during acute pain in awake humans: A 7-Tesla functional magnetic resonance imaging study*. NeuroImage, 259, p.119408. doi: 10.1016/j.neuroimage.2022.119408

- Mendoza FAT, Hughes TE, Robertson RV, Crawford LS, Meylakh N, Macey PM, Macefield VG, Keay KA, & Henderson, L.A. (2022). *Detailed organisation of the human midbrain periaqueductal gray revealed using ultra-high field magnetic resonance imaging.* NeuroImage, p.119828. doi: 10.1016/j.neuroimage.2022.119828

- Young IM, Dadario NB, Tanglay O, Chen E, Cook B, Taylor HM, Crawford LS, Yeung JT, Nicholas PJ, Doyen S, & Sughrue ME. (2023). *Connectivity Model of the Anatomic Substrates and Network Abnormalities in Major Depressive Disorder: A Coordinate Meta-Analysis of Resting-State Functional Connectivity.* Journal of Affective Disorders Reports, p.100478. doi: 10.1016/j.jadr.2023.100478

Conference presentations

2020:

Canadian Pain Society #Weareallinthistogether conference, 11th August (online)

- Free paper presentation: "Brainstem mechanisms of placebo and nocebo pain modulation"
- 3rd prize recipient ($200)

PAIN Conference, 14th-15th November (online)

- Poster presentation: "Subcortical Communication During Acute Pain Modulation: Altered Brainstem Connectivity Underpins Placebo Analgesia and Nocebo Hyperalgesia"

2021:

Australian Pain Society ASM, 19th-20th April (online)

- Poster presentation and oral recording: "CORTICO-BRAINSTEM INFLUENCES OF PLACEBO ANALGESIA: TOP-DOWN CONTACT OF THE MIDBRAIN PERIAQUEDUCTAL GRAY FACILITATES ENDOGENOUS PAIN INHIBITION"

2022:

Australian Pain Society ASM, 10th-13th April (Hobart, Tasmania)

- Invited speaker for topical session presentation: "Placebo and nocebo from the lab to the hospital: State of the field and implementing procedures to drive therapeutic benefits."
- Free paper presentation: "A tale of two connectivities: Cortico-midbrain pathways mediating placebo responsivity in humans"
- Winner of the best free paper award ($2000)

IASP World Congress, 19th-23rd September (Toronto, Canada)

- Poster presentation: "A tale of two connectivities: twin cortico-brainstem mechanisms mediating placebo analgesia"

2023:

Australian Pain Society ASM, 2nd-5th April (Canberra, ACT)

- Poster presentation and rapid communication session: "Pain relief and uncertainty: How pain rating variability shapes the acquisition, expression, and neural representations of placebo analgesia – a Functional Imaging and Magnetic Resonance Spectroscopy study."

List of abbreviations

^{1}H-MRS	–	Proton magnetic resonance spectroscopy
dlPFC	–	Dorsolateral prefrontal cortex
FDA	–	United states food and drug administration
RCT	–	Randomized controlled trial
MCID	–	Minimum clinically important difference
VTA	–	Ventral tegmental area
SN	–	Substantia nigra
CCK	–	Cholecystokinin
PNS	–	Peripheral nervous system
STT	–	Spinothalamic tract
VPL	–	Ventral posterolateral nucleus of the thalamus
S1	–	Primary somatosensory cortex
MD	–	Mediodorsal nucleus of the thalamus
OFC	–	Orbitofrontal cortex
rACC	–	Rostral anterior cingulate cortex
SMA	–	Supplementary motor area
AG	–	Angular gyrus
rTMS	–	Repetitive transcranial magnetic stimulation
tDCS	–	Transcranial direct-current stimulation
NAc	–	Nucleus accumbens
PET	–	Positron emission tomography
PAG	–	Periaqueductal gray

PB	–	Parabrachial complex
LC	–	Locus coeruleus
RVM	–	Rostral ventromedial medulla
SRD	–	Subnucleus reticularis dorsalis
MRI	–	Magnetic resonance imaging
fMRI	–	Functional magnetic resonance imaging
vlPAG	–	Ventrolateral column of the periaqueductal gray
7T	–	7 Tesla
DH	–	Dorsal horn of the spinal cord
SpV	–	Spinal trigeminal nucleus
CB1	–	Cannabinoid receptor type 1
VS	–	Ventral striatum
BOLD	–	Blood oxygen level dependent
SPM	–	Statistical parametric mapping
SUIT	–	Spatially unbiased infratentorial template
HRF	–	Hemodynamic response function
CSF	–	Cerebrospinal fluid
7T	–	Seven tesla
FC	–	Functional connectivity
PPI	–	Psycho-physiological interaction
DCM	–	Dynamic Causal Modelling
NPS	–	Neurological pain signature
vmPFC	–	Ventromedial prefrontal cortex
TPJ	–	Temporoparietal junction

PH	–	Posterior hypothalamus
MeA	–	Medial amygdaloid nucleus
GABA	–	Gamma aminobutyric acid
SpV	–	Spinal trigeminal nucleus
PCC	–	Posterior cingulate cortex
DMN	–	Default mode network
RVLM	–	Rostral ventrolateral medulla
CPM	–	Conditioned pain modulation
SD	–	Standard deviation
VAS	–	Visual analogue scale
HPA	–	Hypothalamic-pituitary-adrenal
DIANA	–	Direct imaging of neuronal activity

Abstract

The human ability to regulate our own pain is governed by specific sites and circuits within the brain which can powerfully inhibit or enhance nociception. Placebo analgesia and nocebo hyperalgesia are the modulatory phenomena which leverage these circuits in the presence of a pharmacologically inert treatment to cause perceived changes in pain. Being pharmacologically inert, treatments which incorporate placebo components, and avoid falling pitfall to nocebo effects, have enormous therapeutic potential to alleviate economic costs, clinical comorbidities, and psychological burdens associated with many active treatments if correctly harnessed. Despite their early discovery and prominence throughout history, a thorough investigation of the neural and potential biochemical inputs which drive placebo analgesia and nocebo hyperalgesia remains to be established. Therefore, the principal aim of this thesis was to utilize recent advancements in high field human brain imaging to assess the responsibility of descending pain-modulatory circuits within the brainstem, as well as the cortical connections which recruit these circuits in the generation of placebo analgesia and nocebo hyperalgesia.

Chapter 2 establishes the brainstem's role in both phenomena. We utilized a response conditioning model and a brainstem-specific imaging pipeline to reveal how activation within discrete nuclei altered depending on the intensity of placebo and nocebo responses. Building on this work, Chapter 3 presents a dual network model of the human cortical sites which regulate brainstem output in the context of placebo analgesia. Relative to chapter 2, this work included a larger sample size, a higher placebo response rate, and analyses sensitive to how cortical connections to the brainstem change across time. Chapter 4 bridges function and biochemistry, circumventing limitations in functional magnetic resonance imaging by incorporating proton magnetic resonance spectroscopy (^{1}H-MRS) to investigate how metabolite concentrations within the dorsolateral prefrontal cortex (dlPFC) - a primary node in the cortical pain system - play a role in the generation of placebo analgesia. I conclude by discussing the clinical and experimental implications of our three studies, with a focus on how further interrogation of the circuits revealed could aid and assist in the development of new approaches that treat chronic pain, by leveraging the neural mechanisms of placebo analgesia and nocebo hyperalgesia.

Chapter 1:

Introduction to Placebo, Nocebo, and Pain Modulation

"Believing in even the possibility of a
happy ending is a powerful thing"
– Cinderella, 1950

1.1 Placebo analgesia and nocebo hyperalgesia: a historic perspective

Placebo analgesia and its negative counterpart, nocebo hyperalgesia, have a rich history extending throughout medical and non-medical contexts. Despite not formally being appreciated by the U.S. Food and Drug Administration (FDA) until 1962 (Katz, 2004), use of the term "placebo" in a medical context extends back to the 18th century, and its original etymology dates even earlier. A Latin mistranslation of Psalm 116:9 provides the first use of the word, the verse reading: "placebo domino in regione vivorum" ("I will please the lord in the land of the living"). A catholic funeral rite – Vespers for the Dead – consisted of mourners unrelated to the departed, who were said to "sing placebos" in their praise in order to earn a spot at the subsequent funeral dinner (Bernstein and Brown, 2017).

Beyond biblical associations, the word "placebo" first appeared medically in the late 18th century, where English physician Alexander Sutherland's used the term to ridicule those at the time practicing in the water cure, calling it "fashionable physician placebo" (Jilch et al., 2020). Within the following decade a major shift occurred – instead of referring to the prescriber, placebo began to refer to the substance being prescribed. It was Scottish pharmacologist William Cullen who lay a framework for the placebo phenomenon, believing that inert substances could be administered to comfort or please a patient, and that the mind-body interaction could provide great curative benefit even if the treatment was sham (Knoff, 1970, Kerr et al., 2008). Indeed, many of his tenets hold true with our understanding of placebo analgesia today. By 1811 the term placebo had become a mainstay in medical jargon, and its inclusion into the Quincey's Lexicon-Medicum, a well-regarded medical dictionary, labelled it as "an epithet given to any medicine adapted more to please than to benefit the patient" (Hooper, 1817).

Throughout 18th and 19th century Europe, the first placebo-controlled trials started being conducted to expose quackery as it arose, halting those seeking to make financial gain from the hope of misinformed patients. Elisha Perkin's patented "perkin's tractors" and Franz Anton Mesmer's "animal magnetism" present two particularly engaging tales – the first led by Dr John Haygarth, a practicing British physician at the time, and the second by Benjamin Franklin, United States founding father and ambassador to France (Lopez, 1993, Lanska, 2019). In investigating 5 patients, Haygarth tested Perkin's famous tractors - metallic wand-like instruments said to draw out "noxious electrical fluid that lay at the root of all suffering" against a placebo instrument - wooden rods painted to replicate the tractors. 4 out of 5 patients responded identically to both sets of instruments, and similar results were observed whether the instruments differed in shape, size, or composition (Hines, 2017). Haygarth published these

results in a wounding pamphlet: "On the Imagination as a Cause and Cure of Disease of the Body exemplified by fictitious Tractors and epidemical Convulsions", citing that the cure itself came not from the tractors, but by a patient's belief in their effectiveness (Booth, 2005).

Franz Anton Mesmer's discreditation also came by the hand of a placebo controlled trial, wielded by Benjamin Franklin and a commission of French scientists in 1782. In a series of experiments, the commission tested Mesmer's theory of "animal magnetism", where an invisible force "*Lebensmagnetismus*" could be called upon to heal any physical ailment (Best et al., 2003). By providing participants with various bowls of water which they believed had been "magnetized" by Mesmer's disciples and observing the varied effectiveness, Franklin and the commission came to conclude that any mitigation of symptoms induced by "animal magnetism" were in fact induced through the power of patients' own minds. Upon publishing their assessment, a pupil of Mesmer, Charles d'Elson famously wrote "the imagination thus directed to the relief of suffering humanity would be a most valuable means in the hands of the medical profession" – a goal still echoing through modern placebo (and nocebo) research (Gravitz, 1994).

It would not be until the 20th century however that this goal was even considered – when American anesthesiologist and war-time physician Henry Beecher asserted the effectiveness of placebo treatments in his controversial yet critical meta-analysis "The Powerful Placebo" (Beecher, 1955). Fifteen studies were included in his analysis, where in 1082 participants, Beecher claimed that 35% were satisfactorily relieved by placebo administration alone. Despite ongoing dispute regarding the interpretation of his analysis, Beecher's work undoubtedly brought placebo into the modern era of research – and indeed was the primary inspiration for the 1962 FDA Kefauver-Harris amendments, which requires any new drug to be tested in a placebo-controlled environment and exceed its effectiveness (Greene and Podolsky, 2012).

Borrowing from placebo's Latin etymology, American clinician Walter Kennedy coined the term "Nocebo" ("I will harm") in his 1961 commentary: "The Nocebo Reaction" (Kennedy, 1961). Whilst mechanistically similar to the placebo effect – relying on expectations and mind-body interactions, the nocebo response stands in exact antithesis to the primary goal of placebo. Instead of alleviating potential symptoms, nocebo responses are a worsening in subjective or physiological symptoms that can arise by the administration of a pharmacologically inert substance. Formal and historical documentation of nocebo responses are scarce relative to its placebo counterpart – however the notion that negative information surrounding a treatment can inadvertently harm a patient's clinical trajectory is well-documented (Long et al., 1989, Tangrea et al., 1994, Drici et al., 1995, Barsky et al., 2002). In one example, Australian physician

Gerald Milton provides an account of his experiences delivering prognoses of melanoma to patients "... there is a small group of patients in whom the realization of impending death is a blow so terrible that they are quite unable to adjust to it, and they die rapidly before the malignancy seems to have developed enough to cause death" (Milton, 1973).

With the advent of human brain imaging, our understanding of the neural underpinnings of placebo and nocebo pain modulatory phenomena has deepened, and now includes an understanding of the cortical and brainstem regions (Freeman et al., 2015) and neurotransmitters involved (Petrovic et al., 2002, Benedetti et al., 2006). This thesis includes three investigations which we have performed that provide advancements to the field in understanding the neural underpinnings of placebo analgesia and nocebo hyperalgesia. Throughout these investigations we assess the specific neural changes – within both the brainstem and associated cortical connections - associated with these phenomena, as well as a potential predetermining factor in how likely an individual is in mounting pain modulatory responses. However, before any recommendations can be made in terms of *how* these studies help advance the field, it is important to first understand *why* we seek to continually update and improve our knowledge on placebo analgesia and nocebo hyperalgesia. That is, their potential to drastically alter how we view and conduct active drug trials and even clinical pain treatments.

1.2 Clinical relevance of placebo (and nocebo) responses

1.2.1 Placebo effects: the curse of Randomized Controlled Trials (RCT)

Placebo effects are complex and multifaceted in their origin – undoubtedly playing a contributing role in a swathe of clinical, psychological, and pharmacological treatment effects. Their prominence has been long debated, with reports and meta-analyses asserting that they contribute either negligibly (Hróbjartsson and Gøtzsche, 2010), or perform better than active treatments in specific scenarios (Howick et al., 2013).

In the words of Wampold et al. (2007), the "villain" of RCT's is the placebo effect. That is, since the induction of the Kefauver-Harris amendments, improvements observed in the placebo group of RCT's have complicated the approval process of drugs which may have been resoundingly effective in preclinical phases, only to show minimal benefit over a placebo arm in human clinical trials. This active- to placebo arm differential must exceed what is known as a "minimum clinically important difference" (MCID), meaning not only must the treatment effect be significant, but it

must be at least as large as the MCID (Chuang-Stein et al., 2011). This means that to reliably discern the effectiveness of an active drug, it is first important to dissect out the exact effect size of the placebo itself. This represents one core goal of placebo research – how effective are sham substances in causing real changes in symptomatology. Due to a number of compounding factors such as natural history (fluctuations in symptoms being tested over time without any treatment), Hawthorne effects (where individuals alter their behaviour or symptom reports when they are aware of their involvement in a clinical trial), or regressions to the mean (a statistical phenomenon where repeated measurements cause data points to become less extreme over time), it is not enough to just compare active to placebo interventions, but to first understand how placebo's work whilst controlling for these *no treatment* effects present in any experimental or patient population (Ernst and Resch, 1995) (Figure 1.1).

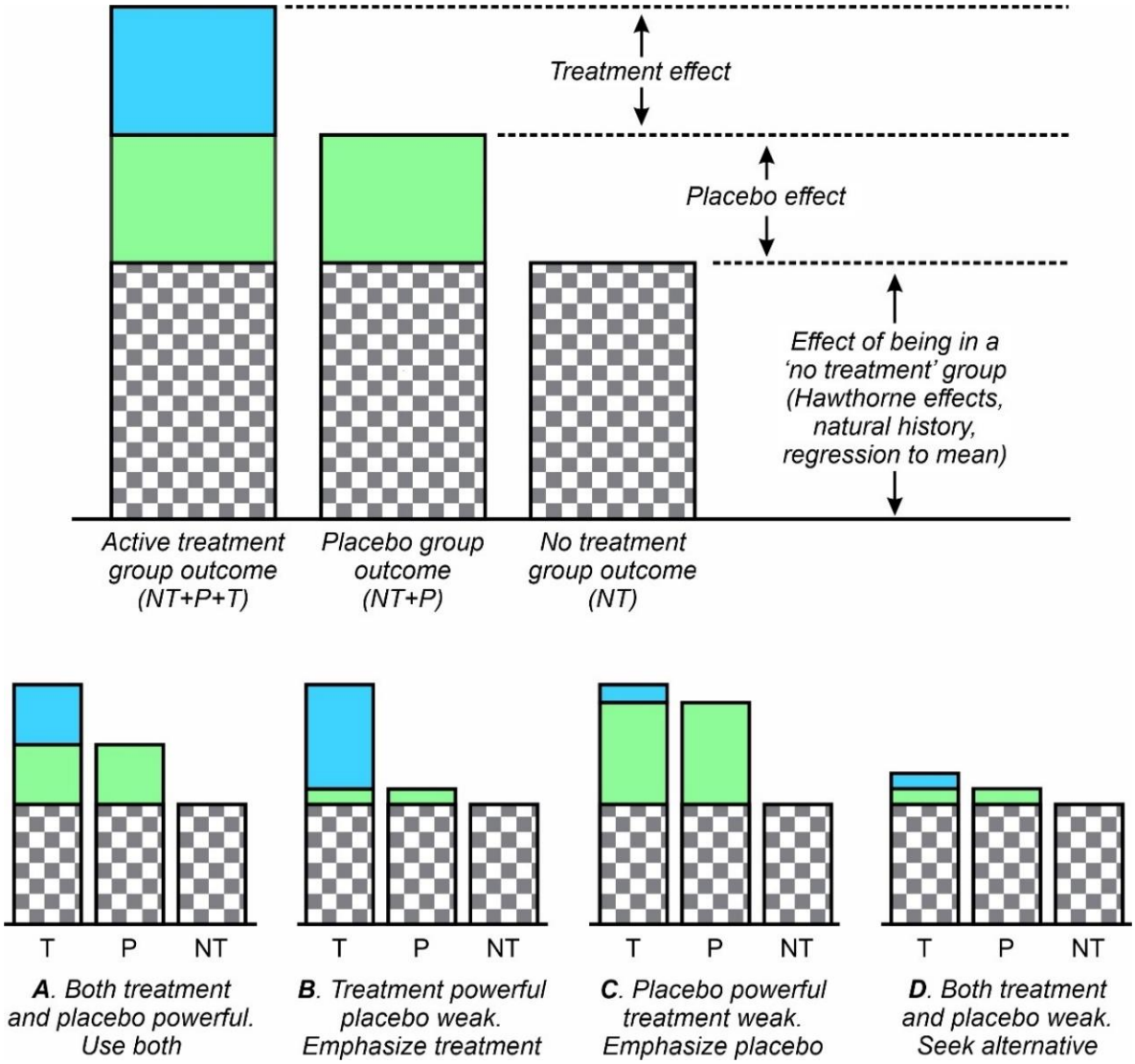

Figure 1.1. Trial design to dissect treatment and placebo effects. Any positive effect induced by a treatment cannot be solely attributed to its active properties, but rather it is an interplay between these active properties with any placebo (positive cues, expectations, prior experiences) and no treatment effects (Hawthorne effects, spontaneous remission / natural history, or regression to the mean) which may be present in any given clinical context or patient population. Integral to understanding the effectiveness of a treatment, and which path of treatment to pursue to maximize therapeutic gain is dissecting out the relative contribution of these three factors. *T – Treatment, P – Placebo, NT – No treatment. Adapted from Howick et al. (2013)*.

Experimental research is uniquely positioned to help advance our understanding of how great a contribution placebo effects offer in the treatment of pain. By being able to precisely control noxious stimulus delivery, participant recruitment, and the model of pain being tested – research settings offer the ability to circumvent confounding factors and specifically test for individual differences in the manifestation of pain modulatory phenomena (Benedetti and Frisaldi, 2014). Typically, reported outcomes (pain rating responses), are compared between a control and placebo (or nocebo) condition within the same set of individuals – so that any difference in pain reported can be clearly attributed to endogenous pain modulatory responses. Studies following protocols of this nature generally support Beecher's original assumptions, identifying significant placebo responses develop in roughly 30-50% of participants (Levine and Gordon, 1984, Benedetti, 1996, Vachon-Presseau et al., 2018, Wang et al., 2022b). What makes a person respond to any given placebo treatment is subject to ongoing debate, however the effectiveness of conditioning procedures, expectations of a treatment's effectiveness, and environmental and genetic factors have emerged as consistent explanations. These three factors will be discussed further in section 1.3 of this thesis, however what ties all three together is that underlying changes in neural activity ultimately drive how an individual perceives pain (Levine et al., 1978, Benedetti et al., 2006, Wager et al., 2007, Scott et al., 2008, Benedetti and Amanzio, 2013, Tinnermann et al., 2017, Schafer et al., 2018).

Interestingly, not only do placebo and "no treatment" effects have an underlying presence in clinical trials, but Nocebo effects can also have a negative impact on the effectiveness of any treatment being tested (McDonald et al., 1983, Whitney and Von Korff, 1992, Hróbjartsson et al., 2011, Goldenholz et al., 2015, Berthelot et al., 2019). Factors such as expectancy and environment can contribute to an anxiogenic environment - making it difficult to accurately measure the effectiveness of a treatment in relieving symptoms. Just as placebo effects have

the potential to disguise the true effectiveness of a treatment by providing added benefit over and above its expected pharmacological potential, nocebo effects may inhibit its true effectiveness, by either exacerbating or creating entirely new symptoms due to inter-individual differences in prior experiences or expectations towards a treatment (Benedetti et al., 2006, Colloca and Miller, 2011). The largest source of nocebo effects in clinical practice comes from the process of informed consent: the requirement of clinicians to detail any potential side-effects which may arise from the administration of active medications. Indeed, in the case of migraine, Amanzio et al. (2009) demonstrated that even in the placebo arm of clinical trials, adverse side effects are often generated matching those which define the active drug under investigation. Similar reports are found in the context of chronic pain, with numerous accounts of clinical intervention to treat chronic pain conditions counter-intuitively causing an exacerbation of pre-existing symptoms (Daniels and Sallie, 1981, Long et al., 1989, Pfingsten et al., 2001).

In a clinical trial setting, Nocebo effects play a negative role with respect to retention rate of patients. Häuser et al. (2012) identified nocebo responses accounted for 72% of mid trial dropouts across 30 RCT's using active drugs to treat fibromyalgia and peripheral neuropathy. Drop out due to adverse events relating to nocebo responses have also been observed in migraine, multiple system atrophy, and have even been proposed to influence vaccination adherence (Mitsikostas et al., 2011, Amanzio et al., 2021, Wang et al., 2022c). Nocebo effects are pervasive across many clinical domains extending far beyond the RCT; however, it is in this particular setting that they have the potential to cause the most significant issues. Not only can their presence impact the perceived efficacy of the real drug being assessed, but they can also incur additional costs associated with managing patient dropout. Nocebo effects are not easily controlled, as the underlying principles which potentiate them - clinical contexts and prior experiences - can vary greatly between individuals and are not easily assessable before starting treatment. It is for this reason that many advocate for the need to better understand the neurobiological mechanisms of the nocebo effect, and minimize their occurrence in the RCT environment (Planès et al., 2016, Jilch et al., 2020).

Modern human brain imaging provides us with the ability to better understanding placebo and nocebo phenomenon. It is important that we begin to define the circuitry underlying the brains' ability to endogenously inhibit or enhance the perception of pain, and how we can potentially harness this circuitry to bolster positive and reduce negative effects in both clinical and experimental settings.

1.2.2 Harnessing placebo responses to aid clinical practice

We have known since the unmasking of Perkins and Mesmer that the human brain is capable of powerfully converting our own expectations, beliefs, and prior experiences into modifications of conscious perception. Experimentally, the placebo is more than just an inert pill, but rather a proverbial key to unlock these factors and cause a shift in the way we process pain. In hospital settings however, the clinician can become the placebo, capable of significantly altering the effectiveness of any given treatment through verbal and nonverbal cues or contexts which may favour either placebo or nocebo responses. Thomas (1987) was one of the first to acknowledge the influence of the patient-practitioner interaction for causing shifts in treatment outcome. In his experiment, 200 patients were assigned to either a "positive" or "non-positive" manner consultation with or without placebo administration. In this 2 x 2 factorial design, a positive manner consultation involved confident statements as to the diagnosis and timeline of symptom improvement, whereas a lack of certainty and a neutral tone defined the non-positive consultations. Somewhat surprisingly, no significant difference in patient improvement or satisfaction was observed between the pooled treated (with a placebo) vs non-treated groups. However, whilst it appeared the placebo alone played a minor role in producing symptom relief, it was the placebo in the hands of a positive clinician that induced the greatest improvements. Sixty four percent of participants in the positive consultation group (compared to 39% in the non-positive consultation) improved after two weeks - advocating for the role of a positive patient-practitioner relationship in enhancing placebo- and minimizing nocebo responses.

Substantial work has now been performed detailing optimal parameters for bolstering placebo responses in clinical medicine. From the white coat of a practitioner providing expectations of improvement, to the colour, size, or branding of a medication inducing greater belief that the treatment will be effective – these ritualistic influences all integrate within the clinical context, manifesting brain-body interactions that produce placebo (and nocebo) effects (Figure 1.2) (Bingel et al., 2011, Blasini et al., 2018, Meissner and Linde, 2018). In the context of pain, the interaction between treatment effects and placebo and nocebo responses can be considered under a Bayesian hierarchy. Current symptoms (priors) are modulated by the relative effects of the patient-practitioner relationship (likelihood) - be it expectations, prior experiences, or other ritualistic effects - to cause neurobiological changes that either positively or negatively influence those symptoms (posterior). These changes, driven by higher cortical regions, alter the way in which pain is being processed in a top-down system involving the cortex, brainstem, and spinal cord (Eippert et al., 2009b, Büchel et al., 2014, Blasini et al., 2017, Geuter et al., 2017).

At the inception of this thesis, whilst a number of experiments had been performed describing the role of cortical sites and various conditioning and expectancy-based protocols for manifesting placebo analgesia and nocebo hyperalgesia, scant literature existed specifically identifying the role of discrete brainstem nuclei underlying these two phenomena. Since the brainstem both reciprocally connects with both the cortex and spinal cord and has been demonstrated consistently in experimental animals to be capable of producing profound changes in pain-related behaviours, leveraging advanced human brain imaging to investigate the interaction between brainstem activation and pain modulatory phenomena was the next step in better understanding their neurobiological tenets (Mayer et al., 1971, Cannon et al., 1982, Le Bars et al., 1992, Zhuo and Gebhart, 1997, Cauzzo et al., 2022). Importantly, if bolstering placebo effects and limiting nocebo effects are to be routinely utilized in patient-settings, a complete understanding of their neural underpinnings, relative rate of occurrence, and any individual predisposing factors are necessary in bringing them into everyday practice.

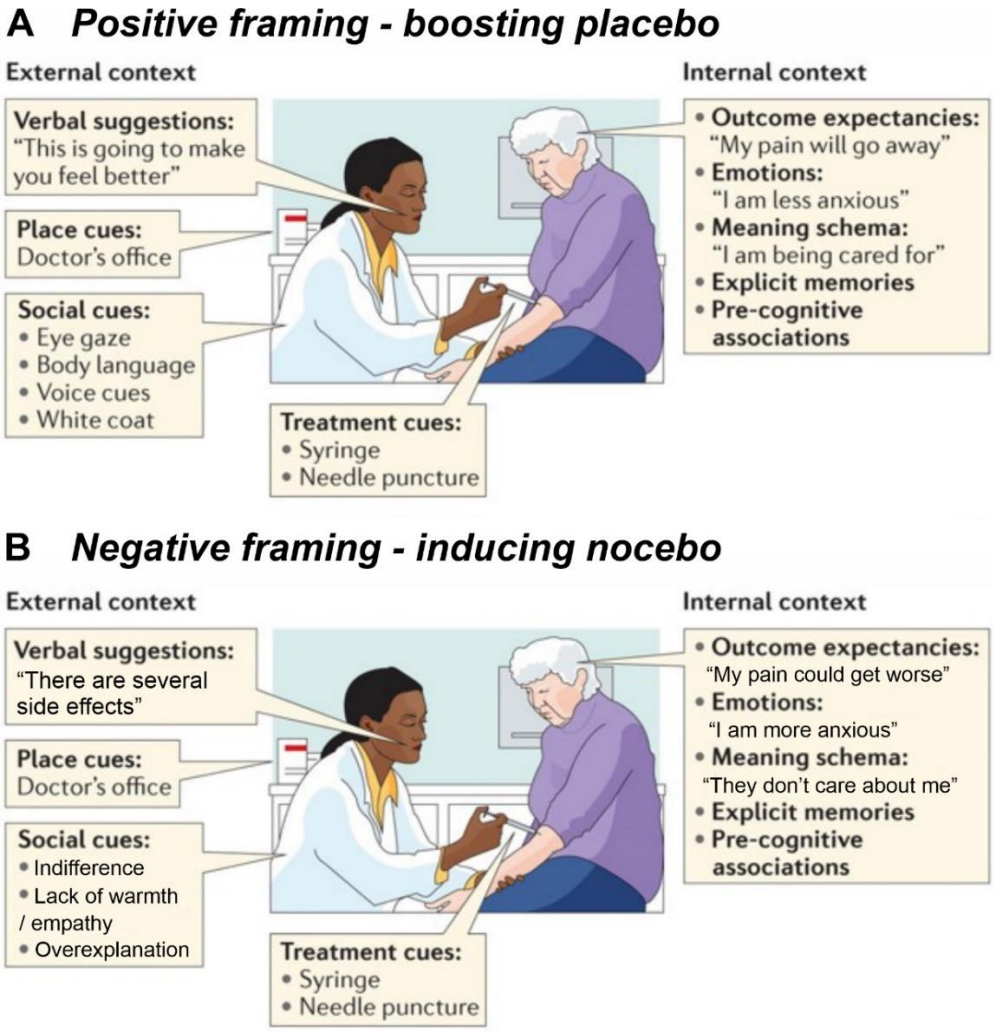

Figure 1.2. Placebo and nocebo effects in the clinic. All patient-practitioner interactions are subject to (whether directly or indirectly generated) placebo and nocebo effects. How a treatment is framed, the demeanour of the physician, and patient attitudes to potential treatment effects and side effects are three prominent sources of these modulatory phenomenon in clinical practice. **A.** Positive verbal suggestions that the treatment will help reduce symptom severity, as well as social warmth and clinical professionalism are all ways to boost the potential for placebo effects. **B.** Inverting these factors – for instance explaining a treatment in terms of its potential side effects and not attempting to connect with patients can create an anxiogenic treatment environment and potentiate nocebo effects. *Adapted from Wager and Atlas (2015)*

1.3 Factors contributing to endogenous pain modulation

1.3.1 Classical conditioning

Whilst Henry Beecher is considered the "father of placebo", one of the three competing theories for the human ability to endogenously modulate our own pain perception draws from one of the founders in the discipline of psychology: classical conditioning and Pavlov's Dog. Conditioning theory dictates that placebo analgesia and nocebo hyperalgesia are generated via associative learning acquired during prior exposures to analgesic drugs and their aversive consequences (Price et al., 2006, Colloca et al., 2010, Planès et al., 2016).

Across a series of experiments, Nicholas Voudouris documented the existence of this phenomenon in healthy humans, demonstrating that the deceptive manipulation of thermal stimuli applied alongside a placebo cream described as a "fast-acting analgesic preparation" could reduce perceived pain relative to an adjacent skin site (Voudouris et al., 1985, Voudouris et al., 1989, Voudouris et al., 1990). This experimental approach, now known as response conditioning, follows canonical *conditioning*, *reinforcement*, and *test* phases which have since been routinely applied to elicit both placebo and nocebo responses in both humans and experimental animals (Jensen et al., 2012, Freeman et al., 2015, Schafer et al., 2015, Bräscher et al., 2017). By first surreptitiously lowering painful stimuli applied to a placebo substance described to hold analgesic properties, participants acquire an association between pain-relief and the placebo (i.e. conditioning and reinforcement), which can then be expressed in a subsequent test session when painful stimuli are raised to match a control condition (typically an adjacent skin site where a non-placebo control substance was applied). This same protocol using surreptitious high

intensity stimuli can also be utilized to trigger nocebo hyperalgesia (Figure 1.3). Recently, Bajcar et al. (2020) demonstrated that deceptive conditioning of heightened pain responses to a visual cue can trigger subsequent hyperalgesia without the involvement of expectations. Their work eloquently controlled for any potential expectancy effects by including both open- and deceptively conditioned groups, where no difference in the elicited nocebo response was identified whether the association between visual cue and incoming painful stimuli was known to participants. Importantly, they also noted no correlates with psychometric data, supporting the sole influence of classical conditioning in this phenomenon.

Both conditioning-based placebo and nocebo responses have now been shown to relate to changes in neural activity, often within largely similar regions of the cortex, brainstem, and spinal cord – suggesting that a shared neural mechanism involving descending cortical influence over spinally-projecting brainstem circuits is likely involved (Eippert et al., 2009a, Yoshida et al., 2013, Tinnermann et al., 2017).

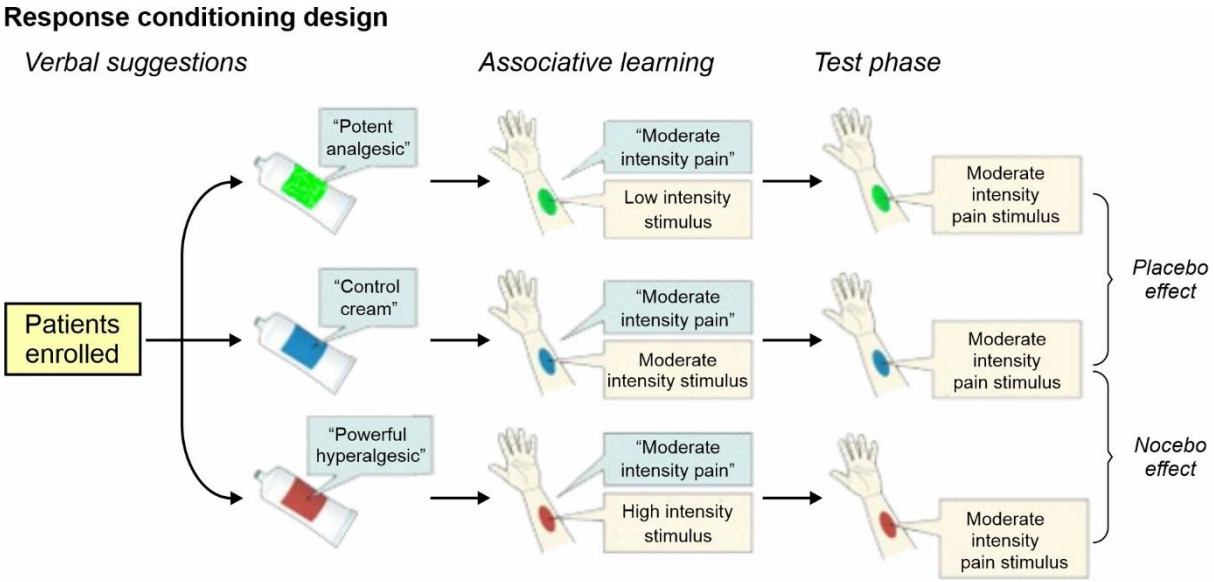

Figure 1.3 Response conditioning. Through a combination of deceptive descriptions and visual cues, response conditioning involves pairing pharmacologically inert substances with lower or higher intensity stimuli relative to a control condition to trigger placebo or nocebo effects, respectively. In these designs, participants are informed that these substances hold analgesic (placebo) or hyperalgesic (nocebo) properties, and that all conditions are receiving identical intensity stimuli. In reality, the experimenter deceptively alters the intensity of stimuli applied to trigger belief that the substances hold their described properties, conditioning participants to respond accordingly in a future test session. In the test session however, both the placebo (or nocebo) and control conditions receive identical moderate intensity stimuli, and differences in reported pain between conditions indicate a magnitude of placebo analgesia or nocebo hyperalgesia. *Adapted from Wager and Atlas (2015).*

1.3.2 Expectancy

The second school of thought is that placebo analgesia and nocebo hyperalgesia manifest as a result of positive and negative expectations towards an inert substance, respectively. Expectance theory differentiates itself from classical conditioning in that prior exposures and deceptive modulation of noxious stimuli are not necessary for triggering the endogenous modulation of pain. Rather, the context of the given treatment combined with situational cues combine to alter an individual's perception of pain. Interestingly, although not explicitly named, in their first experiment Voudouris et al. (1985) established the importance of expectations when attempting to investigate conditioning-based analgesia. That is, even when the cream applied was described to hold *analgesic* properties, if during conditioning the intensity of noxious stimuli applied to that placebo was surreptitiously increased, the violation of positive expectations generated prior to conditioning could trigger heightened pain responses. Indeed, this finding is what motivated early work by Montgomery and Kirsch (1997), who demonstrated that conditioned analgesic responses could be abolished if the expectation of receiving pain relief was removed.

Modern approaches leveraging expectations to produce pain modulatory effects have found that both analgesia and hyperalgesia can be formed devoid from conditioning. When participants observe a confederate acting as if they have experienced profound pain relief from a treatment, placebo analgesia can be generated in some individuals (socially-acquired placebo) at a similar magnitude if a conditioning procedure was used (Colloca and Benedetti, 2009,

Schenk and Colloca, 2019). Similarly, Tinnermann et al. (2017) demonstrated that greater nocebo effects can be generated by modulating participants expectations through value cues and statements. By presenting two different sham nocebo substances: one described as and appearing expensive and the other cheap, they found greater hyperalgesic pain responses to the "expensive" nocebo, alongside altered activation in cognitive pain processing brain regions and in the spinal cord.

Whilst in the past conditioning and expectancy existed as two distinct theories to explain placebo and nocebo effects, it is now widely accepted that the two intermingle, together playing a shared role in inhibiting and enhancing perceived pain (Wager and Atlas, 2015, Schafer et al., 2018, Tu et al., 2022). Indeed, it is difficult to explain the effects of conditioning without conceding that these procedures likely create expectations – either positive or negative - towards the inert treatment. Similarly, even when expectations are generated without employing classical conditioning, an argument can be made that prior experiences, contextual cues, and participant-experimenter interactions play an analogous role to conditioning signals. Several accounts exist which "settle" the argument between these two competing views – the most well-known being Amanzio and Benedetti (1999), who specifically tested the effects of expectation and conditioning for the generation of placebo analgesia in isolation, and then their effects when combined in a third group. By following an open vs hidden design where participants were either made aware or deceptively administered pain relieving substances (morphine or ketorolac) over successive days, these substances were swapped to inert saline on a following day and placebo responses were assessed. Whilst in isolation, placebo effects were observed in the expectation- and conditioning only groups, the greatest change in pain tolerance emerged when the two were combined (Figure 1.4). Indeed, the view of Hoffman et al. (2005) is convincing – '*while a sense of rivalry prevails in the literature between these two perspectives* [conditioning and expectancy], *it is important to realise that they are not actually mutually exclusive*'.

Whilst these two views predominate the literature, with known inter-individual variability that exists in the expression of placebo analgesia and nocebo hyperalgesia, a third view has emerged which may help in explaining how and by which mechanisms pain modulatory effects are generated – that is, what role do personal factors such as biology or environment play in placebo analgesia and nocebo hyperalgesia, and can these factors be predictive of individuals who may show greater pain modulatory responses?

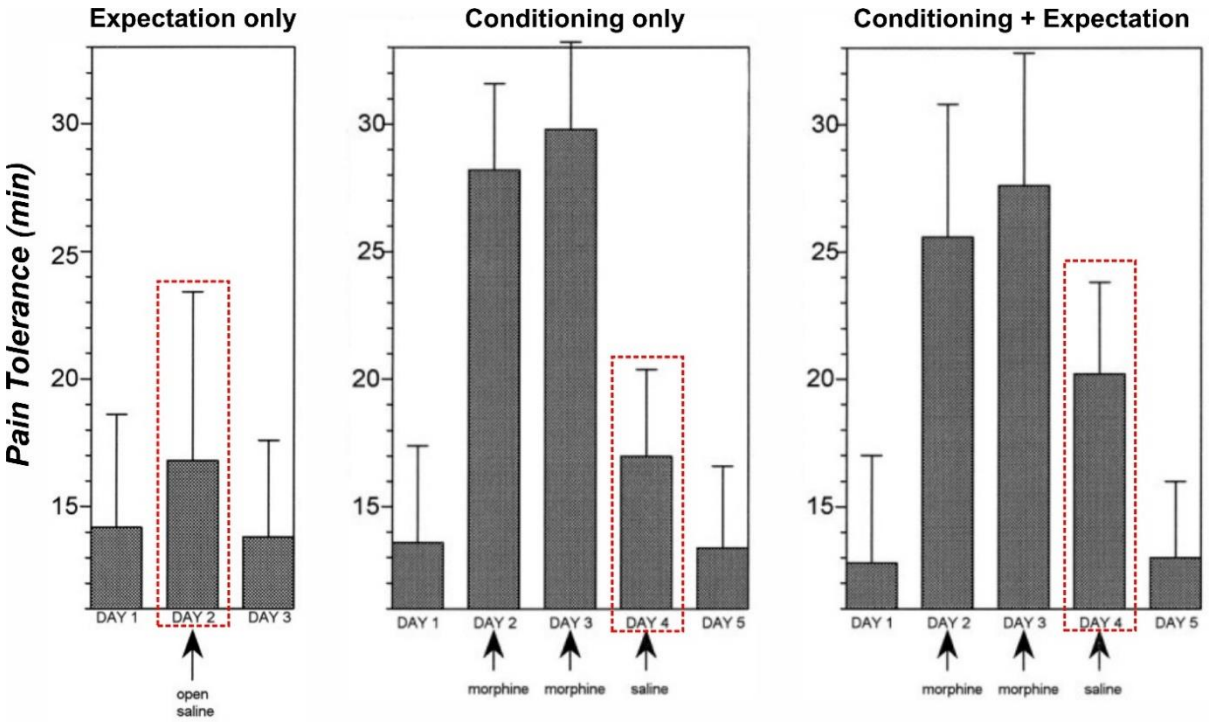

Figure 1.4. Conditioning, expectations, or both? Using a delicate experimental design involving hidden- and open conditioning using an active drug (morphine or ketorolac) or saline solution, Amanzio and Benedetti (1999) investigated the combinative role of expectations and conditioning in the formulation of placebo analgesia. Pain tolerance in response to a pressure task (an experimental model for ischemic arm pain) was their experimental outcome, and participants either received expectations of improvement or conditioning cues only, or a combination of both effects before a pain threshold assessment. In the left most plot, participants were informed they were receiving morphine, when in reality the solution was inert saline (expectation only). To trigger effects of conditioning only, participants received two consecutive days of morphine, followed by a "hidden" injection of saline on a third day – which was described as an antibiotic solution which would not affect pain responses. Finally, to combine conditioning and expectations, participants received the same two successive days of morphine, followed by an injection of saline described as morphine. Red boxes denote the degree of pain tolerance. Note that the largest change in pain tolerance with least variance is found when conditioning and reinforcement are combined. *Adapted from Amanzio and Benedetti (1999).*

1.3.3 Biology and environment

It is well documented that placebo and nocebo responses are not ubiquitous phenomena, and their manifestation and respective influence on the perception of pain can vary greatly between individuals. Whilst this inter-individual variance could simply be explained by the effectiveness of conditioning procedures, or how strongly positive or negative expectations are generated by the inert substance being used; a third argument for the psychosocial underpinnings of placebo analgesia and nocebo hyperalgesia offers a compelling alternate explanation – one which proposes that genetic or trait markers can encode an individual's predisposition to have their pain endogenously modulated.

The "transactional model" of placebo responsiveness defined by Darragh et al. (2015) proposes that an interaction occurs in any placebo context between an individual's dispositional (stable psychological/biological traits) and environmental (experimental designs, patient-practitioner interactions) variables. When these two variables match, a more significant placebo response is observed. This model builds upon a number of prior investigations which have tied factors such as optimism (Geers et al., 2010), goal-seeking (Peciña et al., 2013), and suggestibility (De Pascalis et al., 2002) to greater placebo responses. The identification of "placebo responders" is not just limited to these psychological traits, but is also tied to the neural pathways that are associated with them. A number of the traits listed in the transactional model stem from the dopaminergic system – the neurotransmitter largely responsible for reward processing, decision-making, and learning (Schultz, 2002, Chau et al., 2018). Schweinhardt et al. (2009) investigated the interaction between placebo responses and structural brain correlates of dopamine signalling, demonstrating larger grey matter volume in structures such as the dlPFC, ventral striatum, and insula were associated with both dopamine-related traits and placebo responses. Dopamine too has shown to play a role in the development of nocebo responses – specifically in the ventral striatum where a reduction in dopaminergic neurotransmission has been tied to greater hyperalgesia (Scott et al., 2008). However, dopaminergic release is not limited to the cortex, but is also reflected within the brainstem: namely within the ventral tegmental area (VTA) and substantia nigra (SN). These two structures, and their relation to placebo and nocebo responding remain to be accurately explored.

Genetics too have been reported to play a role in delineating responders from non-responders in endogenous pain modulatory phenomena. The "placebome", a term coined by Hall et al. (2015), proposes that a cacophony of genetic polymorphisms could play a role in an individual's ability to mount a placebo analgesic response. Indeed, biomarkers of the placebo response have

been identified in genetic polymorphisms for dopamine (Rs4680), μ-Opioid (OPRM1), cannabinoid (fatty acid amide hydrolase) and serotonin (Monoamine oxidase A) metabolism (Hall et al., 2012, Tiwari et al., 2013, Peciña et al., 2014, Peciña et al., 2015). As will be described in section *1.5.3* of this thesis, several of these same neurotransmitters share a role in the generation of nocebo responses and are released from discrete brainstem nuclei under strict control from top-down cortical signalling.

Overall, the literature currently available paints a clear picture. Both placebo and nocebo responses alike are highly complicated phenomena – stemming not from one clear origin but an integration of internal and external factors. Whilst no one technique can assess the swathe of potential influences over these modulatory effects, being able to disentangle which systems play a primary role in their manifestation could lead to more focussed efforts in identifying reliable neural substrates for predicting placebo and nocebo responders. However, before any biomarker can be proposed, it is important to understand the neural circuitry for pain – how nociceptive signals ascend from the periphery to the cortex, and how top-down circuits involving the brainstem cause an effect in altering pain perception.

1.4 How the cortex receives pain

1.4.1 #Nociception.

Pain is more than just sensation, and its complexity is shaped by an individual's thoughts, emotions, and motivations. Despite long being considered an extension or sub modality of touch, pain is now considered akin to thirst and hunger, that is, a homeostatic process guiding our behaviour in situations that could cause potential tissue damage (Kandel et al., 2000). Together, sensory, cognitive, and emotional systems are engaged during pain to inform action selection and an appropriate behavioural response (Melzack and Casey, 1968). These can include escaping from further harm, or in certain instances, resting and recovering from tissue damage. Acute instances of pain in a specific environment enable learning for how to avoid similar pain in the future (Melzack and Wall, 1965, Wall, 1979).

Clearly, in some instances pain is a useful perception, and can enforce positive responses to aid in survival. The human ascending and descending pain systems are highly organized and have adapted to drive an optimal response in any given environment. Given that pain responses are learned throughout development, the processes of perceiving and modulating pain are highly

variable between those of different demographics, life experiences, and attitudes (Wilcox et al., 2015). Despite the uniformity of peripheral sensory pathways involved in nociception across individuals, marked inter-individual variations in behavioural responses to acute noxious stimuli have been observed (Fillingim, 2017, Bell, 2018, Mischkowski et al., 2018, Wang et al., 2022a). This insight led to a number of investigations which have pruned and assigned components of the cortical pain system in cognitive-evaluation and producing emotional responses to pain, accounting for its subjectivity (Dubin and Patapoutian, 2010).

1.4.2 #Ascending neural systems: from periphery to cortex.

The detection of noxious stimuli is performed by specialised nerve cell endings, known as nociceptors, distributed throughout the peripheral nervous system (PNS). Nociceptors are highly specific and can either be unimodal, responding uniquely to thermal, chemical or mechanical noxious stimuli, or polymodal, responding to a combination of these stimuli (Mense, 1993).

To transmit noxious information, nociceptors are located on two classes of pseudounipolar sensory nerve fibres: myelinated A-delta fibres, which are responsible for encoding acute, short lasting noxious information, and unmyelinated C fibres, which have a slower conductance rate, and transmit longer lasting pain signals. The nociceptive signal travels to the dorsal horn of the spinal cord where it is transferred to second order neurons located in laminae I, II, and V of the spinal cord at an anatomical junction called the "primary afferent synapse" (Cross, 1994, Millan, 2002). In the dorsal horn, incoming noxious information can be modulated by local inter-neurons as well as from inputs descending from discrete brainstem nuclei (Dubin and Patapoutian, 2010).

These second order neurons then decussate, crossing the midline of the spinal cord at the corresponding dermatome level, before ascending contralaterally within a number of tracts. The primary ascending pain pathway is the Spinothalamic Tract (STT), which synapses in the ventral posterolateral (VPL) nucleus of the thalamus. The VPL thalamus projects third-order neurons containing noxious information to various brain regions responsible for the sensory-discriminative aspects of the pain percept, such as the primary somatosensory cortex (S1) where each spinally-innervated body site is represented and encoded along its extent (Khalid and Tubbs, 2017). In addition, some of the ascending noxious information projects to more medial thalamic nuclei such as the mediodorsal nucleus (MD). From here, noxious information

travels to cortical areas that are thought to code the emotional and cognitive aspects of pain – such as the anterior insula, midline cingulate, and prefrontal cortices (Cross, 1994, Kulkarni et al., 2005) (Figure 1.5). Once noxious information reaches the cortex, it becomes consciously perceived, and is subject to cortical evaluation and modulation by regions involved in emotional and affective processing (Brooks and Tracey, 2005).

Part of this conscious evaluation involves brain regions directing input back towards the where nociceptive information enters the central nervous system, the dorsal horn (DH), in order to either up- or down-regulate pain depending on the required behavioural response. In certain situations, such as when behaviours need to be limited to avoid exacerbating existing injuries, a hyperalgesic response is necessary. In others, such as when pain is unavoidable or during threat responses to initiate escape behaviour, analgesia may be required. These pro- and anti-nociceptive cortical signals leverage discrete brainstem pathways that connect the cortex with the spinal cord to drive subsequent modulation.

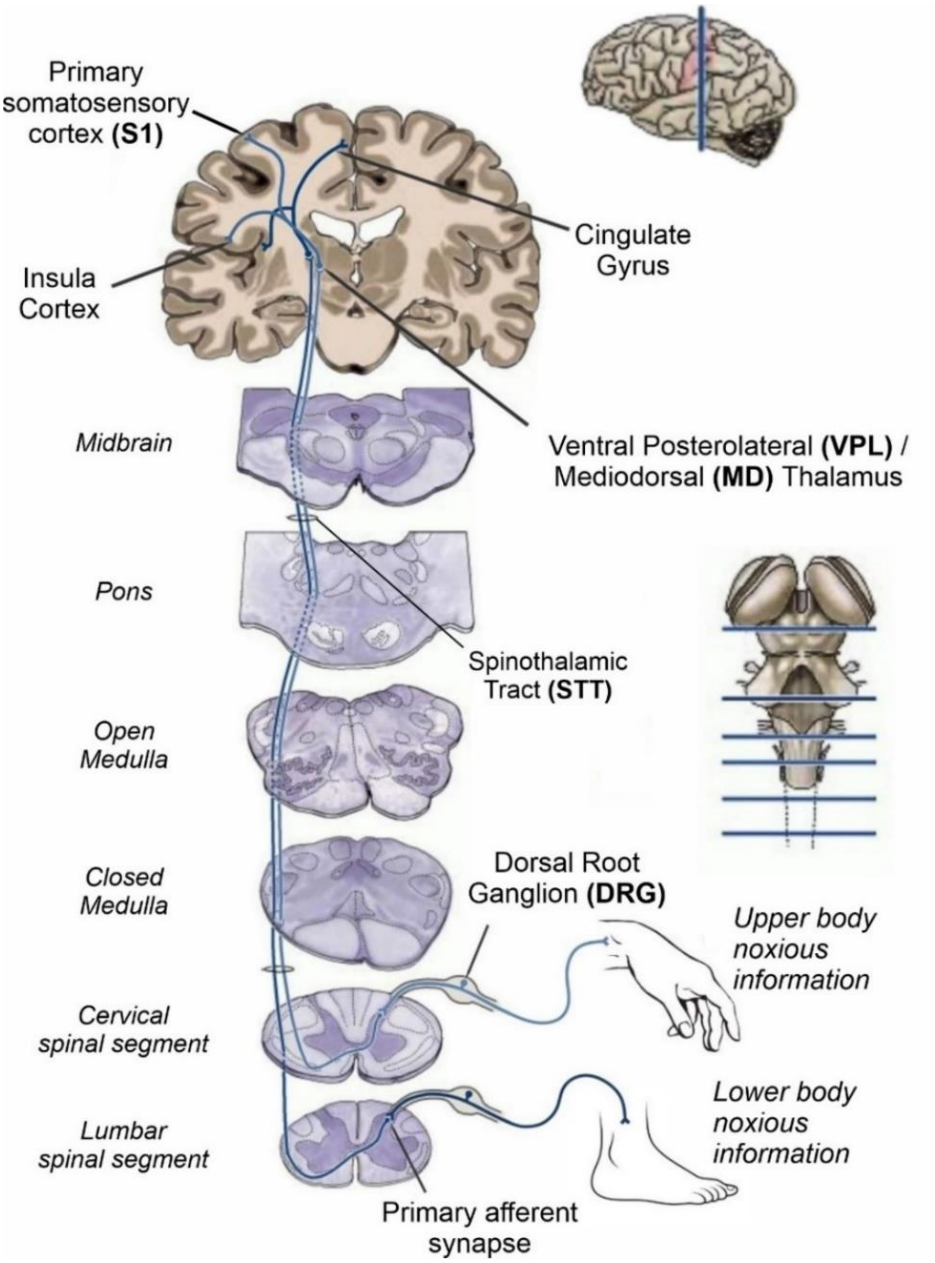

Figure 1.5. Ascending nociceptive information and the spinothalamic tract (STT). Noxious information is first received by peripheral nociceptors distributed throughout the body. Noxious signals then travel medially before synapsing within the spinal cord dorsal horn, at the primary afferent synapse. Second-order neurons then cross the cord and ascend within the contralateral STT before reaching the thalamus. A second synapse is located within the ventral posterolateral and mediodorsal nuclei of the thalamus, and third-order neurons originating from these two nuclei convey nociceptive signals throughout various cortical regions responsible for the sensory-discriminative, and cognitive-evaluative components of pain perception, respectively. Various slices of the brainstem are shown to track the ascending STT. The model brain and brainstem on the right indicate specific levels of slicing. *Adapted from Purves et al. (2001).*

1.5 Brainstem circuits coordinating nociceptive modulation

1.5.1 Routes of cortical engagement towards brainstem circuits.

In addition to exploring pathways involved in the sensory, cognitive, and emotional aspects of pain, recent human brain imaging studies have begun to investigate the circuits responsible for the modulation of pain. A landmark study by Petrovic et al. (2002) was the first to establish candidate regions of the placebo response. Their study consisted of comparing the analgesic effect of a fast-acting opioid, Remifentanil, with a placebo response induced through a combination of positive expectations and pharmacological conditioning. They identified a number of brain regions which were responsive to both opioid administration and altered in activation during placebo analgesia – namely the orbitofrontal (OFC) and rostral portion of the anterior cingulate (rACC) cortices. Moreover, they demonstrated a covariance of activation between the rACC and the brainstem during placebo responses that was absent during the processing of pain – suggesting this cortical site held direct control over brainstem circuitry during the manifestation of analgesic responses. More specifically, the brainstem region that covaried with the rACC was the midbrain periaqueductal gray matter (PAG), a site that was previously shown in experimental animal investigations to reduce incoming nociceptive information (Mayer et al., 1971, Lovick, 1985, Behbehani, 1995), The importance of the rACC in contacting modulatory brainstem structures like the PAG to produce placebo analgesia is now well-described, with this result not only replicated, but also affiliated with its pharmacological blockade. As demonstrated by Eippert et al. (2009a), the direct effect of administering Naloxone to abolish placebo analgesia is associated with impaired coupling between this rACC-PAG connection.

Since Petrovic's original investigation, various human brain imaging utilizing response conditioning have now reinforced the role of discrete prefrontal sites alongside the rACC in driving placebo responses. Placebo responses have also been associated with activity reductions in pain-processing regions such as S1, the VPL and MD thalamus (Wager et al., 2004, Eippert et al., 2009a, Elsenbruch et al., 2012, Freeman et al., 2015, Tu et al., 2021). Indeed, a recent meta-analysis conducted by Zunhammer et al. (2021) combined 603 individual participant data across 20 prominent placebo pain studies, and demonstrated the most consistent cortical activations are observed across various frontoparietal regions, the anterior and posterior insula cortices, the supplementary motor area (SMA), and the angular gyrus (AG). One specific frontal region, the dorsolateral prefrontal cortex (dlPFC), appears critical in establishing a brain state necessary for mounting placebo analgesia. By altering excitability within this site by applying

repetitive transcranial magnetic stimulation (rTMS), Krummenacher et al. (2010) demonstrated that transient disruption of the right dlPFC can abolish expected analgesic effects and subsequent placebo responses. In contrast, Tu et al. (2021) demonstrated the opposite effect. That is, by applying transcranial direct-current stimulation (tDCS) and altering right dlPFC excitability, the authors demonstrated a strengthening of placebo responses, as well as discrete changes in connectivity between the dlPFC and other frontotemporal regions. The dlPFC shares reciprocal connections not only with the rACC, but also the PAG – establishing a cortico-brainstem triumvirate in modulating pain by placebo conditioning and expectations (Tang et al., 2019, Cauzzo et al., 2022).

Interestingly, the cortical sites responsive to nocebo hyperalgesia largely mirror those observed in placebo analgesia. Much like placebo analgesia, nocebo hyperalgesia appears to rely on efferents of the ACC contacting brainstem modulatory centres (Tinnermann et al., 2017), and hyperalgesic phenomena leveraging expectations are associated with changes in brainstem and spinal cord activity (Yoshida et al., 2013, Freeman et al., 2015, Tinnermann et al., 2017). Various frontotemporal sites such as the OFC and dlPFC have additionally been tied with greater nocebo responses, suggesting a diverse role for these circuits in dynamically altering the pain percept (Kong et al., 2008, Schienle et al., 2018, Shi et al., 2021). Furthermore, much like placebo responses, altering excitability within the right dlPFC has been shown to alter the manifestation of nocebo hyperalgesia – with Tu et al. (2021) demonstrating the utility of anodal tDCS applied to this site in *reducing* the phenomenon.

In addition to cortical and brainstem sites, both placebo and nocebo appear to involve subcortical areas, including the nucleus accumbens (NAc) and the amygdala. Utilizing positron emission tomography (PET), Scott et al. (2008) showed that placebo and nocebo responses were associated with increased and reduced dopaminergic and opioid neurotransmission within the NAc and amygdala, respectively. Moreover, the amygdala, which reciprocally connects with the PAG has shown a role in experimental animals alongside the hypothalamus in regulating brainstem excitability (Ongür et al., 1998), and has also demonstrated altered activation in both contexts of reduced and enhanced pain (Atlas and Wager, 2014, Thomaidou et al., 2021). These findings are unsurprising given the role of dopaminergic neurotransmission in reward processing, the limbic system in emotional regulation, and these circuitries shared role in adaptive learning (Haber et al., 2006, Nasser et al., 2017).

Overall, our current understanding of how the cortex is capable of mounting endogenous pain modulation is governed by three principal circuits: 1) An "appraiser": a cognitive-evaluative cortical circuit comprising the dlPFC and associated frontotemporal areas, 2) An "enactor": a direct top-down driving circuit from the rACC to the PAG, and 3) A "state setter": subcortical sites such as the amygdala and NAc which supply the cortex with the necessary neurochemicals to initiate the communication between regions in circuits 1) and 2). These cortical sites, however, would be unable to evoke a change in DH transmission without calling upon modulatory nuclei of the brainstem. There is not one specific brainstem nuclei but many which could ultimately exert pain modulatory effects within the DH, and so understanding their relative roles is critical in a better wholistic understanding of these phenomena.

1.5.2 Key brainstem nuclei, their location, and relevance to placebo and nocebo.

Early preclinical laboratory studies using electrophysiological, pharmacological, and lesioning approaches in cats, rats, and mice identified several pain processing and pain modulatory regions in the brainstem (Basbaum and Fields, 1984, Heinricher and Fields, 2013). These regions include the midbrain PAG; the parabrachial complex (PB) and locus coeruleus (LC) in the pons; and the rostral ventromedial medulla (RVM) and subnucleus reticularis dorsalis (SRD) in the medulla. Neuroanatomical tract tracing revealed that each of these brainstem regions project directly to, or receive projections from, both the DH and spinal trigeminal nucleus (SpV) where spinal and orofacial noxious afferents terminate, respectively (Ma and Peschanski, 1988, Aicher et al., 2012, Velo et al., 2013, Keay and Bandler, 2015, Llorca-Torralba et al., 2016).

The PAG-RVM system: The pain modulatory role of the PAG was first revealed in experimental rodents, where electrical stimulation applied to its dorsolateral aspect produced a profound analgesia (Reynolds, 1969). Soon after this original study, multiple groups demonstrated that both electrical stimulation and opiate microinjection to the ventrolateral aspect of the PAG also evoked profound analgesic responses (Mayer et al., 1971, Gebhart and Toleikis, 1978, Basbaum and Fields, 1984). Although both the dorsolateral and ventrolateral aspects of the PAG can produce analgesia, it was also noted that differential behavioural responses could also be evoked from these two regions, suggesting that this small brainstem structure is not homogenous.

Since in both experimental animals and humans the rostro-caudal extent of the PAG is devoid of any distinct anatomical boundaries, work commenced to define distinct regions of the PAG based on functional territories. Richard Bandler was one of the first to robustly characterise the

functional architecture of the PAG, by combining amino acid microinjection and immunohistochemical techniques. He found a unique columnar organization with distinct longitudinally orientated PAG columns along the length of the cerebral aqueduct. This seminal work resulted in a shift in our understanding of descending analgesic pathways, and we now understand that the PAG is comprised of four functionally separate and anatomically distinct columns: a ventrolateral (vlPAG), lateral (lPAG), dorsolateral (dlPAG), and dorsomedial (dmPAG) column (Bandler and Shipley, 1994). Stimulation of the vlPAG evokes a behavioural response characterized by hyporeactivity and quiescence, essentially animals retreat from their environment and cease any ongoing activity. In contrast, stimulation of the lPAG produces hyper-reactivity, and active defensive behaviours such as flight and fight, essentially shifting the animal into heightened defensive activity. Importantly, stimulation of these two columns are also associated with profound opioid-sensitive and opioid-insensitive analgesia, respectively (Lewis and Gebhart, 1977, Depaulis and Bandler, 2012).

As previously mentioned, the PAG has been linked with both placebo analgesia and nocebo hyperalgesia in humans. Additionally, we understand placebo analgesia to be diverse in its neurochemical origins, being either completely or only partially abolished by the opioid antagonist, naloxone. These findings, suggest that placebo analgesia evoked in different situations may leverage different circuits which include different columns of the PAG. To date however, no single human functional imaging study has employed techniques with the spatial resolution required to identify which column and by extension, which behavioural and neurochemical system is being leveraged to produce the pain modulatory phenomena of placebo analgesia and nocebo hyperalgesia.

Regardless of which PAG column is recruited to produce modulatory effects on pain, the primary route in which these effects are conveyed is not directly to the DH/SpV, but via a region in the lower brainstem, the RVM (Fields and Heinricher, 1985, Heinricher et al., 1989). The RVM encompasses the midline nucleus raphe magnus and adjacent nucleus reticularis gigantocellularis, both of which send direct spinal efferents. Within these structures, three distinct cell populations exist: “ON” cells – which produce pain facilitatory effects upon stimulation and naturally increase in firing rate directly preceding behavioural pain responses; “OFF” cells – which are pain inhibitory and cease firing during pain responses; and “NEUTRAL” cells which adapt to act as either “ON” or “OFF” cell class depending on the nature of noxious stimuli (Fields et al., 1983, Heinricher et al., 1989, Khasabov et al., 2015). The mechanism by which the PAG is capable of dynamically altering the human pain percept is believed to involve

changing the ratio of ON, OFF, and NEUTRAL cell firing, altering the balance of nociceptive transmission within the DH/SpV (Heinricher et al., 1994).

Locus Coeruleus: Located bilaterally against the lateral floor of the fourth ventricle in the lower midbrain and upper pons, the LC is one of the seven major adrenergic cell groups in the brainstem (A6), which sends projection neurons rostrally to pain processing regions of the thalamus and cerebral cortex (Chandler, 2016, Llorca-Torralba et al., 2016), as well as caudally to the RVM and DH (Cross, 1994). Due to its wide array of innervation sites, the LC is believed to hold control over ascending and descending noxious information. Indeed, antinociceptive effects can be produced by direct LC stimulation which can be blocked by administration of an adrenoreceptor antagonist (Kanui et al., 1993, Pertovaara, 2006). Additionally, a pain facilitatory role has been proposed for the LC, in which ascending modulation of medial thalamic nuclei by the LC causes a lingering pronociceptive state, which can also be attenuated by adrenoreceptor antagonists (Zhang et al., 1997).

The LC is capable of finely tuning noxious information via the release of noradrenaline. An array of cortical, brainstem and spinal cord sites contain the two distinct and opposing receptors that noradrenaline acts on, i.e. alpha-1 adrenoreceptors (α 1) which are largely antinociceptive, and alpha-2 adrenoreceptors (α 2) which produce pro-nociceptive effects. Within the spinal cord, the LC produces antinociception through acting presynaptically on α 2-adrenoreceptors, and this antinociception can be attenuated through injection of an α 2-adrenoreceptor antagonist either spinally or directly in the LC (Guo et al., 1996). Interestingly, this antinociception can be enhanced through application of opioids to the LC which inhibit local inhibitory GABAergic inputs, suggesting an interplay between opioid- and noradrenergic systems of pain modulation involving the LC (Pan et al., 2004, Pertovaara, 2006).

The Parabrachial Complex: The PB resides inferiorly to the LC in the dorsolateral midbrain/pons, and consists of a group of nuclei which have collectively been shown to influence nociceptive signals through ascending communication with the hypothalamus (Bester et al., 1999), as well as via descending signals directly to the RVM and spinal cord (Chen et al., 2017). Much like the LC, the PB can produce both anti- and pro-nociceptive effects, through disruption of the activity of "ON" and "OFF" cell firing within the RVM (Chen and Heinricher, 2019a). Human brain imaging studies have provided evidence of changes in functional connectivity between the PB and RVM during painful stimulation of the periphery, and that these connectivity changes correlate with subsequent pain ratings (Stroman et al., 2018). Additionally, in animals, it has been shown that inhibition of the PB attenuates pain-related activation in dopaminergic sites

of the ventral midbrain (substantia nigra and ventral tegmental area), suggesting the PB may interact with dopaminergic signalling sites to influence the cognitive appraisal of painful stimuli and inform prediction error – the process of adjusting future behavioural responses to noxious stimuli (Coizet et al., 2010). Human brain imaging has revealed a distinct role of the PB in conditioned pain modulation (CPM), the phenomenon whereby application of a second concurrent stimulus can reduce pain responses to an initial, acute stimulus. Specifically, reductions in PB activation related to greater CPM efficiency, without engagement of the PAG – suggesting that pain modulatory effects can be conveyed via a number of separate descending pathways (Youssef et al., 2016).

Subnucleus Reticularis Dorsalis: The SRD, also referred to as the dorsal reticular nucleus (DRt) is located in the medulla, extending from the spinomedullary junction to obex. The SRD has reciprocal connections with both the DH and SpV, and neurons of the SRD express c-fos after noxious stimulation – demonstrating a role in pain-processing. The SRD has consistently shown to play a facilitatory role, with direct stimulation increasing pain-related behaviours (Almeida et al., 1996, Martins and Tavares, 2017). These effects can be abolished through direct injection of opioid peptides or quinolinic acid (Almeida et al., 1996, Martins et al., 2008). Alongside the PB, the SRD is believed to critical for mounting CPM responses, with a similar inverse correlation being shown in humans between SRD activation change and CPM magnitude (van Wijk and Veldhuijzen, 2010, Youssef et al., 2016).

At the time of commencing this thesis, preclinical literature had established a fundamental role for each of these nuclei in pain processing, as well as defined the anatomical projection patterns by which their varied effects on pain could be produced. These preclinical studies had also encouraged human imaging studies into pain and modulatory phenomena, and indeed evidence had emerged that the PAG-RVM system and the SRD played a role in placebo analgesia and CPM, respectively. What was lacking however, and indeed what led the focus of the investigation in Chapter 2, was i) a specific brainstem analysis of the role of each nuclei in anti- and pro-nociceptive endogenous pain modulation, and ii) an investigation with the specificity required to resolve the discrete involvement of brainstem subregions – such as the distinct PAG functional columns. Figure 1.6 provides a schematic representation of each of the important pain responsive brainstem nuclei discussed above, as well as their most commonly associated modulatory phenomena.

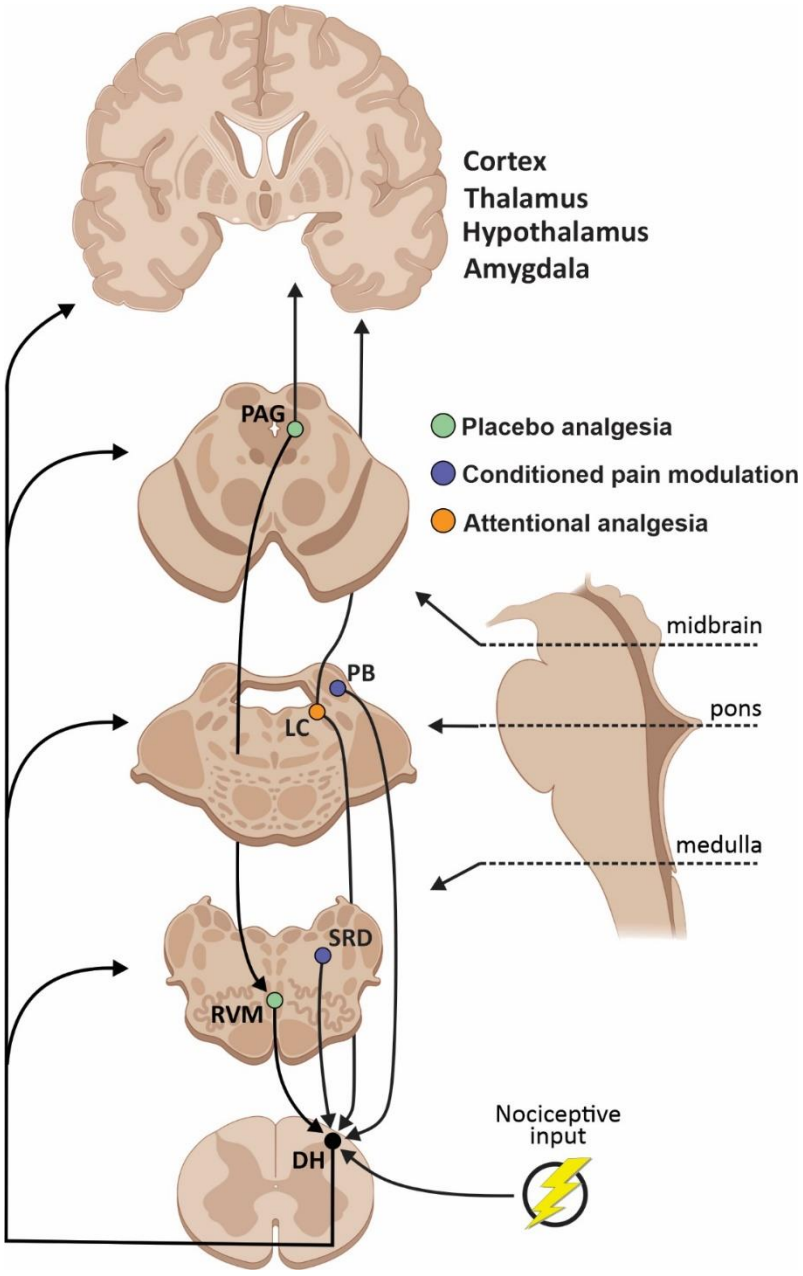

Figure 1.6. Brainstem circuitry involved in the modulation of pain. Nociceptive information is first relayed to the dorsal horn (DH) / spinal trigeminal nucleus (SpV), and subsequently to multiple brainstem and forebrain structures. Descending inputs from the rostroventromedial medulla (RVM), subnucleus reticularis dorsalis (SRD), parabrachial complex (PB), and locus coeruleus (LC) to the DH/SpV can enhance or inhibit incoming noxious information and alter the intensity of perceived pain. Key nodes in this same circuitry are recruited during pain modulatory experimental interventions, including placebo analgesia (green), conditioned pain modulation (blue), and attentional analgesia (orange). **Figure and caption reproduced from Crawford, Boorman, Keay and Henderson (2022).*

1.5.3 Neurotransmitter systems involved in pain modulation.

The initial body of work investigating the neurobiology of human pain modulatory phenomena asserted a primary role of the opioid system in the generation of placebo analgesia. Levine et al. (1978) found that 39% of patients responded to a placebo administration after a painful dental procedure. When naloxone (an opioid antagonist) was subsequently administered, these responses were completely attenuated. The role of endogenous opioids in the generation of placebo analgesia is now well-documented, involving increased receptor binding in a number of pain sensory and evaluative cortical sites such as the ACC, NAc, and anterior insula (AI) to produce the phenomena (Zubieta et al., 2005, Wager et al., 2007, Scott et al., 2008). Whilst the opioid system does appear critical for placebo analgesia, specifically when expectation-based designs are implemented, evidence also exists for nonopioid systems being involved.

Soon after Levine's initial discovery, evidence for a naloxone-resistant placebo analgesia began to emerge (Grevert et al., 1983, Gracely, 1987). In these studies, naloxone triggered only partial antagonism of the placebo response, suggesting that in certain situations, diverse neurobiological mechanisms may contribute to the analgesic phenomena. Mounting evidence now exists that top-down pain modulation involves both the endogenous cannabinoid and opioid systems, dependent on experimental design and individual genetic composition (Benedetti et al., 2013). When placebo analgesia is triggered solely via expectations of improvement, it is accepted that the opioid system plays a primary role (Zubieta et al., 2005). However, in designs utilizing classical conditioning or subliminal (hidden drug administration) cues, the cannabinoidergic system, specifically cannabinoid-receptor 1 (CB1) activation, appears pivotal (Benedetti et al., 2011). Experimental animal investigation have shown that the PAG contains dense concentrations of CB1 and μ-Opioid receptors within the lateral and ventrolateral columns, respectively. These same sites when stimulated produce a profound non-opiate and opiate analgesia, facilitating dynamic pain modulatory responses depending on the nature of noxious stimuli and required behavioural affect (Bandler and Keay, 1996, Keay and Bandler, 2001). Indeed, it is likely that in humans these two distinct neurobiological circuitries are recruited depending on the nature of analgesia elicited.

A third neurobiological system which has been identified as involved in placebo analgesia is dopamine. Dopamine is released by discrete regions of the human cortex and brainstem and is commonly associated with learning mechanisms and cognitive control – both important processes in the formulation of expectations and the appraisal of conditioning cues which are involved in the generation of placebo analgesia. A number of investigations have now been

conducted to ascertain the primary role of dopamine in altering behavioural responses, indicating that dopamine is pivotal in generating anticipatory reward, as well as producing prediction-error signals – the process of matching expected to perceived stimuli (Enck et al., 2008, Nasser et al., 2017). These two functions are critical in mounting an appropriate behavioural state for producing endogenous pain relief, as altering reward expectancy prior to placebo testing can significantly alter its expression (Yu et al., 2014, Schenk et al., 2017). Indeed, both greater gray matter density and dopaminergic neurotransmission within the ventral striatum (VS) (the primary source of cortical dopamine) has been associated with placebo analgesia, as well as in cortical regions that reciprocally connect with the VS. Interestingly, these same sites form the signature believed to activate the brainstem's descending modulatory circuits: the ACC and dlPFC (Scott et al., 2008, Schweinhardt et al., 2009).

Neurobiologically, nocebo hyperalgesia is believed to operate in antithesis to placebo analgesia. Early investigations performed by Benedetti et al. (1997) ascertained that instead of relying on endogenous opioids to produce pain modulatory effects, nocebo responses could be abolished by administering proglumide, an antagonist to CCK – a neurotransmitter which naturally counters the effects of cortical opioids (Benedetti and Amanzio, 2013). Further studies support the idea that nocebo hyperalgesia leverages an anti-opioid system – actively suppressing the neurotransmission of endogenous opioids to produce a pro-nociceptive state (Scott et al., 2008). This mirrored role extends to the dopaminergic system, as reductions in NAc neurotransmission have also been proposed as a potential biomarker of the phenomena (Scott et al., 2008).

Despite having opposite neurobiological action, both placebo analgesia and nocebo hyperalgesia alter pain perception by affecting DH activation, involving neurotransmitters which primarily originate within the brainstem (Eippert et al., 2009b, Tinnermann et al., 2017). Whilst evidence exists supporting cortical neurochemical differences between the two phenomena, we currently lack understanding as to how and specifically which brainstem nuclei are engaged during placebo analgesia and nocebo hyperalgesia – which could help us better understand the discrete roles of these neurotransmitter systems in potentiating their effects. To fully understand the neural underpinnings placebo analgesia and nocebo hyperalgesia, a targeted investigation of how the brainstem is involved in their manifestation is needed. Due to its non-invasive nature and recent advances in spatial resolution, MRI emerges as the candidate technique to disentangle these complicated phenomena.

1.6 Neural imaging of pain

1.6.1 BOLD, what is it good for?

MRI has been used to image the human body and brain for over half a century, but it was not until Seiji Ogawa and his team at Bell laboratories developed functional magnetic resonance imaging (fMRI) in 1990 that we gained the capability to observe changes in regional brain function over time (Ogawa et al., 1990, Filler, 2009). Due to the properties of active neurons, in a process known as "neurovascular coupling", local changes of blood flow and the relative ratio of oxygenated to deoxygenated blood mark areas of altered neuronal activity. fMRI measures these changes within three dimensional pixels, known as voxels and generates brain maps of Blood Oxygen Level Dependent (BOLD) signal that represent changes in neural activity (Figure 1.7) (Hillman, 2014).

The BOLD signal has become a staple in the pain neuroscientist's wheelhouse, with the ability to visualize discrete brain regions responsive to pain, relative to periods of no pain (baseline). Typically, an event-related paradigm is employed, such that over the course of an fMRI scan, multiple short lasting painful events are triggered, separated by baseline periods. Event-related paradigms are considered gold standard in assessing neural responses to acute pain experiences, and the images fMRI generates from these designs can then be modelled post-hoc within software packages such as Statistical Parametric Mapping (SPM) (Friston, 2003).

Whilst a number of processing steps are included within packages such as SPM to account for potential artefacts or properties of blood flow such as hemodynamic delay functions (for a review, see Smith (2004)), a major limitation of standard field strength (3-Tesla) MRI is the size of raw voxels collected. For smaller structures, such as those found within brainstem descending modulatory pathways, higher field strengths are beneficial as they provide significantly greater spatial acuity and counter partial volume effects and confounding BOLD signal from adjacent regions (Napadow et al., 2019). In addition, specific processing pipelines tailored to resolving brainstem BOLD signal have also been developed, one of these being the Spatially Unbiased Infratentorial Template (SUIT) toolbox (Diedrichsen, 2006).

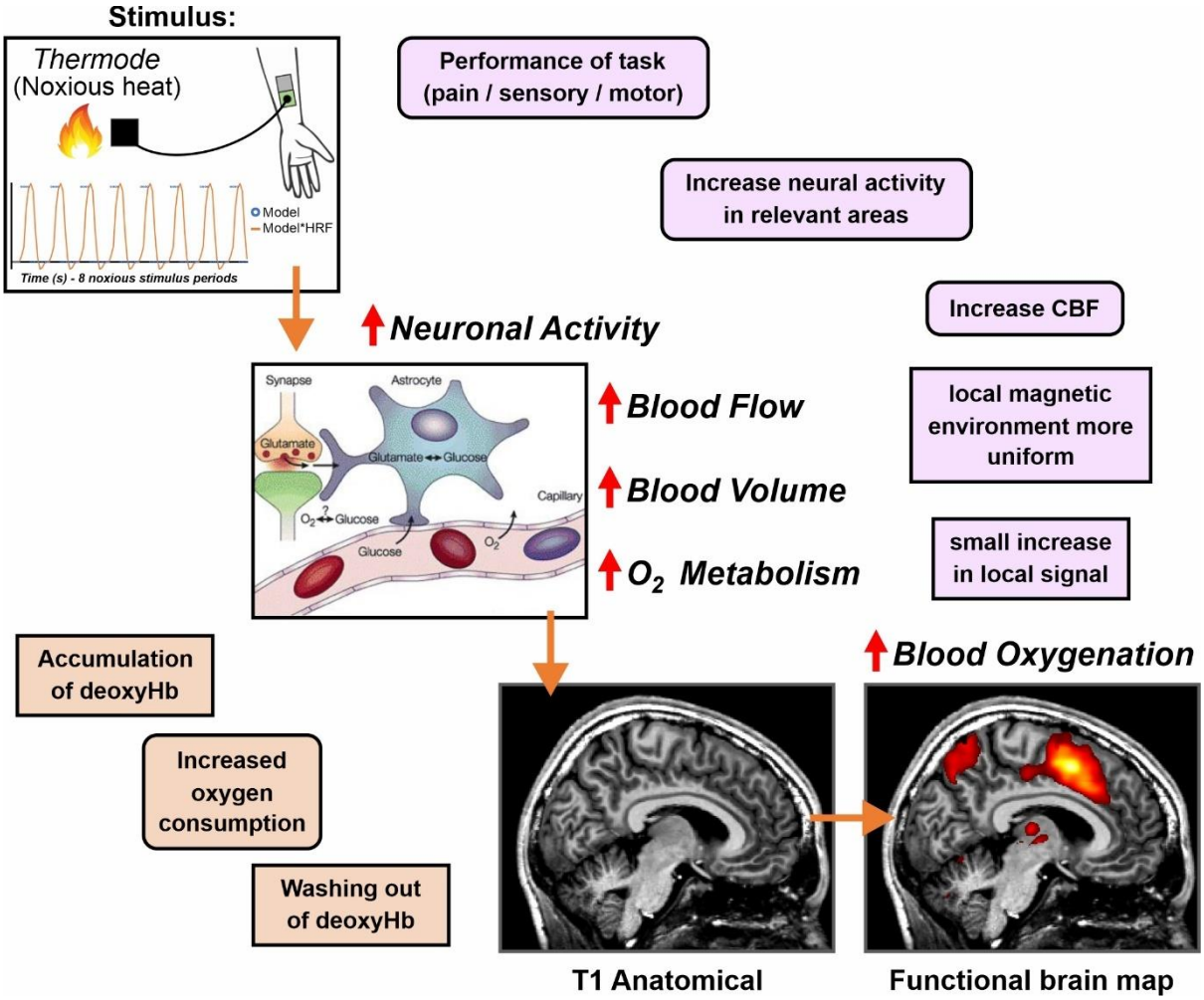

Figure 1.7. Generation of the BOLD signal. Visualizing areas of the brain that are responsive to pain involves the construction of an experimental design which can be both tolerated by participants and conducted within the confined environment of a Magnetic Resonance Imaging scanner. Typically, short lasting noxious stimuli (for example heat pain), are applied to a participant's periphery in succession whilst a functional MRI scan is recorded. The timing of these stimuli are recorded and subsequently modelled, applying a hemodynamic response function (HRF) to account for the time taken for blood to reach and exit discrete cortical regions. When pain is being applied, compared to intertrial intervals, altered neuronal activity in regions responsive to pain occurs, involving changes in blood flow and volume, as well as oxygen metabolism. These processes can be detected by an altered ratio of oxygenated to deoxygenated (deoxyHb) hemoglobin, representing the Blood-Oxygen-Level-Dependent (BOLD) signal. Across the cortex and brainstem, the BOLD signal can be quantified and visualized across the scanning period, allowing for a representation of relative activation within areas of the brain that respond to pain. *Adapted from Vaghela et al. (2010).*

1.6.2 Technique: brainstem-specific analysis using SUIT.

To acquire complete coverage with a reasonable repetition time, most standard field MRI scanners require a raw voxel size in the order of 30mm^3. For investigating larger cortical regions involved in pain perception and modulation - such as the S1 or rACC, this spatial acuity is sufficient, and event-related signal is unlikely to be significantly confounded under a standard image preprocessing protocol. However, brainstem nuclei – which are critical in mounting analgesic and hyperalgesic phenomena, present an entirely different challenge. Their small size and proximity to adjacent tissue and cerebrospinal fluid (CSF) mean that additional precautions must be taken before any interpretations can be made from functional data. Indeed, whilst collecting raw data at a higher field strength (7T) can help by reducing the raw voxel size and improving spatial acuity (Colizoli et al., 2022) to around the 1mm^3 range, structural inconsistencies still account for roughly 25% of within-subject variance in BOLD responses. As such there is a need for, especially within the brainstem, advanced registration techniques and tailored templates to explore the neural associations of discrete brainstem nuclei (Dukart and Bertolino, 2014).

The SUIT toolbox and its associated brainstem and cerebellum template, first released in 2006 and receiving substantial updates since, is a cerebellar- and brainstem-specific segmentation and normalization pipeline designed to preserve the brainstem's structural architecture as well as improve signal-to-noise ratio in subsequently co-registered functional brain data (Diedrichsen, 2006, Diedrichsen et al., 2009, Diedrichsen et al., 2011). In their original manuscript, Diedrichsen (2006) observed a roughly 15% increase in peak activation values using the SUIT pipeline within template regions compared to whole brain analyses, demonstrating the utility of this high resolution template in separating brainstem nuclei from adjacent tissue, accentuating fMRI signal within these nuclei of great importance to pain modulatory phenomena.

1.6.3 Analysis: Functional connectivity – nonspecific interactions between brain regions.

Despite individual roles being prescribed to regions of the cortex and brainstem during the perception and modulation of pain, it is well understood that these regions also interact with one another to produce anti- and pro-nociceptive effects. These interactions can be measured in a technique known as functional connectivity, allowing for the visualization of how closely fluctuations in neural activation overlap between two or more brain regions over time (Biswal

et al., 1995, van den Heuvel and Hulshoff Pol, 2010). A number of studies have since demonstrated the effect of pain of the brain's intrinsic functional connectivity, highlighting the influence of ascending sensory-discriminative and salience processing pathways, together forming a "pain processing network" (Zaki et al., 2007, Iannetti and Mouraux, 2010, Wiech, 2016). Stepwise, the methodology for this analysis involves: *1)* electing a "seed" region and extracting its timeseries of BOLD signal change over time. *2)* attaching that seed timeseries as a regressor to functional brain data, allowing statistical comparisons. *3)* comparing each other voxel timeseries within the brain for interactions with the regressor. "Interactions" can be either positive or negative, with a significant positive value indicating that two regions are functionally coupled and are communicating with each other across time. Alternatively, a significant negative value indicates anti-correlation, which can indicate that two regions are working in direct opposition, and are functionally segregated, or are connected yet perform opposing roles in a similar task (Figure 1.8) (Fox et al., 2005, Fox et al., 2009).

Typically, these interactions are assessed at rest – with altered coupling between two regions indicative of underlying differences in neural processing that may predispose an individual to show greater analgesic or hyperalgesic responses. Recently, Spisak et al. (2020) defined a resting-state brain network encoding pain sensitivity in healthy humans – demonstrating that ongoing fluctuations between frontal, temporal, and subcortical sites could be assessed to predict interindividual differences in pain processing. A similar network-based system tailored to placebo analgesia was also defined by Wagner et al. (2020), defining two anticorrelated brain networks at rest, the first consisting of canonical pain-sensitive regions, and the second consisting of emotional processing regions such as the amygdala and rACC. Utilizing resting state functional connectivity, the authors demonstrated that greater anticorrelation between these two systems was related to greater analgesic effects. Tailored approaches to neural dynamics such as these demonstrate that ongoing fluctuations between brain regions can not only affect a participant or patient's baseline pain responses, but also their ability to mount pain-modulatory phenomena. Interestingly, regardless of the chronicity of pain, changes in underlying coupling between subcortical dopaminergic sites (e.g. the VS) and both the dlPFC and the rACC emerge as particularly important to its modulation (Hashmi et al., 2014, Tétreault et al., 2016, Shi et al., 2021).

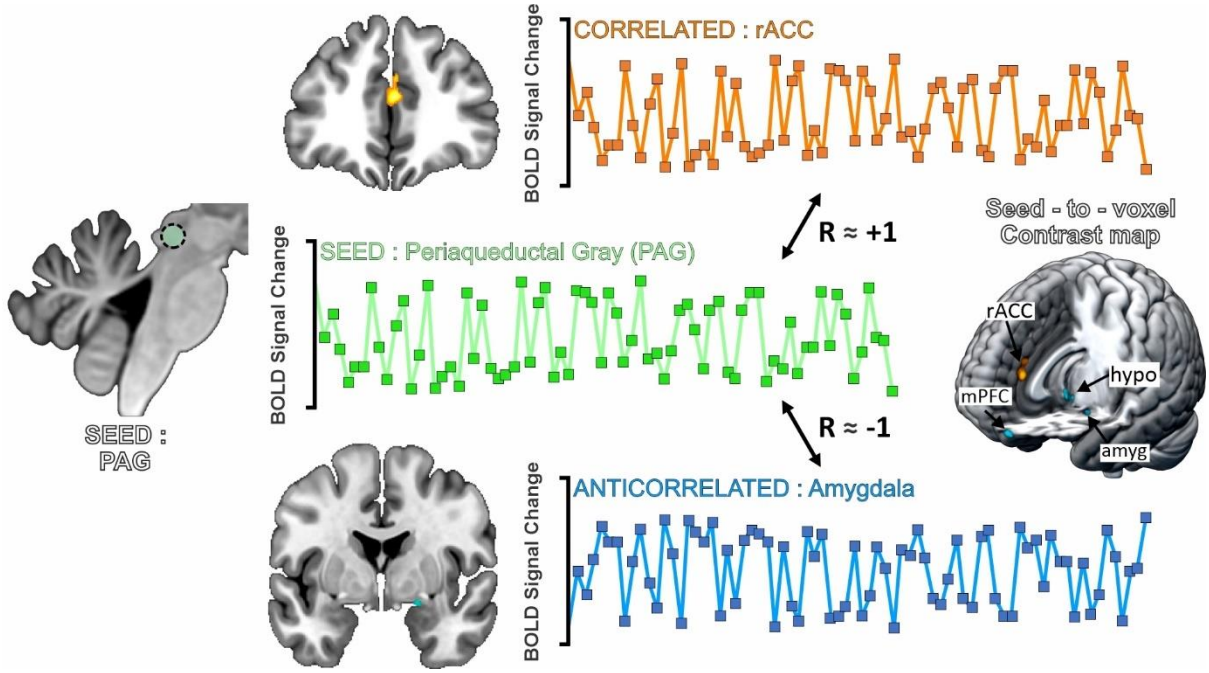

Figure 1.8. Functional connectivity for assessing inter-regional BOLD correlations independent to task-related effects. By first selecting an appropriate seed region (in this example the midbrain periaqueductal gray), functional connectivity is conducted by first extracting a trace of the BOLD signal within that seed across the course of the entire functional scan. This timetrend, is then used as a regressor in subsequent analyses, producing a value for each voxel in the brain representing correlative strength with this timetrend. Following thresholding and correction, areas can be visualized which either correlate (i.e., increase and decrease in BOLD signal at the same timepoints as the seed) or anti-correlate (i.e., decrease in BOLD signal as the seed increases and vise versa) over time – indicating that both the seed and identified cluster are engaged in a similar role, or, are connected.

1.6.4 Analysis: Psychophysiological interaction – pain-related connectivity.

Another method, proposed by Karl Friston in 1997, sought to leverage the potential for conducting event-related paradigms in fMRI to investigate changes in brain connectivity specifically during event- compared to baseline periods. This analysis, psycho-physiological interaction (PPI), builds upon functional connectivity by convolving a seed's timeseries with event timings, creating a new regressor, the "interaction term", prior to comparing each other voxel timeseries within the brain for seed-to-voxel relationships (O'Reilly et al., 2012). This interaction term allows us to identify significant voxels of the brain which demonstrate heightened correlation or anti-correlation with a seed, specifically during a task relative to baseline periods (Figure 1.9). In the context of placebo analgesia and nocebo hyperalgesia, this technique enables the ability to investigate specifically during a painful stimulus - where an individual is expressing the behavioural response of having pain either endogenously inhibited or enhanced - which regions alter in coupling as markers of the pain-modulatory response.

Early studies employing PPI and placebo suggestions reinforced the role of the rACC in recruiting descending analgesic networks of the brainstem when pain is experienced, but pain relief is expected. Bingel et al. (2006) first defined increased activation within the rACC as a marker for greater analgesic expression, and subsequently used this region as their seed for PPI analyses. Both the amygdala and PAG were observed to increase their coupling with the rACC during the placebo response, suggesting that emotional processing of the pain response was critical in mounting these responses via top-down recruitment of brainstem pain modulatory nuclei. Similar studies have linked the importance of pain-related connectivity involving the rACC and emotional systems to nocebo hyperalgesia, with a study from Shi et al. (2020) comparing the two phenomena. Compared to placebo analgesia, the authors used PPI to demonstrate the cortical hallmarks of Nocebo hyperalgesia consisted of changes in pain-related coupling between the rACC, dlPFC, and Insula Cortex - suggesting that this phenomenon in particular may hijack and modulate an anxiety-driven circuit involving frontotemporal cortical systems. Importantly, these anxiety-driven circuits still seem capable of tapping into descending brainstem pathways, as the expected effectiveness of a nocebo substance (i.e. value) directly modulates pain-related connectivity between both the rACC and PAG, and the PAG and spinal cord (Tinnermann et al., 2017).

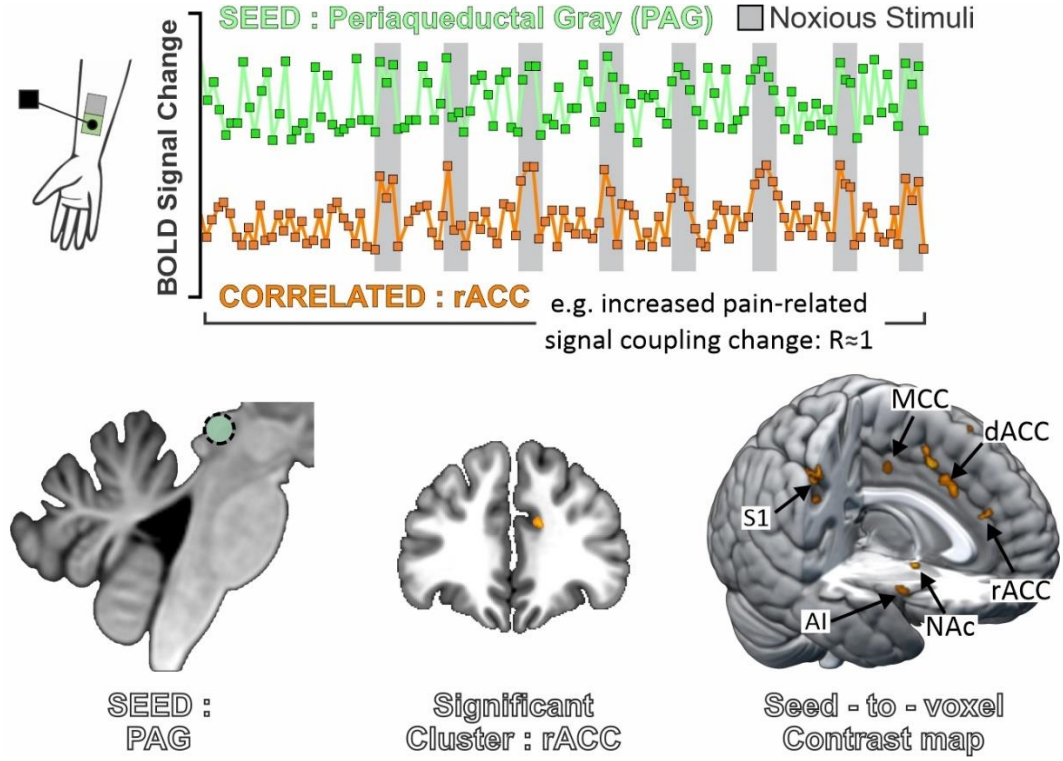

Figure 1.9. Psychophysiological Interaction (PPI) for assessing inter-regional BOLD correlations relating to experimental tasks or stimuli. Similar to functional connectivity, PPI involves first selecting an appropriate seed and extracting its BOLD timeseries. Here however, the analyses diverge, as before adding the seed timeseries as a regressor of interest, it is first convolved with the model timing originally included in the fMRI analysis (e.g. When noxious stimuli were applied throughout the scan). This modified regressor, or interaction term, enables the identification of voxels which alter in connectivity with the seed as a function of the task being performed (noxious stimuli being applied). This "task-related" functional connectivity searches for other regions of the brain which alter in connectivity dependent on psychological context (the task), and the physiological state of the seed region (the timeseries).

1.6.5 Analysis: Determining directionality and variable relationships with Dynamic Causal Modelling and Mediation.

Despite both functional connectivity and PPI providing valuable information on how neural systems interact to produce pain modulatory phenomena, a major shortcoming is commonly associated with these analyses: directionality. That is, if an elected seed should fall in the PAG, and the end result of the analysis is a significant cluster in the rACC increasing in pain-related coupling to that seed, neither of our connectivity analyses can definitively inform whether an ascending mechanism is at play or a top-down influence (i.e. is the PAG modulating the rACC or is the rACC modulating the PAG). Whilst strong hypotheses and supporting literature can bolster assumptions that indeed the changes in connectivity observed relate to descending modulatory input to the DH, additional analyses can also be employed to statistically test the relationships between two regions, and if the patterns of connectivity observed exert influence in a unidirectional manner.

The first of these, Dynamic Causal Modelling (DCM), is a technique proposing that all neural connections exist in a model system – with Bayesian model comparison capable of informing the dependencies between two brain regions, whether they are interacting in a forward, reverse, or reciprocal direction (Marreiros et al., 2008, Friston et al., 2013). Consider the example regions: the rACC and PAG as two nodes in a system. Part of the output of both functional connectivity and PPI informed us that these two regions timeseries were related and demonstrated correlative interactions. However, they gave us no information on the temporal relationships between these two timeseries. DCM, through a number of transformation and approximation equations (see Marreiros et al. (2010) for a complete list) informs how the timeseries of one node can be caused by the timeseries of another. DCM can be further adjusted to include model timings, indicating which connections are altered by event-related responses. These factors make it a flexible analysis for determining connections which alter in coupling either intrinsically, or are dependent on the application of a task or painful stimuli to drive directed modulatory effects.

Due to its complexity, only a select few studies have been conducted utilizing DCM to investigate changes in directed connectivity during pain modulatory phenomena. The best example comes from Sevel et al. (2015), who combined a conditioning-based paradigm with DCM to demonstrate the modulatory influence of the dlPFC over the PAG during the expression of placebo analgesia – reaffirming hypotheses shared throughout the community that it is indeed

top-down recruitment of brainstem pathways rather than ascending modulation of cortical sites by the PAG that primarily drives this phenomenon.

Whilst not specifically designed to measure directed connectivity, single-path and multilevel mediation analyses also offer an alternative solution for measuring the dependencies between two variables. That is, mediation informs whether two variables are directly related, or are mediated by a third, interacting variable (MacKinnon et al., 2007). Mediation relies on first establishing that two variables are indeed related – for example the temperature of noxious stimuli applied relating to the intensity of perceived pain. For pain to be perceived however, it must be received by distinct sensory-discriminative nodes within the cortex – making the respective activation within these nodes mediating variables between stimulus temperatures and reported pain responses. Woo et al. (2015) established this mediating relationship, leveraging the Neurological Pain Signature (NPS) designed by Wager et al. (2013) to demonstrate how distinct neural activation underpinned our ability to perceive alterations in noxious stimulus intensity. They extended on this work, further supporting that the connectivity between cortical sites can also act as mediators in cognitive-appraisal and evaluative responses to pain. By preceding noxious stimuli with verbal instructions to participants to either up- or down-regulate their own pain, the degree to which a participant was capable of accomplishing this task was mediated by distinct connectivity between a fronto-striatal circuit consisting of the amygdala, NAc, and ventromedial prefrontal cortex (vmPFC).

Additionally, mediation has further been employed to support the role of the dlPFC in updating expected outcomes to generate placebo analgesia. In a social-observation model, Schenk and Colloca (2019) asked participants to watch as an experimenter experienced pain relief by a placebo cream. Coupling between the dlPFC and the Temperoparietal Junction (TPJ), a region involved in metalizing, memory recall, and attention (Mars et al., 2012, Igelström et al., 2016) mediated the relationship between condition (the placebo or an adjacent control cream site being stimulated) and magnitude of placebo analgesia – demonstrating that mediation can be an effective tool in determining the critical nature of cortico-cortical connectivity in mounting pain modulatory responses.

Figure 1.10 provides a schematic representation of these two analyses, the questions they specifically answer to determine the importance of brain connectivity, and how they can be leveraged to better understand the formation of human pain modulatory responses.

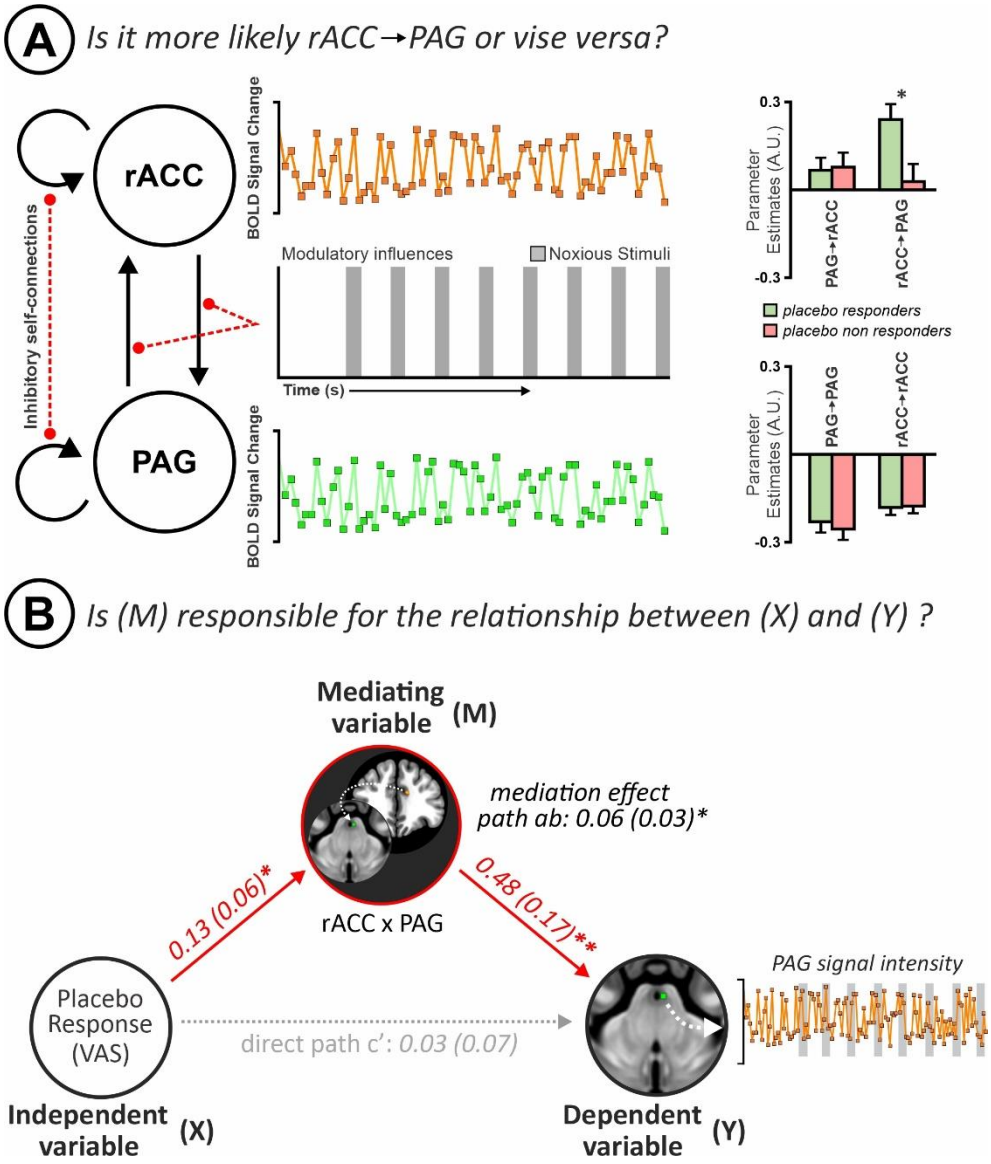

Figure 1.10. Dynamic Causal Modelling (DCM) and Mediation to assess directed connectivity in functional brain data. A major pitfall of both functional connectivity and PPI is that they are not designed to demonstrate directionality of information flow between brain regions, but rather demonstrate that two regions are communicating. Two methods which can shed light on directionality and build on information gleaned from the two connectivity analyses are DCM and mediation. A. DCM involves first entering an accurate model (full model) of which regions in a threshold functional brain map connect, and anatomically, the accepted view of which direction information can travel between them. The next step involves entering the timeseries of each region into this full model and predicting which connections any modulatory effects may be acting on (e.g. pain change / placebo responsivity or the timing of noxious stimulus application). Finally, DCM conducts model selection, pruning connections between regions where timeseries

that are not predictive of one another in either a forward or reverse direction. Those connections remaining represent the "optimal model", and parameter estimates representing the likelihood that region A is driving the BOLD signal change in region B can be extracted and visualized for statistical significance between experimental groups. B. Mediation tests for whether the interaction between two related variables (X and Y) is direct (path c / c'), or is mediated by a third variable (M). In terms of directed connectivity, mediation can be employed to test whether the BOLD signal change in a seed region relating to placebo responsiveness is acting in isolation or is mediated by the coupling between that region and another cortical site it is connected with – as informed by either functional connectivity or PPI.

1.7 Aims.

Given what is currently understood about human pain modulatory responses and what is still to be answered, this thesis presents experiments which sought to explore three core aims:

A1) To define the role of discrete brainstem nuclei during placebo analgesia and nocebo hyperalgesia.

A2) To explore how cortical sites recruit brainstem pain modulatory nuclei to evoke changes in pain during placebo analgesia.

A3) To identify the involvement of cortical biochemistry relating to an individual's ability to mount pain modulatory responses.

Hypotheses tied which these specific aims are highlighted both within the summary and text of each experimental chapter.

Chapter 2:

Altered activation within discrete brainstem nuclei drives placebo and nocebo responses

"Remember, it's not how big you are,
it's how big you play"
– Coach skip, 2000

Chapter 2: Overview

This chapter contains the following publication: Crawford LS, Mills EP, Hanson T, Macey PM, Glarin R, Macefield VG, Keay KA, & Henderson LA. (2021). *Brainstem mechanisms of pain modulation: a within-subjects 7T fMRI study of placebo analgesic and nocebo hyperalgesic responses*. Journal of Neuroscience, 41(47), pp.9794-9806.

This study was the first of its kind to combine ultra-high field (7-Tesla) functional magnetic resonance imaging (fMRI) and a within-subjects design for placebo and nocebo pain modulation. The spatial acuity afforded by 7-Tesla fMRI allowed us to assess the role of brainstem nuclei in the endogenous modulation of pain under placebo and nocebo manipulation. The study involved three sessions: conditioning, reinforcement, and test – conducted over two successive days. Participants were deceptively conditioned to believe a placebo "lidocaine" and nocebo "capsaicin" cream were modulating their pain relative to a control vaseline cream. Whilst collecting fMRI, all three creams received identical thermal noxious stimuli, so that any difference in reported pain reflected a placebo or nocebo response. Importantly, our experimental design had participants report an expectation of pain immediately prior to each series of noxious stimuli, as well as rate their pain continuously during conditioning and throughout scanning – overcoming prior limitations of series-position or experimenter biases associated with participants being asked to reflect on their previously experienced pain. 27 healthy participants completed the study, and placebo and nocebo responses were successfully elicited in 36% and 56% of individuals, respectively.

In direct assessment of Aim 1, we sought to define a subcortical network associated with both placebo and nocebo responses. With the spatial acuity provided by 7-Tesla functional imaging, we were further able to assess the specific involvement of functional subdivisions within these nuclei, namely within the midbrain periaqueductal gray (PAG) where both opioid- and non-opioid analgesia can be expressed depending on which area is activated. We hypothesized due to the short-lasting and localized nature of noxious stimuli applied throughout the experiment that a non-opioid system would be engaged, centred on the lateral PAG column, and that responses in brainstem pathways would be divergent between placebo and nocebo responses to reflect the opposing effects on the pain percept induced in these phenomena.

Behavioral/Cognitive

Brainstem Mechanisms of Pain Modulation: A within-Subjects 7T fMRI Study of Placebo Analgesic and Nocebo Hyperalgesic Responses

Lewis S. Crawford,[1] Emily P. Mills,[1] Theo Hanson,[1] Paul M. Macey,[2] Rebecca Glarin,[3] Vaughan G. Macefield,[4,5] Kevin A. Keay,[1] and Luke A. Henderson[1]

[1]School of Medical Sciences Neuroscience Program and Brain and Mind Centre, University of Sydney, New South Wales 2050, Australia, [2]School of Nursing and Brain Research Institute, University of California, Los Angeles, Los Angeles, California 90095, [3]Melbourne Brain Centre Imaging Unit, University of Melbourne, Melbourne, Victoria 3050, Australia, [4]Baker Heart and Diabetes Institute, Melbourne, Victoria 3004, Australia, and [5]Department of Anatomy and Physiology, University of Melbourne, Victoria 3050, Australia

Pain perception can be powerfully influenced by an individual's expectations and beliefs. Although the cortical circuitry responsible for pain modulation has been thoroughly investigated, the brainstem pathways involved in the modulatory phenomena of placebo analgesia and nocebo hyperalgesia remain to be directly addressed. This study used ultra-high-field 7 tesla functional MRI (fMRI) to accurately resolve differences in brainstem circuitry present during the generation of placebo analgesia and nocebo hyperalgesia in healthy human participants (N = 25, 12 male). Over 2 successive days, through blinded application of altered thermal stimuli, participants were deceptively conditioned to believe that two inert creams labeled lidocaine (placebo) and capsaicin (nocebo) were acting to modulate their pain relative to a third Vaseline (control) cream. In a subsequent test phase, fMRI image sets were collected while participants were given identical noxious stimuli to all three cream sites. Pain intensity ratings were collected and placebo and nocebo responses determined. Brainstem-specific fMRI analysis revealed altered activity in key pain modulatory nuclei, including a disparate recruitment of the periaqueductal gray (PAG)–rostral ventromedial medulla (RVM) pathway when both greater placebo and nocebo effects were observed. Additionally, we found that placebo and nocebo responses differentially activated the parabrachial nucleus but overlapped in engagement of the substantia nigra and locus coeruleus. These data reveal that placebo and nocebo effects are generated through differential engagement of the PAG–RVM pathway, which in concert with other brainstem sites likely influences the experience of pain by modulating activity at the level of the dorsal horn.

Key words: **analgesia; hyperalgesia; nocebo; nociception; pain modulation; placebo**

Significance Statement

Understanding endogenous pain modulatory mechanisms would support development of effective clinical treatment strategies for both acute and chronic pain. Specific brainstem nuclei have long been known to play a central role in nociceptive modulation; however, because of the small size and complex organization of the nuclei, previous neuroimaging efforts have been limited in directly identifying how these subcortical networks interact during the development of antinociceptive and pro-nociceptive effects. We used ultra-high-field fMRI to resolve brainstem structures and measure signal change during placebo analgesia and nocebo hyperalgesia. We define overlapping and disparate brainstem circuitry responsible for altering pain perception. These findings extend our understanding of the detailed organization and function of discrete brainstem nuclei involved in pain processing and modulation.

Received Apr. 14, 2021; revised Sep. 23, 2021; accepted Sep. 28, 2021.

Author contributions: L.S.C., E.P.M., K.A.K., and L.A.H. designed research; L.S.C., E.P.M., T.H., V.G.M., and L.A.H. performed research; P.M.M. and R.G. contributed unpublished reagents/analytic tools; L.S.C., T.H., and P.M.M. analyzed data; L.S.C. wrote the paper.

This work was supported by a research collaboration agreement with Siemens Healthineers. This work was also supported by the "NWG Macintosh Memorial Grant", obtained by the author E.P.M. We thank the facilities and the scientific and technical assistance of the Australian National Imaging Facility at the Melbourne Brain Centre Imaging Unit, University of Melbourne, and Assoc. Prof. Bradford Moffatt for developing the visual analog scale for use in the scanner.

The authors declare no competing financial interests.

Correspondence should be addressed to Luke A. Henderson at luke.henderson@sydney.edu.au.

https://doi.org/10.1523/JNEUROSCI.0806-21.2021

Introduction

The perceived intensity of pain can be strongly influenced by expectations. For example, when an individual expects pain relief, an inert treatment can produce analgesic responses, that is, placebo analgesia. Conversely, if an individual expects pain intensification, an inert treatment can produce hyperalgesic responses, that is, nocebo hyperalgesia. These phenomena are thought to be mediated by descending neural pathways (Vanegas and Schaible, 2004; Eippert et al., 2009) originating within the cortex that are recruited in response to a combination of an

individual's expectations (Kirsch et al., 2014; Frisaldi et al., 2015; Egorova et al., 2019), conditioning effects (Voudouris et al., 1989, 1990; Medoff and Colloca, 2015; Babel et al., 2018), and environmental associations (Finniss et al., 2010; Hansen et al., 2017; Tinnermann et al., 2017). Although the phenomena of placebo analgesia and nocebo hyperalgesia are well documented, the basic circuitry underpinning their expression, in particular the circuits within the brainstem, remain largely undefined.

Given that expectation is critical for both placebo and nocebo responses, it is not surprising that human brain imaging investigations have reported changes in signal intensity during placebo and nocebo in higher brain regions including the prefrontal, cingulate, insular, and somatosensory cortices (Petrovic et al., 2002; Wager et al., 2004; Craggs et al., 2007; Frisaldi et al., 2015; Sevel et al., 2015; Schienle et al., 2018; Hibi et al., 2020; Schenk and Colloca, 2020). Additionally, there is evidence that these same higher brain regions recruit brainstem pain modulatory circuitry to mediate placebo and nocebo effects, most notably via a connection between the anterior cingulate cortex (ACC) and the periaqueductal gray (PAG), which is functionally altered during placebo (Wager et al., 2004; Bingel et al., 2006; Eippert et al., 2009) and nocebo (Tinnermann et al., 2017) responses.

Within the brainstem, the best described pain modulatory circuitry arises from neurons of the PAG, which project via a relay in the rostral ventromedial medulla (RVM) to neurons of the superficial dorsal horn (DH) of the spinal cord. Although some human brain imaging studies have reported signal change encompassing the PAG during experimental analgesic responses (Petrovic et al., 2002; Wager et al., 2004; Eippert et al., 2009; Grahl et al., 2018; Oliva et al., 2021), no study has accurately and robustly defined the complete brainstem circuits responsible for either placebo analgesic or nocebo hyperalgesic responses. Preclinical studies have established that opioid-mediated analgesic responses can be evoked from neurons in the ventrolateral column of the caudal PAG (vlPAG), whereas, a nonopioid analgesia can be triggered from neurons in the lateral PAG (lPAG) and dorsolateral PAG (dlPAG) columns (Bandler and Shipley, 1994; Coulombe et al., 2016; Sims-Williams et al., 2017).

As the administration of the opioid antagonist naloxone can attenuate placebo analgesia in humans (Amanzio and Benedetti, 1999; Eippert et al., 2009), it has been hypothesized that the vlPAG in particular plays a critical role in its expression. However, limited spatial acuity in previous imaging studies has prevented exact localization of signal changes within specific PAG columns, which raises doubt over whether within the human brainstem this phenomenon is potentiated by opioidergic projections that arise from the vlPAG. Additionally, depending on the method of conditioning, placebo analgesia has shown to be naloxone resistant (Vase et al., 2005; Benedetti et al., 2011), which suggests alternative brainstem systems outside of or including adjacent PAG columns may play a key role in the expression of placebo analgesia. Similarly, the question of whether specific PAG columns play a role in nocebo responses also remains to be addressed experimentally.

The development of ultra-high-field-strength (7 tesla) MRI has made precise identification of brainstem circuitry possible and provides the opportunity to resolve the PAG at a columnar level (Satpute et al., 2013). Indeed, 7 tesla investigations have already successfully identified specific patterns of PAG columnar recruitment during respiratory control (Faull et al., 2015) and shifting cognitive load (Kragel et al., 2019). Despite our understanding that pain modulatory circuits originating in the vlPAG are strongly modulated by opioids, whereas those originating in the dlPAG and lPAG are nonopioidergic (Palazzo et al., 2010; Linnman et al., 2012), no studies have been performed at 7 tesla to identify specific PAG columnar associations of placebo analgesia and nocebo hyperalgesia.

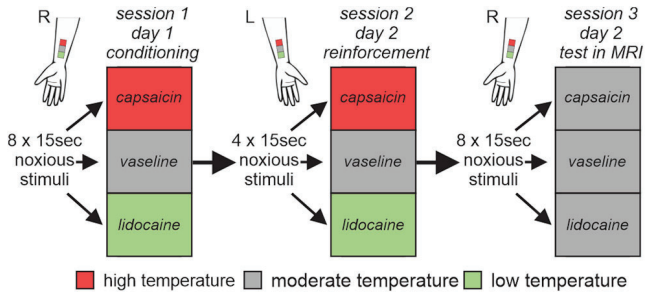

Figure 1. Experimental protocol. Conditioning and reinforcement were conducted by surreptitiously applying a series of individually calculated low-intensity thermal noxious stimuli to the lidocaine cream site, moderate intensity stimuli to the control cream site, and high intensity stimuli to the capsaicin cream site despite informing participants that all three cream sites were receiving identical intensity stimuli. An expectation of pain rating was collected before each series of stimuli as a measure of belief that the creams were acting to modulate participants' perceived pain. During the test phase, all three creams actually received identical moderate intensity thermal stimuli, and placebo and nocebo responsiveness were determined by calculating the difference in reported pain among the three cream sites.

The aim of this study was to use ultra-high-field functional MRI (fMRI) to identify the brainstem circuitry mediating placebo analgesia and nocebo hyperalgesia in healthy humans. We hypothesized that placebo and nocebo responses would be characterized by different activation patterns and columnar recruitment of the PAG-RVM pathways and that each response would elicit significant signal changes in other key nuclei linked to pain modulation and perception.

Materials and Methods

Ethics

All experimental procedures were approved by the University of Sydney Human Research Ethics Committee and were consistent with the Declaration of Helsinki. Written informed consent was obtained from participants at the beginning of each session. Participants were also provided with an emergency buzzer while inside the scanner so that they could stop the experiment at any time. At the conclusion of testing, participants were informed both verbally and through a written statement of the necessary deception and true methodology of the experiment and were invited to seek clarification of what they had just experienced.

Participants

Twenty-seven healthy control participants were recruited for the study (13 male, 14 female; mean age, 22.7 ± 0.7 years ± SEM; range, 19–33 years). To evaluate the necessary number of participants required for this study, an a priori power analysis (Faul et al., 2007) was performed using results from a previous imaging study investigating analgesic brainstem mechanisms (Youssef et al., 2016). This revealed a total sample size of 21 would be necessary to detect similar effect sizes with 95% power (d = 0.84, α = 0.05, $power$ = 0.95). Before beginning the study, participants completed a data sheet recording current medication(s) and any alcohol or caffeine ingested in the 24 h before testing.

Experimental design

The study included three sessions occurring on 2 successive days–a conditioning session on day 1, and a reinforcement and MRI scanning session on day 2 (Fig. 1). Throughout the study, noxious stimuli were administered to the volar surface of participants' left and right forearms using a 3 × 3 cm MR-compatible Peltier element thermode, which delivered a heat stimulus at a preprogrammed temperature via a Thermal

Sensory Analyzer (TSA-II, Medoc). Each stimulus lasted 15 s, including a ramp-up period (4° per second), a plateau period at a noxious temperature, and a ramp-down period (4° per second). Each stimulus was separated by a 15 s interstimulus-interval at a nonpainful baseline temperature of 32°C. Throughout conditioning, participants rated their pain on-line using a horizontal 10 cm visual analog scale (VAS) ranging between 0 and 100, where 0 was described as no pain and 100 as the worst pain imaginable. During scanning, participants used an MR-compatible button box to continuously report their pain perception. The VAS scale was shown on a reflected digital screen at the end of the magnet bore, and participants controlled the position of a slider to report their pain continuously by holding the left button (moved slider toward 0) or right button (moved slider toward 10) with their left middle and index fingers.

Day 1–conditioning protocol. Session 1 was conducted outside the MRI and consisted of two rounds of a conditioning protocol. Participants were first informed both verbally and via a written statement that the study was designed to investigate the modulatory effects of two active creams: a topical anesthetic containing lidocaine, which had been shown to provide pain relief in some individuals, and a hyperalgesic containing capsaicin, which had been shown to increase thermal sensitivity. A third cream was stated to be purely Vaseline and was described as a negative control to evaluate typical pain responses. In reality, all three creams contained purely Vaseline and only differed in color and their described properties. We then conducted a determination of moderate pain test, where 10 randomized stimuli ranging from 44 to 48.5°C in 0.5°C intervals were delivered to the volar aspect of the left forearm. Participants were informed that we were interested in recording a temperature that elicited a moderate subjective pain response (40–50 VAS rating) and that this temperature would be used throughout the remainder of the experiment. However, using the ratings provided during the determination of moderate pain, we delivered the following three different temperature stimuli: a low pain temperature (20–30 VAS rating), a moderate pain temperature (40–50 VAS rating), and a high pain temperature (60–70 VAS rating). These three temperatures were then deceptively applied to the different cream sites throughout the remainder of sessions 1 and 2.

Creams were then applied to three adjacent 3 × 3 cm squares on the volar surface of the participants' right forearm. To increase believability that the creams contained active substances, false labels were attached to the cream bottles, and green or red food coloring was added to the lidocaine and capsaicin creams, respectively. The Vaseline control cream always occupied the central square, and the green lidocaine and red capsaicin creams were counterbalanced between participants to occupy either the distal or proximal squares to reduce sensitivity effects. Ten minutes following cream application, we conducted two rounds of conditioning. Participants believed they would receive eight identical moderate thermal stimuli and were instructed to report their perceived pain intensity using the VAS. Participants were also asked before each set of stimuli for an average expectation of the pain they would experience, which acted both to measure belief that the creams were working to modulate their subjective pain and to reinforce the pain relieving and enhancing qualities of the creams. During the two conditioning rounds we deceptively applied a moderate temperature to the central control cream site, a low temperature to the green lidocaine cream site, and a high temperature to the red capsaicin cream site.

Day 2–reinforcement and test protocols. At approximately the same time on the following day, sessions 2 and 3 were conducted with participants inside the MRI machine and consisted of a reinforcement protocol (session 2) and a test protocol (session 3). The creams were applied to the volar surface of both left and right forearms, in the same order and locations as session 1, and once again described to hold powerful pain modulatory effects. Reinforcement was conducted by applying four noxious stimuli at the same low, middle, and high temperatures that were used throughout session 1 to the participants' left volar forearm. This reinforcement protocol was conducted to ensure that despite the change of day and immediate environment (inside the MRI), all participants continued to report different expectations and subjective pain across the three cream sites.

Following this reinforcement protocol, we waited 15 min for residual pain and sensitivity to dissipate before beginning the test protocol. During this 15 min period structural brain scans were collected. Unlike in sessions 1 and 2, the test protocol consisted of all three cream sites on the volar surface of the participant's right volar forearm receiving identical moderate intensity stimuli. We asked each participant for an average expectation of pain intensity directly before stimulation and instructed each participant to report the pain intensity experienced over the duration of the scan using the button box and the projected digital VAS. Each participant received four consecutive series of eight stimuli, with a separate functional series collected during each set of stimuli. The control cream site was always stimulated during the first and third series, and the lidocaine and capsaicin cream sites were stimulated during the second and fourth series, so that half of the participants received the placebo analgesia condition before the nocebo hyperalgesia condition, and the other half received a nocebo hyperalgesia condition before the placebo analgesia condition. This procedure ensured that each of the lidocaine and capsaicin stimulation periods were compared with an independent control cream site stimulation period. Furthermore, the counterbalanced condition presentation reduced the potential for order effects (Fig. 1).

fMRI data acquisition and preprocessing

Brain images were acquired using a whole-body Siemens MAGNETOM 7T MRI system with a combined single-channel transmit and 32-channel receive head coil (Nova Medical). Participants were positioned supine with their head in the coil and sponges supporting the head laterally to minimize movement. A T1-weighted anatomic image set covering the whole brain was collected (repetition time = 5000 ms, echo time = 3.1 ms, raw voxel size = 0.73 × 0.73 × 0.73 mm, 224 sagittal slices, scan time = 7 min). The four fMRI acquisitions each consisted of a series of 134 gradient-echo echo-planar measurements using blood oxygen level-dependent (BOLD) contrast covering the entire brain. Images were acquired in an interleaved collection pattern with a multiband factor of four and an acceleration factor of three (repetition time = 2500 ms, echo time = 26 ms; raw voxel size = 1.0 × 1.0 × 1.2 mm, 124 axial slices, scan time = 6 min and 25 s).

Image preprocessing and statistical analyses were performed using SPM12 (Penny et al., 2011) and custom software. Functional images were slice-time corrected, and the resulting six directional movement parameters were inspected to ensure that all fMRI scans had no more than 1 mm of linear movement or 0.5° of rotation movement in any direction. Images were then linearly detrended to remove global signal changes, and physiological noise relating to cardiac and respiratory frequency was removed using the DRIFTER toolbox (Särkkä et al., 2012), and the six-parameter movement-related signal changes were modeled and removed using a linear modeling of realignment parameters procedure. Using the spatially unbiased infratentorial template (SUIT) toolbox (Diedrichsen, 2006) for both the fMRI and T1 image sets, the brainstem and cerebellum were isolated and then normalized to the brainstem- and cerebellum-only template in Montreal Neurologic Institute (MNI) space. During this process, both the T1 structural and functional image sets were resliced into 0.5 mm isotropic voxels, and these images were spatially smoothed using a 1 mm full-width at-half maximum Gaussian filter. Data were upsampled, and a small smoothing kernel was applied to align with recommendations from Sclocco et al. (2018) to enhance the accurate investigation of signal intensity changes within small brainstem nuclei.

Placebo and nocebo responders versus nonresponders

Participants were grouped as either a responder or nonresponder separately for placebo and nocebo based on the 2 SDs method described previously by Youssef et al. (2016). Briefly, for the eight noxious stimuli delivered during the control (Vaseline) scan, the SD of the eight pain intensity ratings was calculated. During the subsequent lidocaine and capsaicin cream scans, the average pain intensity rating was calculated, and if this rating was either 2 SDs of the control average above for the capsaicin cream scan or 2 SDs below for the lidocaine cream scan, the participant was considered a responder. If not, the participant was considered a

nonresponder. Additionally, the average change in pain intensity ratings was also calculated for each participant during the lidocaine and capsaicin scans relative to the immediately previous control scan, which informed their placebo and nocebo ability, respectively. Significant differences between groups with respect to expected changes in pain intensities immediately before testing were determined using paired t tests (two tailed, $p < 0.05$). Because participants were grouped into either responder or nonresponder categories based on their perceived pain intensities during the fMRI scans (session 3), we did not assess significant differences between groups for the perceived pain intensity changes. A single-factor ANOVA ($p < 0.05$) was used to determine whether there were differences in the temperatures applied or pain intensity ratings reported between responder and nonresponder groups during the two control scans.

fMRI statistical analysis

To determine significant changes in signal intensity during each noxious stimulation period, a repeating boxcar model convolved with a canonical hemodynamic response function was applied to each of the four fMRI series. The first five volumes of each scan were removed from the model because of excessive signal saturation from the scanner. The contrast images generated for each functional image series were then used in group analyses.

We conducted three separate analyses to determine differences in brainstem activity during the placebo and nocebo responses, as well as the specific PAG columnar recruitment during these phenomena. In analysis 1, significant signal intensity changes within brainstem regions of responder and nonresponder groups were determined for both the lidocaine (placebo analgesia) and capsaicin (nocebo hyperalgesia) cream scans compared with the immediately preceding control (Vaseline) cream scans using random effects, paired voxel-by-voxel analyses. In analysis 2, significant relationships between regional brainstem activity changes (lidocaine–control β maps or capsaicin–control β maps) and the magnitude of placebo analgesic and nocebo hyperalgesic responses (mean pain intensity change relative to the control scan) were determined using random effects, voxel-by-voxel analyses. In analysis 3, PAG columnar and RVM rostrocaudal organization of placebo analgesic and nocebo hyperalgesic responses was explored. For the PAG, masks encompassing the dorsomedial (dmPAG), dlPAG, lPAG, and vlPAG columns as defined by Bandler and Keay (1996) were created at 1 mm intervals throughout the PAG's rostrocaudal extent (MNI z coordinates, −3 to −11) and for the RVM, dorsal (dRVM), middle (mRVM), and ventral (vRVM), masks were created at 1 mm intervals throughout the RVM's rostrocaudal extent ($z = -39$ to -51). The mean ± SEM number of voxels in each mask at each rostrocaudal level were the following: vlPAG 18 ± 0, lPAG 18 ± 0, dlPAG 18 ± 0, dmPAG 16 ± 0, dRVM 126 ± 1, mRVM 130 ± 10, and vRVM 142 ± 11. The number of 0.5 mm^3 voxels that were significantly positively or negatively correlated with either placebo or nocebo were then determined for each mask and plotted as a percentage of the total volume of each mask.

Analyses 1 and 2 were initially visualized at a threshold of $p < 0.005$, uncorrected with a cluster extent threshold of five contiguous voxels. Cluster-level correction for multiple comparisons was performed on resulting clusters ($p < 0.05$) to reduce the likelihood of type I errors. The locations of significant clusters in MNI space were tabulated, and β values extracted to determine the directions of signal changes. For display purposes, significant clusters were overlaid onto a mean T1-weighted anatomic of all 25 participants. So that the brainstem axial slices were aligned to the plane of standard human brainstem atlases (Paxinos and Huang, 2013), we altered the tilt of the display images so that the long axis of the brainstem was vertically oriented. This was achieved by tilting the overlays by 0.4 radians. The MNI coordinates of significant clusters were derived before this rotation.

Results

Psychophysics results

Data from two participants were excluded because of excessive variability in pain ratings during the test phase, which resulted in ceiling and floor effects and consequently an inability to accurately measure placebo analgesic or nocebo hyperalgesic effects. Data from 25 participants were included in the final psychophysical and functional image analyses. For the placebo analgesia protocol, 9 participants were classified as responders (36%) and 16 as nonresponders (64%), and for the nocebo hyperalgesia protocol 14 participants were classified as responders (56%) and 11 as nonresponders (44%). Of the 25 participants tested, six were categorized as both placebo and nocebo responders, and eight as both placebo and nocebo nonresponders.

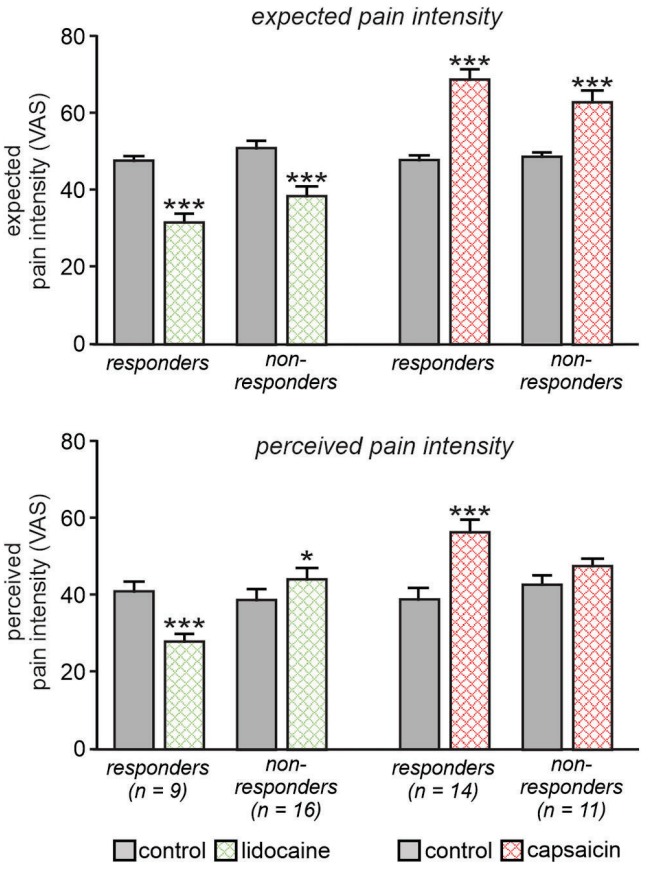

Figure 2. Expected and perceived pain intensities. Plots of mean (± SEM) expected pain intensity (top) and perceived pain intensity (bottom) during noxious stimuli delivered during the test phase. Note that both responder and nonresponder groups expected a pain reduction during stimulation of the lidocaine site and an increase during stimulation of the capsaicin site compared with stimulation of the control site. However, although responders' expectations were met by perceived pain intensity reductions or increases during actual stimulation of lidocaine and capsaicin sites, respectively, in the nonresponder groups expectation and perceived changes in pain were not met. That is, nonresponder groups did not experience a modulatory response to match their expectations and reported similar pain across the three cream sites. $^*p < 0.05$, $^{***}p < 0.001$.

Participants' expectations of pain directly before each of the test scans revealed that all four groups expected the creams to significantly alter pain intensity (Fig. 2). That is, both placebo responders and nonresponders expected their pain to be significantly inhibited during lidocaine cream stimulation compared with control (mean ± SEM VAS responder: control 47.2 ± 1.4, lidocaine 31.1 ± 2.5; nonresponder: control 50.4 ± 2.3, lidocaine 38.1 ± 3.5; both $p < 0.001$). Likewise, both nocebo responders and nonresponders expected significantly enhanced pain during capsaicin cream stimulation compared with control (responder: control 48.3 ± 0.9, capsaicin 68.3 ± 2.9; nonresponder: control 48.0 ± 1.9, capsaicin 62.5 ± 3.1; both $p < 0.001$).

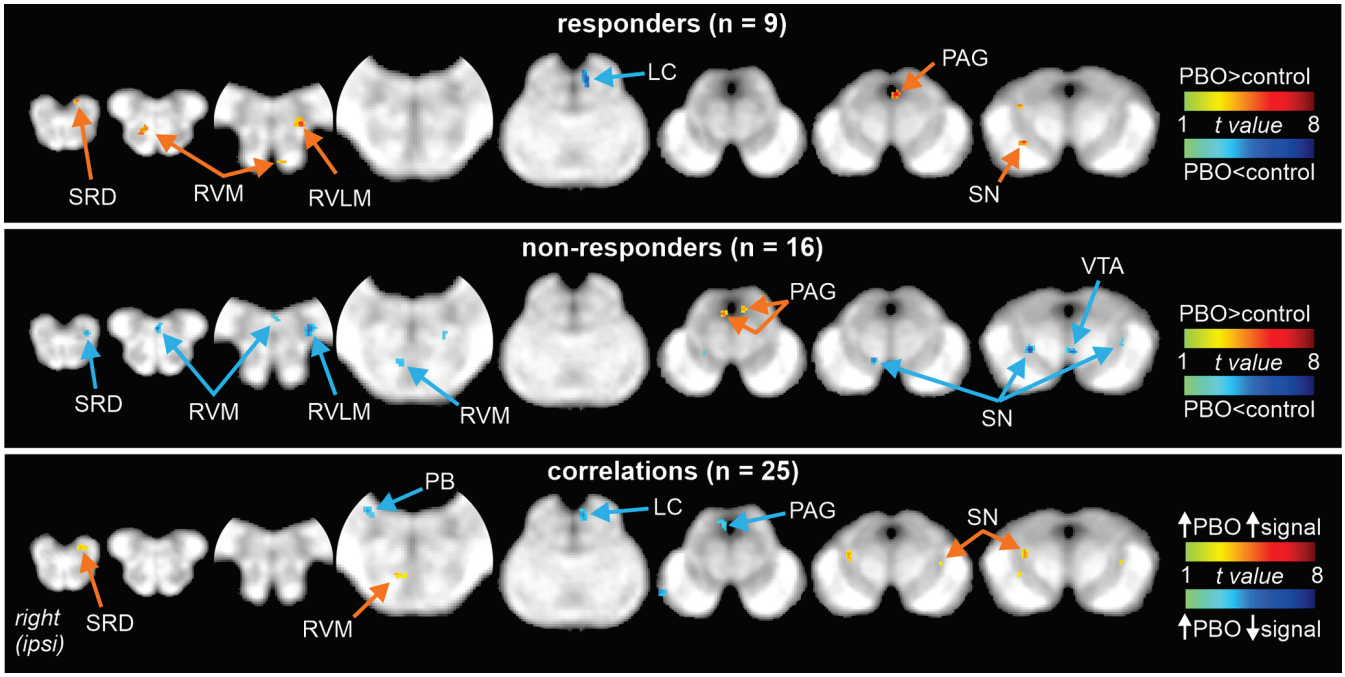

Figure 3. Signal changes in placebo responders, nonresponders, and sites where signal change correlated with placebo ability. Brainstem activity differed significantly between the control and lidocaine (placebo, PBO) scans in several pain modulatory nuclei. In responders (top), significant signal decreases (cool color scale) occurred in the region of the contralateral (to stimulation) LC, and signal increases (hot color scale) occurred in the ipsilateral SN, the contralateral ventrolateral PAG, RVLM, SRD, and RVM. In nonresponders (middle), signal increases occurred in the PAG bilaterally, and signal decreases in the SN bilaterally, the contralateral RVLM, the VTA and RVM. Signal intensity changes significantly correlated with placebo ability (bottom) were found to be positively correlated (hot color scale) in the SN bilaterally, the contralateral SRD, and RVM and negatively correlated (cool color scale) in the PAG, ipsilateral PB and contralateral LC. LC = locus coeruleus, SN = substantia nigra, PAG = periaqueductal gray, RVLM = rostral ventrolateral medulla, SRD = subnucleus reticularis dorsalis, RVM = rostral ventromedial medulla.

In contrast, and consistent with the categorization of each participant, during actual stimulation of the lidocaine site, placebo responders reported reduced pain intensities compared with the preceding control site (Mean ± SEM VAS control 40.7 ± 2.5, lidocaine 27.3 ± 2.3), and placebo nonresponders reported an increase in pain intensity during stimulation on the lidocaine site (control 38.6 ± 3.3, lidocaine 44.6 ± 3.1; Fig. 2). In contrast, nocebo responders reported increased pain during stimulation of the capsaicin relative to the preceding control site (control 38.7 ± 3.3, capsaicin 56.0 ± 3.6), whereas nocebo nonresponders reported little change in pain intensity (control 42.9 ± 2.8, capsaicin 47.8 ± 2.6).

Stimulation of the central control cream site before both the placebo and nocebo sites gave two independent preconditions to which the placebo and nocebo responses were compared. Average pain intensity ratings for all subjects between control series 1 and 2 were not significantly different (mean ± SEM VAS: control placebo: 39.4 ± 2.3; control nocebo: 40.6 ± 2.3; $p = 0.41$; paired t test). Additionally, neither test temperature nor pain intensity ratings during stimulation of the control sites differed between responders and nonresponders for either the placebo (mean ± SEM test temperature °C: responders 47.4 ± 0.3; nonresponders 46.9 ± 0.2; $F_{(2,23)} = 0.83$, $p = 0.28$; mean ± SEM VAS control: $F_{(2,23)} = 0.17$, $p = 0.69$) or nocebo groups (test temperature: responders 47.4 ± 0.1; nonresponders 46.8 ± 0.3, $F_{(2,23)} = 3.61$, $p = 0.07$; VAS control: $F_{(2,23)} = 0.85$, $p = 0.37$). Additionally, in all subjects, there were no significant linear relationships between placebo and nocebo abilities; that is, changes in average VAS responses ($r = 0.09$, $p = 0.67$), between placebo expected and perceived pain changes ($r = 0.05$, $p = 0.81$), or nocebo expected and perceived pain changes ($r = 0.41$, $p = 0.06$).

fMRI results

Placebo analgesia

Comparison of signal intensity changes during control site versus lidocaine site stimulation in responders and nonresponders revealed that the placebo response was associated with signal intensity changes in several distinct brainstem nuclei, including the PAG and the RVM. In placebo analgesia responders, placebo-related signal intensity increases, that is, signal increases during lidocaine and decreases during control site stimulation, occurred contralateral to the stimulated forearm in the vlPAG and the rostral ventrolateral medulla (RVLM), ipsilateral to the stimulated forearm in the substantia nigra (SN), and on the midline in the rostral ventromedial medulla (RVM). Signal intensity in the contralateral subnucleus reticularis dorsalis (SRD) increased during lidocaine but did not change during control site stimulation. A significant placebo-analgesia-related signal decrease occurred in the region encompassing the locus ceruleus (LC) contralateral to the noxious stimulation (Fig. 3; Table 1). Compared with control site stimulation, nonresponders showed bilateral signal increases in the vlPAG; however, they showed signal decreases in the RVM, in the contralateral RVLM and SRD, bilaterally in the SN, and in the region of the ventral tegmental area (VTA) during lidocaine site stimulation.

Correlation analysis of signal intensity change differences between control and lidocaine scans and average change in pain intensity, that is, placebo ability, in all 25 participants revealed a similar pattern of brainstem signal changes to that of the individual responder and nonresponder groups. That is, signal intensity changes were negatively correlated with placebo ability, meaning, as placebo magnitude increased, signal change differences (lidocaine–control) were smaller in the regions of the ipsilateral dl/lPAG and contralateral LC as well as in a region encompassing the

Table 1. Location, level of significance and cluster size of significant clusters in each of the placebo groups and correlation analyses

	MNI coordinates						Beta value change (mean ± SEM)	
	x	*y*	*z*	*t* value	Cluster size[a]	Volume (mm^3)	Control scan	Lidocaine scan
Placebo responders								
PBO > control								
Contralateral PAG	−1	−30	−6	6.33	46	5.75	−0.38 ± 0.36	1.70 ± 0.44
Ipsilateral SN	12	−27	−6	4.86	20	2.5	−0.58 ± 0.31	0.68 ± 0.35
	11	−19	−8	5.47	31	3.875		
Contralateral RVLM	−5	−38	−42	5.78	39	4.875	−0.25 ± 0.16	0.74 ± 0.26
RVM	−2	−30	−46	4.18	16	2	−1.01 ± 0.34	0.74 ± 0.26
	5	−35	−47	4.80	27	3.375		
Contralateral SRD	−2	−46	−53	4.79	13	1.625	−0.01 ± 0.13	1.07 ± 0.17
Control > PBO								
Contralateral LC	−1	−33	−18	6.32	103	12.875	1.03 ± 0.26	−0.70 ± 0.41
Placebo nonresponders								
PBO > control								
Ipsilateral PAG	3	−33	−9	3.91	17	2.125	−0.59 ± 0.22	0.79 ± 0.19
Contralateral PAG	−2	−32	−9	4.56	28	3.5	−0.15 ± 0.22	0.78 ± 0.18
Control > PBO								
Ipsilateral SN	10	−21	−6	5.91	59	7.375	1.19 ± 0.16	−0.08 ± 0.19
Contralateral SN	−12	−21	−7	4.36	45	5.625	0.82 ± 0.21	−0.32 ± 0.17
VTA	−1	−20	−6	4.80	64	8	1.01 ± 0.24	−0.21 ± 0.29
Contralateral RVLM	−6	−38	−42	4.78	123	15.375	0.87 ± 0.17	−0.22 ± 0.15
RVM	2	−31	−42	5.62	21	2.625	0.94 ± 0.28	−0.07 ± 0.24
	1	−41	−46	5.34	29	3.625		
Contralateral SRD	−1	−41	−53	3.91	29	3.625	0.74 ± 0.21	−0.27 ± 0.13
Placebo correlations								
Negative correlation							*r* values	
Ipsilateral PAG	2	−34	−9	4.14	54	6.75	−0.65	
Contralateral LC	−1	−36	−18	4.35	64	8	−0.61	
Ipsilateral PB	9	−42	−37	3.95	50	6.25	−0.62	
Positive correlation								
Ipsilateral SN	10	−24	−6	5.51	135	16.875	0.62	
Contralateral SN	−11	−23	−7	3.34	23	2.875	0.56	
RVM	4	−31	−42	3.54	37	4.625	0.61	
Contralateral SRD	−3	−43	−53	3.54	48	6	0.66	

Coordinates are in MNI space.
[a]Cluster sizes are reported in resliced 0.5 mm^3 voxels.

parabrachial nucleus (PB). Conversely, signal changes positively correlated with placebo ability in the ipsilateral and contralateral SN, the RVM, and in the contralateral SRD (Figs. 3, 5; Table 1).

Nocebo hyperalgesia

Analysis of signal intensity changes associated with nocebo responses revealed similar overall patterns of brainstem changes. In nocebo hyperalgesia responders, nocebo-induced decreases (i.e., signal intensity decreased from baseline during capsaicin and increased during control site stimulation) were found bilaterally in the lPAG, in the SN contralateral to the stimulated forearm, and again on the midline in the RVM (Fig. 4; Table 2). In contrast, in nonresponders, capsaicin cream stimulation evoked signal intensity increases in the contralateral lPAG; bilaterally in the SN, RVM, and VTA; ipsilateral LC; and in the region of the nucleus cuneiformis (NCF). Additionally, a signal intensity decrease was found in the ipsilateral PB.

Correlation analysis revealed that nocebo ability was positively correlated with the change in signal between the control and capsaicin scans in the dl/lPAG and PB ipsilateral to the forearm stimulation, and bilaterally in the SN. It was negatively correlated with signal changes in the RVM and ipsilateral LC (Figs. 4, 5; Table 2).

Figure 5 shows a summary of the significant signal intensity changes correlated to placebo and nocebo ability for all 25 participants. It is clear that within the PAG and RVM, placebo and nocebo have opposing effects. That is, greater placebo ability was associated with less PAG and greater RVM signal change, whereas greater nocebo ability was associated with greater PAG and less RVM signal change. A similar relationship was also seen in PB activity, which correlated negatively and positively with placebo and nocebo ability, respectively. In contrast, two other brainstem regions displayed similar signal relationships with both placebo and nocebo; that is, in the SN, signal changes were positively correlated and in the LC signal negatively correlated with both placebo and nocebo abilities.

PAG and RVM organization of placebo and nocebo responsiveness

Detailed analysis of the PAG revealed that apart from a group of voxels at one rostrocaudal level of the ipsilateral PAG, the vast majority of voxels that significantly correlated with placebo or nocebo abilities were located in the contralateral PAG (Fig. 6). For placebo, negatively correlated voxels were located at relatively caudal levels and primarily in the dlPAG and lPAG columns. No significantly correlated voxels were found in the vlPAG. For nocebo, the vast majority of positively correlated voxels were also located in the lPAG, although smaller numbers were also found in the remaining three columns at more rostral levels than those of placebo. With regard to the RVM, for both placebo and nocebo, significantly correlated voxels were located primarily at rostral levels in the middle

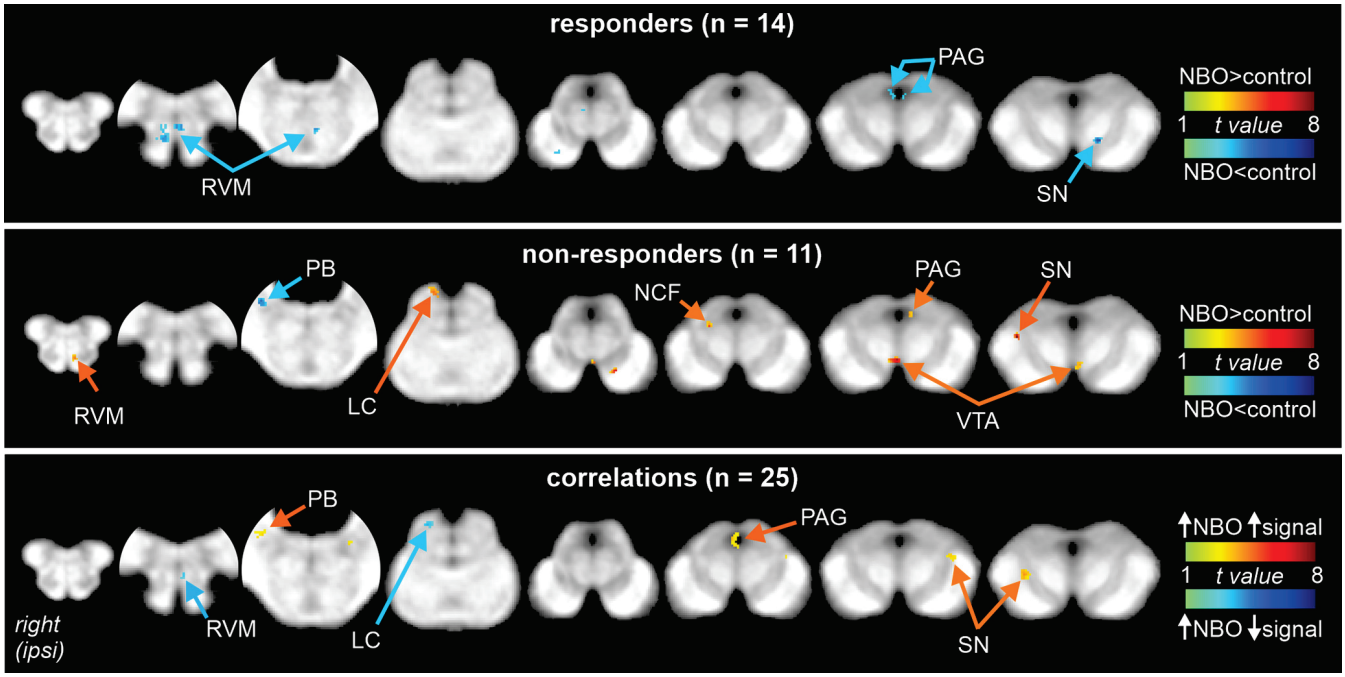

Figure 4. Signal changes in nocebo responders, nonresponders, and sites where signal change correlated with nocebo ability. Brainstem activity differed significantly between the control and capsaicin (nocebo, NBO) scans in several pain modulatory nuclei. In responders (top) signal decreases (cool color scale) occurred in the midbrain PAG bilaterally, contralateral SN, and midline RVM. In nonresponders (middle), signal increases (hot color scale) occurred in the contralateral PAG, the SN bilaterally, ipsilateral LC, and NCF, VTA, and RVM, and signal deceases occurred in the PB ipsilaterally. Signal intensity changes significantly correlated with nocebo ability (bottom) were found to be positively correlated (hot color scale) in the ipsilateral lateral PAG and PB, and SN bilaterally, and negatively correlated (cool color scale) in the ipsilateral LC and the RVM. PAG = periaqueductal gray, SN = substantia nigra, RVM = rostral ventromedial medulla, LC = locus coeruleus, NCF = nucleus cuneiformis, VTA = ventral tegmental area, PB = parabrachial nucleu.

and ventral aspects, although positively correlated for placebo and negatively correlated for nocebo.

Discussion

Our data reveals the first in-depth exploration of the detailed human brainstem circuitry involved in generating placebo and nocebo responses. These divergent pain modulatory responses were characterized by different activation patterns in the PAG and RVM, which together form a core brainstem pain modulatory circuit that regulates incoming noxious information at the level of the primary afferent synapse (Fields et al., 1983; Zhang et al., 1997; Fields, 2004; Tracey and Mantyh, 2007). Within the PAG, placebo and nocebo responsivities were correlated to signal changes within the lateral and dorsolateral PAG but not within the ventrolateral PAG column. Additionally, we found that other brainstem pain modulatory sites such as the LC, PB, RVLM, SRD, and SN act collectively with the PAG-RVM axis to produce both placebo and nocebo effects.

Despite the expectations of all subjects that the lidocaine and capsaicin creams would modulate pain intensity, only a proportion of individuals showed a significant change in perceived pain intensity. This is consistent with the fact that pain modulation is highly variable (Tétreault et al., 2016) as it depends on the concordance between expectation and experience (precision) of prior painful events (Grahl et al., 2018). Fewer participants responded to placebo (36%) than nocebo trials (56%), only 24% of participants responded to both nocebo and placebo, and there was no significant relationship between placebo and nocebo abilities. These proportions are similar to those reported in other investigations (Levine et al., 1979; Grevert et al., 1983; Levine and Gordon, 1984; Wager et al., 2004; Meister et al., 2020). Because perceived pain intensities can vary within individuals to repeated presentations of the same noxious stimulus, we used the two SD threshold to define significant pain change and categorize individuals as responders and nonresponders. Investigating responders and nonresponders separately allowed us to highlight potential differences in an individual's ability to engage pain modulatory circuits.

Even with limited evidence, PAG–RVM–DH circuitry is assumed to be the final common pathway through which placebo analgesia and nocebo hyperalgesia are mediated (Bingel et al., 2006; Eippert et al., 2009; Tinnermann et al., 2017; Schafer et al., 2018). Indeed, our investigation supports that this brainstem circuit is a pivotal component in the potentiation of placebo and nocebo responses. We found opposing activity changes in this circuitry, with greater placebo ability associated with reduced signal changes in the dorsolateral and lateral PAG and increased signal changes in the RVM, and greater nocebo ability associated with increased signal changes in the dorsolateral and lateral PAG and reduced signal changes in the RVM. It is well established from experimental animal investigations that the RVM contains off and on neurons that inhibit and facilitate neurotransmission at the primary nociceptive synapse, respectively (Fields, 2004; Vanegas and Schaible, 2004; Benarroch, 2008; Heinricher et al., 2009; Ossipov et al., 2010). Activation of on cells is typically observed during prolonged exposure to noxious stimuli and leads to enhanced nociception (Morgan and Fields, 1994), whereas activation of off cells is believed to be sufficient to produce pronounced analgesia (Cheng et al., 1986; Ossipov et al., 2010). Our data suggest that when short duration stimuli are applied, reduced synaptic activity within the PAG results in an increase in the overall balance of RVM off-cell compared with on-cell firing, which in turn results in increased inhibition of incoming nociceptive drive at the dorsal horn and a placebo

Table 2. Location, level of significance and cluster size of significant clusters in each of the nocebo groups and correlation analyses

	MNI coordinates						Beta-value change (mean ± SEM)	
	x	*y*	*z*	*t* value	Cluster size[a]	Volume (mm^3)	Control scan	Capsaicin scan
Nocebo responders								
NBO < controls								
Ipsilateral PAG	2	−30	−4	3.85	16	2	1.25 ± 0.30	−0.84 ± 0.40
Contralateral PAG	−1	−28	−3	3.46	8	1	1.73 ± 0.41	−0.74 ± 0.46
Contralateral SN	−6	−18	−6	5.98	69	8.625	0.89 ± 0.20	−0.08 ± 0.20
RVM	4	−33	−45	5.92	186	23.25	0.72 ± 0.17	−0.33 ± 0.15
	1	−35	−43	4.37	26	3.25		
Nocebo nonresponders								
NBO > controls								
Contralateral PAG	−2	−28	−3	4.49	16	2	−0.73 ± 0.26	0.42 ± 0.35
Ipsilateral NCF	7	−28	−7	5.24	17	2.125	−0.67 ± 0.27	0.53 ± 0.31
Ipsilateral SN	15	−24	−3	7.91	43	5.375	−0.35 ± 0.22	1.02 ± 0.28
Contralateral SN	−5	−19	−12	8.50	23	2.875	−0.69 ± 0.26	0.91 ± 0.37
RVM	0	−31	−48	5.68	64	8	−0.60 ± 0.24	0.42 ± 0.15
Ipsilateral LC	5	−37	−17	4.58	57	7.125	−0.13 ± 0.20	1.22 ± 0.25
VTA	2	−19	−7	7.92	66	8.25	−1.41 ± 0.30	0.16 ± 0.24
NBO < controls								
Ipsilateral PB	12	−41	−35	4.70	57	7.125	0.33 ± 0.25	−1.13 ± 0.24
Nocebo correlations								
Negative correlation							*r* values	
Ipsilateral LC	6	−35	−16	3.69	22	2.75	−0.61	
RVM	0	−36	−43	3.30	9	1.125	−0.57	
Positive correlation								
Ipsilateral PAG	2	−30	−5	3.57	63	7.875	0.57	
Ipsilateral SN	13	−21	−4	4.53	83	10.375	0.63	
Contralateral SN	−12	−26	−6	3.65	58	7.25	0.65	
Ipsilateral PB	12	−41	−34	3.53	42	5.25	0.63	

Coordinates are in MNI space.
[a]Cluster sizes are reported in resliced 0.5 mm^3 voxels.

analgesic response, and conversely for hyperalgesic responses. The human RVM is difficult to localize anatomically as it is a large and complex structure extending through the caudal pons and a large section of the midline medulla. However, the clusters we identify as RVM are consistent with those identified previously in studies of placebo and attentional analgesia (Eippert et al., 2009; Oliva et al., 2021), suggesting that combined with the changes observed in the PAG, both these phenomena involved altered recruitment along this central pain modulatory pathway.

As placebo analgesic responses have been shown to be opioid mediated (Amanzio and Benedetti, 1999; Zubieta et al., 2005; Wager et al., 2007; Scott et al., 2008; Zhang et al., 2013), and vlPAG-evoked analgesic responses are also opioid mediated (McNally et al., 2004; Loyd and Murphy, 2009), it has been hypothesized that placebo analgesic responses are likely mediated by the vlPAG. However, we found that both placebo- and nocebo-related signal changes occurred in the lPAG and dlPAG but not the vlPAG column. Experimental animal investigations have shown that lPAG stimulation produces a nonopioid analgesia coupled with active defensive behaviors (Bandler et al., 2000) that are mediated by brainstem circuits including via lPAG–RVM projections (Mantyh, 1983; Petrovic et al., 2004; Hohmann et al., 2005; Loyd and Murphy, 2009; Mokhtar and Singh, 2021). It appears that higher brain regions involved in conditioning and expectation recruit the lPAG and dlPAG to produce placebo and nocebo responses. Indeed, the anterior cingulate cortex and amygdala have been shown to be recruited during placebo analgesia (Tracey and Mantyh, 2007; Eippert et al., 2009; Freeman et al., 2015), and it is possible that the opioid-mediated nature of placebo results from the actions of opioids on these regions and not the PAG.

Furthermore, because opioid-mediated antinociception is generally prolonged and not easily reversible (Atlas and Wager, 2012), it is possible that a long-lasting placebo analgesia involves persistent recruitment of opioid mechanisms at the level of the vlPAG, whereas analgesia generated in response to brief acute stimuli rely on alternate mechanisms. Placebo analgesia, which is conditioned through administration of the nonsteroidal anti-inflammatory drug Ketoralac, is blocked by the cannabinoid receptor antagonist Rimonabant (Benedetti et al., 2011), and the dlPAG contains a dense concentration of CB1 cannabinoid receptors (Wilson-Poe et al., 2012), raising the possibility that this may be the neurochemical system mediating both our observed placebo and nocebo effects.

In addition to the PAG and RVM, a region encompassing the ipsilateral PB also displayed opposing signal intensity changes during placebo and nocebo responses; that is, signal changes negatively correlated with placebo ability but positively correlated with nocebo ability. Experimental animal studies have reported that the PB is a key integration site of nociceptive information, including relaying noxious inputs to higher brain areas (Loewy and Spyers, 1990; Petrovic et al., 2004) as well as providing descending modulatory influences over the PAG, RVM, and dorsal horn via the spinoparabrachial and spino-bulbo-spinal pathways, respectively (Gauriau and Bernard, 2002; Mainero et al., 2007; Roeder et al., 2016; Chen et al., 2017; Stroman et al., 2018; Bannister, 2019). Blocking the ipsilateral (to applied noxious stimuli) PB attenuates behavioral hyperalgesia in animals (Chen and Heinricher, 2019), and PB stimulation evokes aversive

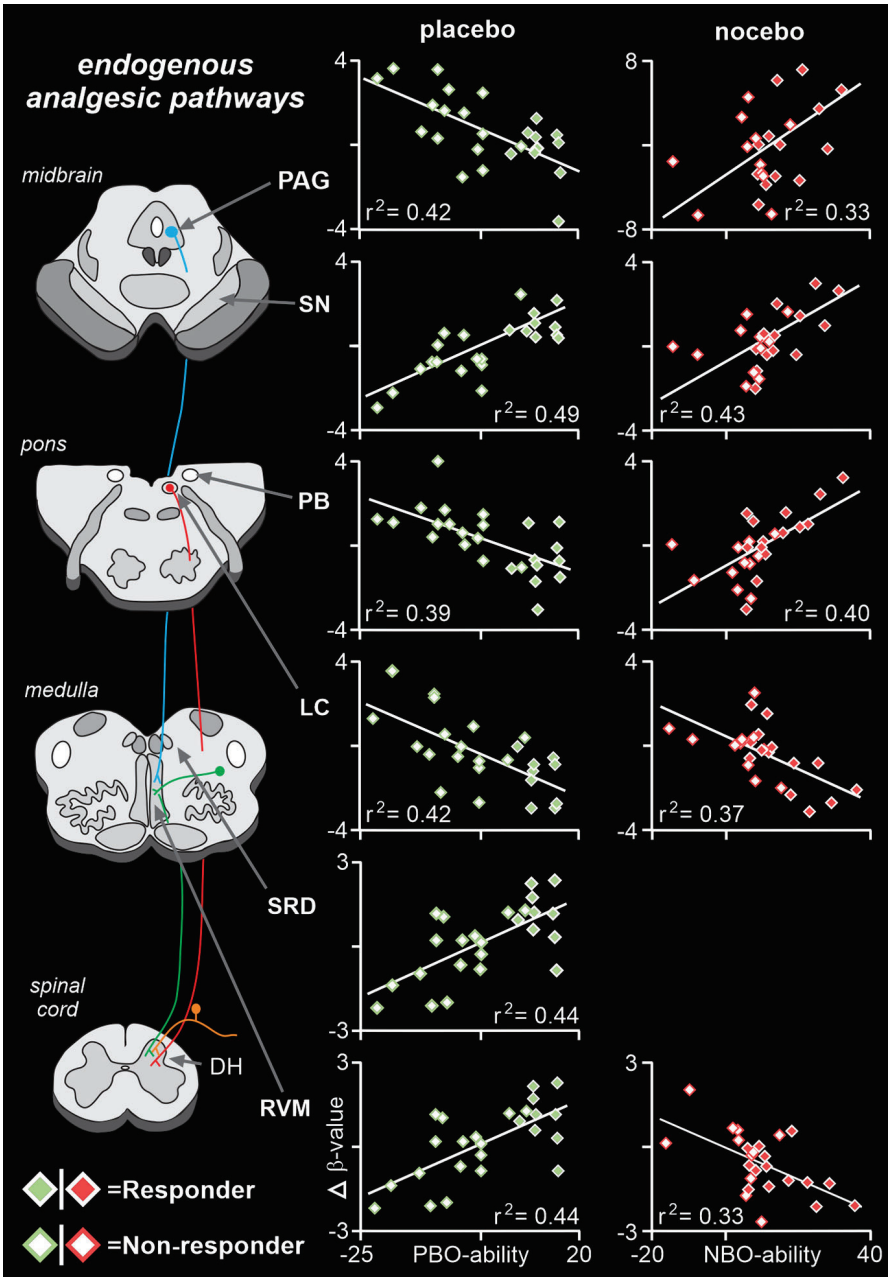

Figure 5. Divergent recruitment of brainstem sites. A summary of brainstem regions in which signal intensity changes are significantly correlated to placebo or nocebo abilities reveals that the midbrain PAG–RVM pathway displays opposing responses. That is, signal changes in the PAG are negatively correlated with placebo ability but positively correlated with nocebo ability and vice versa for the RVM. In direct contrast, brainstem regions such as the SN and LC display similar relationships with both placebo and nocebo abilities, suggesting they may be involved in aspects of placebo and nocebo that are not directly related to altering pain perception. Colored lines indicate major descending pathways within the brainstem; blue = PAG–RVM axis, green = reticular-spinal cord projections, and red = spinal projections from the LC.

behaviors in response to painful stimuli (Rodriguez et al., 2017). In addition to a role in placebo and nocebo, we have previously shown PB signal intensity changes during conditioned pain modulation analgesia, suggesting that the PB can modulate pain under a variety of paradigms (Youssef et al., 2016). Of course, because the PB also receives ascending noxious information from the dorsal horn, it is possible that changes in PB signal during placebo and nocebo reflect alterations in ascending drive because of the descending modulatory effects of the PAG–RVM on incoming noxious information at the dorsal horn (Yasui et al., 1989; Jasmin et al., 1997; Hunt and Mantyh, 2001; Gauriau and Bernard, 2002).

In contrast to the differential signal changes in the PAG, RVM, and PB, we found that signal within the LC was negatively correlated, SN positively correlated with both placebo and nocebo abilities, and VTA changes occurred in nonresponders only. The location of the LC cluster labeled in this study is consistent with the lateral extent of this nucleus as defined by Paxinos and Huang (2013) and previous human brain imaging investigations (Sclocco et al., 2016; Brooks et al., 2017). Although preclinical studies have shown that LC stimulation can produce a profound nonopioid analgesia (Hodge et al., 1983; Viisanen and Pertovaara, 2007), the LC, along with the SN and VTA, may be involved in an alternative aspect such as attentional or stimulus-response processes. Ascending dopaminergic circuitry can facilitate learning effects and encode prediction error, and phasic activity of midbrain dopamine neurons may be responsible for the expectations of future pain toward appetitive and aversive stimuli (Pauli et al., 2015; Nasser et al., 2017; Henderson et al., 2020) as well as updating them when expected rewarding or punishing responses are challenged (Schultz, 2002; Wager et al., 2006). The VTA plays a crucial role in coding unexpected responses to valanced predicted events, with unexpected rewarding events eliciting increased VTA firing and unexpected punishments decreasing VTA activity (Romo and Schultz, 1990; Jhou et al., 2009). Consistent with these findings, placebo and nocebo nonresponders demonstrated decreased and increased VTA signal, respectively, signal changes that may reflect unexpected punishment or reward. Additionally, the SN has been linked to unvalanced prediction error signal, enabling further processing of unexpected stimuli and cognitive flexibility within the cortex (Matsumoto and Hikosaka, 2009). Our results support SN as a pivotal driver for both positive and negative pain modulatory effects, likely through its ascending dopaminergic projections.

Finally, we found SRD and RVLM signal changes associated with placebo but not nocebo. Stimulation of the PAG produces analgesic responses that are attenuated by lesions encompassing the RVLM, which projects directly to the dorsal horn (Lovick, 1985; Siddall et al., 1994), and the SRD is critical in the expression of conditioned pain modulation (Youssef et al., 2016). It has been proposed that the RVM, SRD, and RVLM form an interconnected reticular triad that receives input from a variety of cortical and brainstem regions to balance nociceptive signaling (Martins and

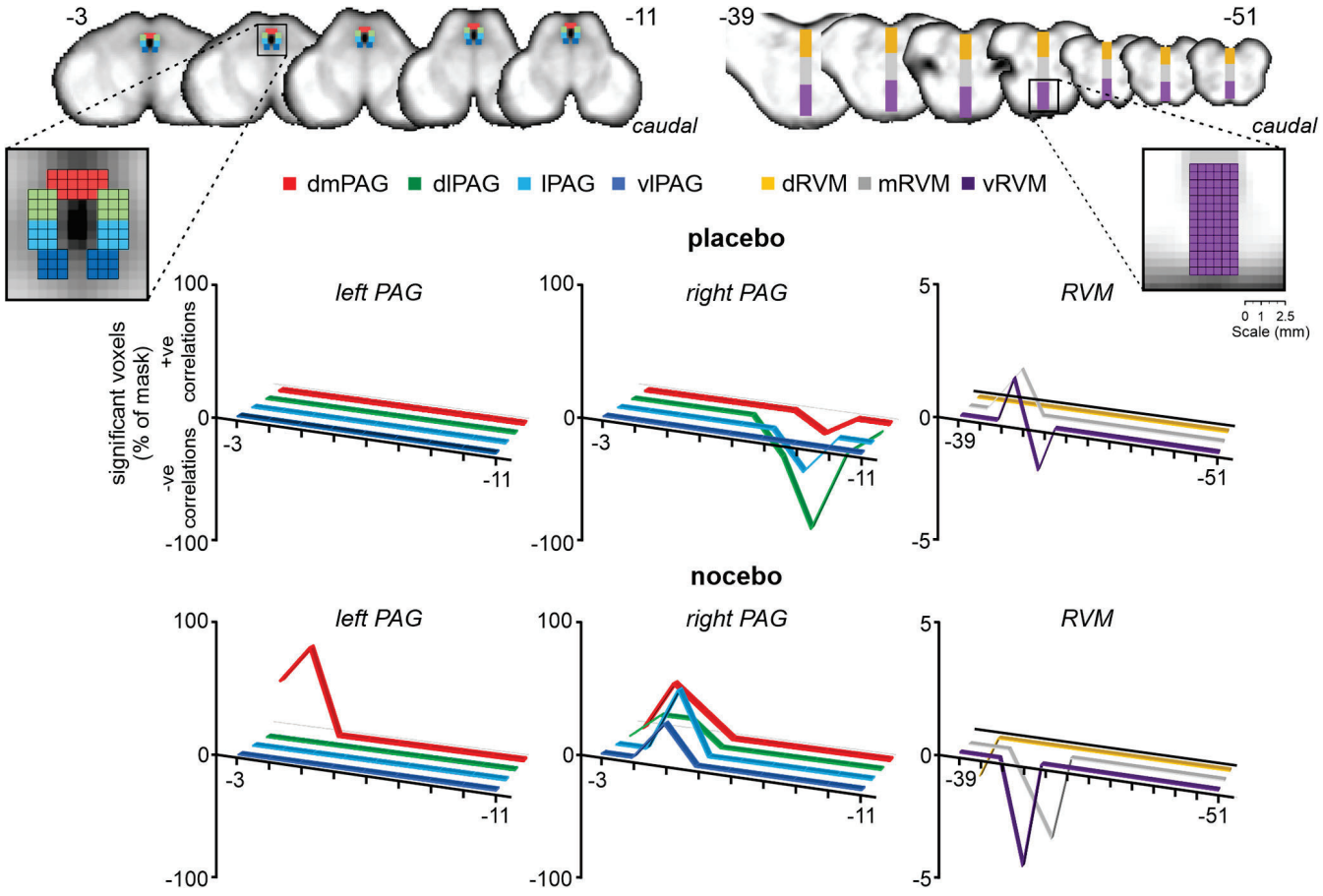

Figure 6. Regional correlations in the midbrain periaqueductal gray and rostral ventromedial medulla. Top left, Inset shows the individual masks in dmPAG (red), dlPAG (green), lPAG (light blue), and vlPAG (dark blue). Top right, Inset shows the individual masks in dRVM (orange), mRVM (gray), and vRVM (purple). The *z* coordinates in Montreal Neurological Institute space are indicated at the top right. Bottom, Plots depicting the percentage of voxels in each masked region that positively or negatively correlate with placebo or nocebo ability. Note that placebo and nocebo abilities are largely correlated with signal intensity changes in voxels located in discrete rostrocaudal levels of the dlPAG and lPAG as well as the middle and ventral RVM.

Tavares, 2017). Our results suggest that activation of this reticular triad underpins placebo analgesia and that when these regions are deactivated, expected analgesic effects are attenuated. Interestingly, we found only the RVM had a role in generating nocebo hyperalgesia, suggesting that placebo effects may involve a more widespread brainstem circuitry than is required for the expression of nocebo hyperalgesia.

It is important to note some limitations. First, conditioning-based models of pain modulation are prone to response bias (Hróbjartsson et al., 2011), so we asked participants to rate their pain on-line during the scan instead of afterward to reduce such potential bias. This protocol also reduced the potential for series-position effects (Murdock, 1962). Second, as the experimental design required pairing potentially modulated with nonmodulated responses, it was not possible to fully counterbalance the ordering of stimuli. We did, however, counterbalance the location and stimulation order of the lidocaine and capsaicin cream sites, reducing the likelihood of an ordering effect or location-based sensitivity. Third, although dichotomizing participants as responders and nonresponders was important for evaluating individual variations in brainstem recruitment, this may have introduced a selection bias that could influence the overall interpretation. Although the 2 SD band method constrains group assignment to individual pain responses on the same dermatome, it could be interpreted that participants with lesser pain sensitivity on the placebo-treated site would be more likely to be placebo responders and the inverse for nocebo responders. If so, an alternative interpretation could be that the PAG–RVM circuit and the PB were responsive to the intensity of noxious stimuli rather than the manifestation of modulatory phenomena. By extension, habituation and sensitization effects to heat pain are both spatially and temporally dependent (Jepma et al., 2014). We found that pain responses for all subjects between control series 1 and 2 were not significantly different, suggesting that habituation and/or sensitization was absent during the test phase. Furthermore, although we included a 24 h period between conditioning and reinforcement, at least a 15 min period between reinforcement and test phases, and reinforcement and test phases were conducted on opposite forearms, some stimulus history effects may have remained. Fourth, we used an initial threshold of $p < 0.005$ uncorrected for multiple comparisons with a cluster extent threshold of five contiguous voxels. Our results were largely limited to brainstem nuclei previously stated to play a functional role in pain modulation, and we further performed cluster-level thresholding to reduce the potential for type 2 errors. Given that the wealth of experimental animal investigations into the brainstem sites responsible for pain modulation have identified the PAG–RVM–DH as the critical circuit, one might have hypothesized that areas such as the SRD, PB, LC, and NCF play somewhat more minor roles. However, our data do not show this with regard to overall significance, although the precise role of each of these regions in placebo and nocebo responses remains to be ascertained. Finally, although the enhanced spatial resolution provided by 7 tesla imaging allowed us to describe voxel

peaks within anatomically meaningful areas using the brainstem atlas from Paxinos and Huang (2013), we appreciate that even with increased spatial resolution we are not able to precisely identify small brainstem nuclei. Given this, the described cluster locations need to be appreciated with some caution.

Conclusions

Using ultra-high-field fMRI, we have shown, for the first time, that specific nuclei within the brainstem mediate changes in the perceived intensity of noxious stimuli to produce placebo and nocebo responses. In support of prevailing models asserting the PAG–RVM axis as the central pathway for descending pain modulatory effects, we have shown that the rostral ventromedial medulla is positively related and the periaqueductal gray inversely related to the placebo response, whereas the reverse is true for nocebo responses. We further suggest that this central circuitry alone is not solely responsible for subsequent pain modulation, but rather a more widespread engagement of pathways involving the parabrachial complex, locus ceruleus, and substantia nigra are pivotal drivers in producing significant antinociceptive and pro-nociceptive effects. The specific roles and cortical connectivity profiles of each of these brainstem regions in modulating perceived pain intensity remains to be determined and would be a valuable future investigation if we are to fully understand these complex phenomena.

References

Amanzio M, Benedetti F (1999) Neuropharmacological dissection of placebo analgesia: expectation-activated opioid systems versus conditioning-activated specific subsystems. J Neurosci 19:484–494.

Atlas LY, Wager TD (2012) How expectations shape pain. Neurosci Lett 520:140–148.

Babel P, Adamczyk W, Swider K, Bajcar EA, Kicman P, Lisinska N (2018) How classical conditioning shapes placebo analgesia: hidden versus open conditioning. Pain Med 19:1156–1169.

Bandler R, Shipley MT (1994) Columnar organization in the midbrain periaqueductal gray: modules for emotional expression? Trends Neurosci 17:379–389.

Bandler R, Keay KA (1996) Columnar organization in the midbrain periaqueductal gray and the integration of emotional expression. Prog Brain Res 107:285–300.

Bandler R, Keay KA, Floyd N, Price J (2000) Central circuits mediating patterned autonomic activity during active vs. passive emotional coping. Brain Res Bull 53:95–104.

Bannister K (2019) Descending pain modulation: influence and impact. Curr Opin Physiol 11:62–66.

Benarroch EE (2008) Descending monoaminergic pain modulation: bidirectional control and clinical relevance. Neurology 71:217–221.

Benedetti F, Amanzio M, Rosato R, Blanchard C (2011) Nonopioid placebo analgesia is mediated by CB1 cannabinoid receptors. Nat Med 17:1228–1230.

Bingel U, Lorenz J, Schoell E, Weiller C, Büchel C (2006) Mechanisms of placebo analgesia: rACC recruitment of a subcortical antinociceptive network. Pain 120:8–15.

Brooks JCW, Davies W-E, Pickering AE (2017) Resolving the brainstem contributions to attentional analgesia. J Neurosci 37:2279–2291.

Chen Q, Heinricher MM (2019) Plasticity in the link between pain-transmitting and pain-modulating systems in acute and persistent inflammation. J Neurosci 39:2065–2079.

Chen Q, Roeder Z, Li MH, Zhang Y, Ingram SL, Heinricher MM (2017) Optogenetic evidence for a direct circuit linking nociceptive transmission through the parabrachial complex with pain-modulating neurons of the rostral ventromedial medulla (RVM). eNeuro 4:ENEURO.0202-17.2017.

Cheng ZF, Fields HL, Heinricher MM (1986) Morphine microinjected into the periaqueductal gray has differential effects on 3 classes of medullary neurons. Brain Res 375:57–65.

Coulombe MA, Erpelding N, Kucyi A, Davis KD (2016) Intrinsic functional connectivity of periaqueductal gray subregions in humans. Hum Brain Mapp 37:1514–1530.

Craggs JG, Price DD, Verne GN, Perlstein WM, Robinson MM (2007) Functional brain interactions that serve cognitive-affective processing during pain and placebo analgesia. Neuroimage 38:720–729.

Diedrichsen J (2006) A spatially unbiased atlas template of the human cerebellum. Neuroimage 33:127–138.

Egorova N, Benedetti F, Gollub RL, Kong J (2019) Between placebo and nocebo: response to control treatment is mediated by amygdala activity and connectivity. Eur J Pain 24:580–592.

Eippert F, Bingel U, Schoell ED, Yacubian J, Klinger R, Lorenz J, Büchel C (2009) Activation of the opioidergic descending pain control system underlies placebo analgesia. Neuron 63:533–543.

Faul F, Erdfelder E, Lang AG, Buchner A (2007) G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods 39:175–191.

Faull OK, Jenkinson M, Clare S, Pattinson KTS (2015) Functional subdivision of the human periaqueductal grey in respiratory control using 7 tesla fMRI. Neuroimage 113:356–364.

Fields H (2004) State-dependent opioid control of pain. Nat Rev Neurosci 5:565–575.

Fields HL, Bry J, Hentall I, Zorman G (1983) The activity of neurons in the rostral medulla of the rat during withdrawal from noxious heat. J Neurosci 3:2545–2552.

Finniss DG, Kaptchuk TJ, Miller F, Benedetti F (2010) Biological, clinical, and ethical advances of placebo effects. The Lancet 375:686–695.

Freeman S, Yu R, Egorova N, Chen X, Kirsch I, Claggett B, Kaptchuk TJ, Gollub RL, Kong J (2015) Distinct neural representations of placebo and nocebo effects. Neuroimage 112:197–207.

Frisaldi E, Piedimonte A, Benedetti F (2015) Placebo and nocebo effects: a complex interplay between psychological factors and neurochemical networks. Am J Clin Hypn 57:267–284.

Gauriau C, Bernard JF (2002) Pain pathways and parabrachial circuits in the rat. Exp Physiol 87:251–258.

Grahl A, Onat S, Büchel C (2018) The periaqueductal gray and Bayesian integration in placebo analgesia. Elife 7:e32930.

Grevert P, Albert LH, Goldstein A (1983) Partial antagonism of placebo analgesia by naloxone. Pain 16:129–143.

Hansen E, Zech N, Meissner K (2017) [Placebo and nocebo: how can they be used or avoided? Internist (Berl) 58:1102–1110.

Heinricher MM, Tavares I, Leith JL, Lumb BM (2009) Descending control of nociception: specificity, recruitment and plasticity. Brain Res Rev 60:214–225.

Henderson LA, Di Pietro F, Youssef AM, Lee S, Tam S, Akhter R, Mills EP, Murray GM, Peck CC, Macey PM (2020) Effect of expectation on pain processing: a psychophysics and functional MRI analysis. Front Neurosci 14:6–6.

Hibi D, Takamoto K, Iwama Y, Ebina S, Nishimaru H, Matsumoto J, Takamura Y, Yamazaki M, Nishijo H (2020) Impaired hemodynamic activity in the right dorsolateral prefrontal cortex is associated with impairment of placebo analgesia and clinical symptoms in postherpetic neuralgia. IBRO Rep 8:56–64.

Hodge CJ, Apkarian AV, Owen MP, Hanson BS (1983) Changes in the effects of stimulation of locus coeruleus and nucleus raphe magnus following dorsal rhizotomy. Brain Res 288:325–329.

Hohmann AG, Suplita RL, Bolton NM, Neely MH, Fegley D, Mangieri R, Krey JF, Walker JM, Holmes PV, Crystal JD, Duranti A, Tontini A, Mor M, Tarzia G, Piomelli D (2005) An endocannabinoid mechanism for stress-induced analgesia. Nature 435:1108–1112.

Hróbjartsson A, Kaptchuk TJ, Miller FG (2011) Placebo effect studies are susceptible to response bias and to other types of biases. J Clin Epidemiol 64:1223–1229.

Hunt SP, Mantyh PW (2001) The molecular dynamics of pain control. Nat Rev Neurosci 2:83–91.

Jasmin L, Burkey AR, Card JP, Basbaum AI (1997) Transneuronal labeling of a nociceptive pathway, the spino-(trigemino-)parabrachio-amygdaloid, in the rat. J Neurosci 17:3751–3765.

Jepma M, Jones M, Wager TD (2014) The dynamics of pain: evidence for simultaneous site-specific habituation and site-nonspecific sensitization in thermal pain. J Pain 15:734–746.

Jhou TC, Geisler S, Marinelli M, Degarmo BA, Zahm DS (2009) The mesopontine rostromedial tegmental nucleus: a structure targeted by the lateral habenula that projects to the ventral tegmental area of Tsai and substantia nigra compacta. J Comp Neurol 513:566–596.

Kirsch I, Kong J, Sadler P, Spaeth R, Cook A, Kaptchuk T, Gollub R (2014) Expectancy and conditioning in placebo analgesia: separate or connected processes? Psychol Conscious (Wash D C) 1:51–59.

Kragel PA, Bianciardi M, Hartley L, Matthewson G, Choi J-K, Quigley KS, Wald LL, Wager TD, Feldman Barrett L, Satpute AB (2019) Functional Involvement of human periaqueductal gray and other midbrain nuclei in cognitive control. J Neurosci 39:6180–6189.

Levine JD, Gordon NC (1984) Influence of the method of drug administration on analgesic response. Nature 312:755–756.

Levine JD, Gordon NC, Bornstein JC, Fields HL (1979) Role of pain in placebo analgesia. Proc Natl Acad Sci U S A 76:3528–3531.

Linnman C, Moulton EA, Barmettler G, Becerra L, Borsook D (2012) Neuroimaging of the periaqueductal gray: state of the field. Neuroimage 60:505–522.

Loewy AD, Spyer KM (1990) Vagal preganglionic neurons. In:Central regulation of Autonomic Functions (Loewy AD, Spyer KM eds), pp 68–87. New York: Oxford University Press.

Lovick TA (1985) Ventrolateral medullary lesions block the antinociceptive and cardiovascular responses elicited by stimulating the dorsal periaqueductal grey matter in rats. Pain 21:241–252.

Loyd DR, Murphy AZ (2009) The role of the periaqueductal gray in the modulation of pain in males and females: are the anatomy and physiology really that different? Neural Plast 2009:462879.

Mainero C, Zhang W-T, Kumar A, Rosen BR, Sorensen AG (2007) Mapping the spinal and supraspinal pathways of dynamic mechanical allodynia in the human trigeminal system using cardiac-gated fMRI. Neuroimage 35:1201–1210.

Mantyh PW (1983) Connections of midbrain periaqueductal gray in the monkey. II. Descending efferent projections. J Neurophysiol 49:582–594.

Martins I, Tavares I (2017) Reticular formation and pain: the past and the future. Front Neuroanat 11:51.

Matsumoto M, Hikosaka O (2009) Two types of dopamine neuron distinctly convey positive and negative motivational signals. Nature 459:837–841.

McNally GP, Pigg M, Weidemann G (2004) Opioid Receptors in the midbrain periaqueductal gray regulate extinction of pavlovian fear conditioning. J Neurosci 24:6912–6919.

Medoff ZM, Colloca L (2015) Placebo analgesia: understanding the mechanisms. Pain Manag 5:89–96.

Meister R, Abbas M, Antel J, Peters T, Pan Y, Bingel U, Nestoriuc Y, Hebebrand J (2020) Placebo response rates and potential modifiers in double-blind randomized controlled trials of second and newer generation antidepressants for major depressive disorder in children and adolescents: a systematic review and meta-regression analysis. Eur Child Adolesc Psychiatry 29:253–273.

Mokhtar M, Singh P (2021) Neuroanatomy, periaqueductal gray. Treasure Island, FL: StatPearls.

Morgan MM, Fields HL (1994) Pronounced changes in the activity of nociceptive modulatory neurons in the rostral ventromedial medulla in response to prolonged thermal noxious stimuli. J Neurophysiol 72:1161–1170.

Murdock BB Jr (1962) The serial position effect of free recall. J Exp Psychol 64:482–488.

Nasser HM, Calu DJ, Schoenbaum G, Sharpe MJ (2017) The dopamine prediction error: contributions to associative models of reward learning. Front Psychol 8:244–244.

Oliva V, Gregory R, Davies W-E, Harrison L, Moran R, Pickering AE, Brooks JCW (2021) Parallel cortical-brainstem pathways to attentional analgesia. Neuroimag 226:117548.

Ossipov MH, Dussor GO, Porreca F (2010) Central modulation of pain. J Clin Invest 120:3779–3787.

Palazzo E, Luongo L, de Novellis V, Rossi F, Maione S (2010) The role of cannabinoid receptors in the descending modulation of pain. Pharmaceuticals (Basel) 3:2661–2673.

Pauli WM, Larsen T, Collette S, Tyszka JM, Seymour B, O'Doherty JP (2015) Distinct contributions of ventromedial and dorsolateral subregions of the human substantia nigra to appetitive and aversive learning. J Neurosci 35:14220–14233.

Paxinos G, Huang X-F (2013) Atlas of the human brainstem. Burlington, VT: Elsevier Science.

Penny WD, Friston KJ, Ashburner JT, Kiebel SJ, Nichols TE (2011) Statistical parametric mapping: the analysis of functional brain images. Amsterdam, Netherlands: Elsevier.

Petrovic P, Kalso E, Petersson KM, Ingvar M (2002) Placebo and opioid analgesia– imaging a shared neuronal network. Science 295:1737–1740.

Petrovic P, Petersson KM, Hansson P, Ingvar M (2004) Brainstem involvement in the initial response to pain. Neuroimage 22:995–1005.

Rodriguez E, Sakurai K, Xu J, Chen Y, Toda K, Zhao S, Han BX, Ryu D, Yin H, Liedtke W, Wang F (2017) A craniofacial-specific monosynaptic circuit enables heightened affective pain. Nat Neurosci 20:1734–1743.

Roeder Z, Chen Q, Davis S, Carlson JD, Tupone D, Heinricher MM (2016) Parabrachial complex links pain transmission to descending pain modulation. Pain 157:2697–2708.

Romo R, Schultz W (1990) Dopamine neurons of the monkey midbrain: contingencies of responses to active touch during self-initiated arm movements. J Neurophysiol 63:592–606.

Särkkä S, Solin A, Nummenmaa A, Vehtari A, Auranen T, Vanni S, Lin F-H (2012) Dynamic retrospective filtering of physiological noise in BOLD fMRI: DRIFTER. NeuroImage 60:1517–1527.

Satpute AB, Wager TD, Cohen-Adad J, Bianciardi M, Choi JK, Buhle JT, Wald LL, Barrett LF (2013) Identification of discrete functional subregions of the human periaqueductal gray. Proc Natl Acad Sci U S A 110:17101–17106.

Schafer SM, Geuter S, Wager TD (2018) Mechanisms of placebo analgesia: a dual-process model informed by insights from cross-species comparisons. Prog Neurobiol 160:101–122.

Schenk LA, Colloca L (2020) The neural processes of acquiring placebo effects through observation. Neuroimage 209:116510.

Schienle A, Höfler C, Ûbel S, Wabnegger A (2018) Emotion-specific nocebo effects: an fMRI study. Brain imaging Behav 12:180–187.

Schultz W (2002) Getting formal with dopamine and reward. Neuron 36:241–263.

Sclocco R, Beissner F, Desbordes G, Polimeni JR, Wald LL, Kettner NW, Kim J, Garcia RG, Renvall V, Bianchi AM, Cerutti S, Napadow V, Barbieri R (2016) Neuroimaging brainstem circuitry supporting cardiovagal response to pain: a combined heart rate variability/ultrahigh-field (7 T) functional magnetic resonance imaging study. Phil Trans R Soc A 374:20150189.

Sclocco R, Beissner F, Bianciardi M, Polimeni JR, Napadow V (2018) Challenges and opportunities for brainstem neuroimaging with ultrahigh field MRI. NeuroImage 168:412–426.

Scott DJ, Stohler CS, Egnatuk CM, Wang H, Koeppe RA, Zubieta JK (2008) Placebo and nocebo effects are defined by opposite opioid and dopaminergic responses. Arch Gen Psychiatry 65:220–231.

Sevel LS, Craggs JG, Price DD, Staud R, Robinson ME (2015) Placebo analgesia enhances descending pain-related effective connectivity: a dynamic causal modeling study of endogenous pain modulation. J Pain 16:760–768.

Siddall PJ, Polson JW, Dampney RAL (1994) Descending antinociceptive pathway from the rostral ventrolateral medulla: a correlative anatomical and physiological study. Brain Res 645:61–68.

Sims-Williams H, Matthews JC, Talbot PS, Love-Jones S, Brooks JC, Patel NK, Pickering AE (2017) Deep brain stimulation of the periaqueductal gray releases endogenous opioids in humans. NeuroImage 146:833–842.

Stroman PW, Ioachim G, Powers JM, Staud R, Pukall C (2018) Pain processing in the human brainstem and spinal cord before, during, and after the application of noxious heat stimuli. Pain 159:2012–2020.

Tétreault P, Mansour A, Vachon-Presseau E, Schnitzer TJ, Apkarian AV, Baliki MN (2016) Brain connectivity predicts placebo response across chronic pain clinical trials. PLOS Biol 14:e1002570.

Tinnermann A, Geuter S, Sprenger C, Finsterbusch J, Büchel C (2017) Interactions between brain and spinal cord mediate value effects in nocebo hyperalgesia. Science 358:105–108.

Tracey I, Mantyh PW (2007) The cerebral signature for pain perception and its modulation. Neuron 55:377–391.

Vanegas H, Schaible HG (2004) Descending control of persistent pain: inhibitory or facilitatory? Brain Res Brain Res Rev 46:295–309.

Vase L, Robinson ME, Verne GN, Price DD (2005) Increased placebo analgesia over time in irritable bowel syndrome (IBS) patients is associated with

desire and expectation but not endogenous opioid mechanisms. Pain 115:338–347.
Viisanen H, Pertovaara A (2007) Influence of peripheral nerve injury on response properties of locus coeruleus neurons and coeruleospinal antinociception in the rat. Neuroscience 146:1785–1794.
Voudouris NJ, Peck CL, Coleman G (1989) Conditioned response models of placebo phenomena: further support. Pain 38:109–116.
Voudouris NJ, Peck CL, Coleman G (1990) The role of conditioning and verbal expectancy in the placebo response. Pain 43:121–128.
Wager TD, Rilling JK, Smith EE, Sokolik A, Casey KL, Davidson RJ, Kosslyn SM, Rose RM, Cohen JD (2004) Placebo-induced changes in FMRI in the anticipation and experience of pain. Science 303:1162–1167.
Wager TD, Matre D, Casey KL (2006) Placebo effects in laser-evoked pain potentials. Brain Behav Immun 20:219–230.
Wager TD, Scott DJ, Zubieta JK (2007) Placebo effects on human mu-opioid activity during pain. Proc Natl Acad Sci U S A 104:11056–11061.
Wilson-Poe AR, Morgan MM, Aicher SA, Hegarty DM (2012) Distribution of CB1 cannabinoid receptors and their relationship with mu-opioid receptors in the rat periaqueductal gray. Neuroscience 213:191–200.
Yasui Y, Saper CB, Cechetto DF (1989) Calcitonin gene-related peptide immunoreactivity in the visceral sensory cortex, thalamus, and related pathways in the rat. J Comp Neurol 290:487–501.
Youssef AM, Macefield VG, Henderson LA (2016) Pain inhibits pain; human brainstem mechanisms. Neuroimage 124:54–62.
Zhang C, Yang SW, Guo YG, Qiao JT, Dafny N (1997) Locus coeruleus stimulation modulates the nociceptive response in parafascicular neurons: an analysis of descending and ascending pathways. Brain Res Bull 42:273–278.
Zhang R-R, Zhang W-C, Wang J-Y, Guo J-Y (2013) The opioid placebo analgesia is mediated exclusively through μ-opioid receptor in rat. Int J Neuropsychopharmacol 16:849–856.
Zubieta J-K, Bueller JA, Jackson LR, Scott DJ, Xu Y, Koeppe RA, Nichols TE, Stohler CS (2005) Placebo effects mediated by endogenous opioid activity on μ-opioid receptors. J Neurosci 25:7754–7762.

Chapter 2: Summary

This chapter presented my first attempt at demonstrating discrete brainstem involvement in pain modulation: specifically, during placebo analgesia and nocebo hyperalgesia. In line with our central hypotheses, we found that the lateral PAG column was primarily involved in both phenomena, and that largely similar brainstem nuclei were involved, however showing opposing changes in activation between placebo and nocebo respectively.

Two participants were removed from the experimental cohort due to demonstrating ceiling and floor effects to noxious stimuli. In the remaining 25 participants, the proportion of individuals in which placebo and nocebo were elicited was similar to that reported throughout previous literature, supporting that the two-standard deviation band method which we used to delineate "responder" and "non-responder" groups was effective.

Interestingly, we found that regardless of group assignment, both responder and nonresponder groups *expected* significant pain-modulatory effects by the placement of our placebo "lidocaine" and nocebo "capsaicin" creams. Additionally, ANOVA and linear regression revealed no significant differences or interactions between pain expectations, temperature of stimuli applied, or placebo and nocebo responses. This suggested that the type of pain modulation we had elicited throughout this experiment primarily relied on the effectiveness of the conditioning procedure.

Also supporting our core hypotheses, we found a divergent recruitment of core brainstem nuclei underlying greater placebo and nocebo responses. Specifically, signal change within the midbrain PAG, pontine parabrachial complex (PB), and rostral ventromedial medulla (RVM) was disparate between the two phenomena. Within two other sites however: the substantia nigra (SN) and locus coeruleus (LC), we identified similar patterns of significant activation change between placebo analgesia and nocebo hyperalgesia. The SN and LC are the primary brainstem sites of the dopaminergic and noradrenergic systems, respectively. These results led to our discussion that their involvement reflected similar cognitive processes associated with error-prediction, memory retrieval, and attention to incoming noxious stimuli shared between the two phenomena, rather than opposing top-down pain modulatory signals. That is, these processes would be shared regardless of the directionality of pain change. Overall, our findings suggest that brainstem involvement in pain modulation extends far beyond the classical PAG-RVM-spinal cord pathway, rather engaging a circus of subcortical sites to alter an individual's perceived pain.

Chapter 3:

Placebo analgesia relies on two distinct brain networks: imaging pain and context of improvement

"From the crawling ant to the
leaping antelope. We are all connected
in the great Circle of Life."
– Mufasa, 1994

Chapter 3: Overview

This chapter contains the following publication: Crawford LS, Meylakh N, Macey PM, Macefield VG, Keay KA, & Henderson LA. (2023). *Stimulus-independent and stimulus-dependent neural networks underpin placebo responsiveness in humans*, which was submitted to Biology Communications on 20 January 2023 and is currently under review. The submitted manuscript, in its entirety, is reproduced in this chapter.

Whilst the previous chapter provided direct evidence for discrete nuclei within the brainstem playing a role in perceived changes in pain associated with placebo analgesia and nocebo hyperalgesia, a major limitation of the study was that we limited our investigation to only the brainstem. Whilst this was necessary to first establish that activation in descending pain modulatory pathways indeed played a role in manifesting these pain modulatory phenomena, as discussed in section 1.5, these pathways are held under strict influence from top-down projections originating within the cortex.

To improve the interpretations of our findings within Chapter 2, this investigation sought to identify how discrete changes in cortical coupling with the midbrain PAG played a role in the development of placebo analgesia. That is, we combined two separate connectivity analyses, as well as dynamic causal modelling and mediation to define a complete cortical architecture which contacted the brainstem to drive endogenous pain modulation, thus addressing Aim 2.

The study involved an overhaul of the experimental design, removing the nocebo component and recruiting a broader sample size (n=47) to improve our capacity of identifying specific projection pathways from the cortex to the brainstem. A similar method of response conditioning was conducted, with an identical rating system to assess placebo responses. Interestingly, this methodological amendment appeared to increase the ratio of placebo responders : nonresponders, and we successfully elicited significant placebo responses in 48% of individuals.

We hypothesized that top-down influence from limbic sites would be present in placebo responders outside periods of noxious stimuli – in line with preclinical investigations demonstrating these regions' regulatory role over brainstem circuitry. Additionally, we hypothesized that a separate network, comprised of cingulate and prefrontal sites would contact the brainstem during noxious stimulation to drive analgesic output within the PAG.

Title: Stimulus-independent and stimulus-dependent neural networks underpin placebo responsiveness in humans.

L.S. Crawford[1], N. Meylakh[1], P.M. Macey[2], V.G. Macefield[3], K.A. Keay[1] and L.A. Henderson[1]

[1]School of Medical Sciences (Neuroscience), Brain and Mind Centre, University of Sydney, Australia, 2006; [2]UCLA School of Nursing, University of California, Los Angeles, USA; [3]Department of Neuroscience, Monash University, Australia

Correspondence: Luke A. Henderson, University of Sydney, Australia. luke.henderson@sydney.edu.au (email); +612 9351 7063 (Tel) +612 9351 6556 (Fax)

Acknowledgements: The authors acknowledge the facilities, scientific and technical assistance from the National Imaging Facility, a National Collaborative Research Infrastructure Strategy capability, at the Melbourne Brain Centre Imaging Unit, The University of Melbourne. This work was supported by the University of Melbourne, funding from the Australian National Health and Medical Research Council, and the NGW Macintosh memorial fund. The authors declare no competing financial interests.

Abstract: The neural circuits that regulate placebo analgesia responsivity are unknown, although engagement of brainstem pain modulatory regions is likely critical. In 47 participants, we identify differences in neural circuit connectivity's in placebo responders versus non-responders. We distinguish stimulus-independent and stimulus-dependent neural networks that display altered connections between the hypothalamus, anterior cingulate cortex, and midbrain periaqueductal gray matter. This dual regulatory system underpins an individual's ability to mount placebo analgesia.

Main text: Placebo analgesia is a powerful phenomenon in which an inert substance or visual cue that provokes positive expectations [1], conditioning effects [2], or environmental associations [3-5] evokes pain inhibition. It is thought that placebo analgesia involves the recruitment of descending projections from prefrontal and cingulate cortices to the brainstem pain modulating centre, the midbrain periaqueductal gray matter (PAG) [4,6,7]. Since placebo analgesic effects can be reduced by opioid antagonists and stimulation of the ventrolateral column of the PAG (vlPAG) produces opioid-mediated analgesia [5,7], it has long been thought that the vlPAG is responsible for placebo analgesia. However, a recent ultra-high field functional magnetic resonance imaging (fMRI) study demonstrated that it is the lateral PAG (lPAG), which produces non-opiate mediated analgesia, and not the vlPAG that is critical for placebo analgesia [8].

Preclinical investigations have revealed that lPAG stimulation evokes emotional coping behaviours, of which analgesia is an integral component [9]. While the lPAG can produce these behaviours without input from higher centres, it was shown over half a century ago that the sensitivity of lPAG is tonically regulated by hypothalamic inputs [10-13]. In humans, the hypothalamus forms part of the lower pain control system and is involved in both pain control and maintaining autonomic homeostasis via its reciprocal connection with the lPAG [14,15]. Additionally, both hypothalamic and midbrain activation has been observed during placebo analgesia, suggesting a phylogenetically conserved system of pain control exists consisting of subcortical and brainstem structures including the hypothalamus and PAG [16].

Importantly, placebo analgesic responses are not expressed in all individuals and what determines placebo analgesia responsivity remains unknown. Given the abovementioned

preclinical and human data, it is possible that on-going modulation of the lPAG by the hypothalamus determines whether or not an individual expresses placebo analgesia. Placebo analgesia is also associated with noxious stimulus-evoked activity changes in higher brain regions including the rostral anterior cingulate cortex (rACC) and dorsolateral prefrontal cortex (dlPFC) [6,7]. These activation patterns are accompanied by heightened μ-opioid binding in the rACC and coupling between the rACC and PAG [17,18], and the expression of placebo analgesia can be blocked by the administration of the opioid antagonist naloxone. When naloxone is administered, the attenuation of these responses is associated with a reduction in rACC-PAG connectivity [7].

Given these observations, we hypothesised a two-network model of brain regulation of placebo analgesia. That is, placebo responsivity will depend on lPAG regulation by two distinct networks: i) a *stimulus-independent network* that includes the hypothalamus, and tonically regulates lPAG sensitivity, and ii) a *stimulus-dependent network* that includes the rACC, and phasically alters lPAG activity to produce placebo-mediated reductions in perceived pain intensity.

By deceptively applying different intensity short-lasting thermal stimuli onto sites on the arm, we conditioned healthy participants to believe a placebo cream (labelled “lidocaine”) was acting to reduce their pain relative to an adjacent control cream (labelled “vaseline”). In a subsequent session, whilst collecting ultra-high-field (7 Tesla), high-resolution (1x1x1.2mm voxel) fMRI, we applied identical intensity stimuli to both creams (“vaseline"/control; “lidocaine”/placebo) and recorded subjective pain responses in 47 participants (25 male; mean±SD age 24.0±3.8) (Fig 1A). We classified individuals as responder (n=23) or non-responder (n=24) using the two-

standard deviation band method [19] (Fig 1B), and conducted group-level analyses using SPM12 and custom software to explore changes in signal intensity, stimulus-independent connectivity (functional connectivity), and stimulus-dependent (psychophysiological interaction) connectivity associated with placebo responses. Although it has long been proposed that top-down recruitment of analgesic brainstem pathways underpins placebo analgesia, information on directionality of seed-to-voxel relationships cannot be gleaned from these connectivity analyses alone [20]. As such we additionally conducted Dynamic Causal Modelling (DCM) and a multiple mediation analysis to determine directed connectivity between cortical and subcortical regions (i.e., if placebo analgesia was associated with top-down or bottom-up projections), as well as determine which regions were working either independently or as a system to drive the relationship between lPAG activity and placebo responses.

Throughout the experiment, participants rated their pain continuously by sliding a cursor connected to a visual analogue scale (VAS), extending from 0 (no pain) to 100 (worst pain imaginable). Despite both groups expecting reduced pain on the placebo lidocaine-treated site (*mean±SEM expectation* responder: vaseline = 49.3±0.8, lidocaine = 33.5±1.6, $p < 0.001$; non-responder: vaseline = 51.7±1.8, lidocaine = 37.1±1.6, $p < 0.001$), only 23 of the 47 participants demonstrated a significant pain reduction when identical intensity stimuli were applied to both sites (*mean±SEM VAS* responder: vaseline = 45.2±1.5, lidocaine = 32.9±1.9, $p < 0.001$; non-responder: vaseline = 42.2±2.8, lidocaine = 45.9±2.4, $p = 0.09$) (Fig 1C). Pain rating responses to the control Vaseline-site did not differ between response and non-responder groups ($F_{2,46} = 2.59$, $p = 0.22$). Additionally, inspection of the low and moderate temperatures applied throughout conditioning and test phases revealed no differences between placebo responder

and non-responder groups (Supplementary table 1). Group-level analyses of placebo responder and non-responder groups revealed a significant and differential engagement of the lPAG, consistent with a previous report (*mean±SEM change in β value* responder: -0.56±0.33; non-responder: 1.15±0.24; $p < 0.001$) (Fig 1D). A 1mm radius sphere at the peak of this cluster was used as a seed region for subsequent connectivity analyses.

Next, by conducting functional connectivity (FC) and psychophysiological interaction (PPI) analyses, we investigated the existence of *stimulus-independent* and *stimulus-dependent* networks, respectively. As hypothesised, we identified a stimulus-independent network in which placebo responders displayed marked decreases in functional connectivity between the bilateral posterior hypothalamus (PH) and the lPAG (Figure 2E, Table 2). Whilst PH-lPAG coupling was strong during the control vaseline-site scan, it was negligible during the placebo lidocaine-site scan. In addition, similar coupling changes occurred between the lPAG and both the medial nucleus of the amygdala (MeA) and medial prefrontal cortex (mPFC). Placebo-related connectivity increases were observed between the lPAG and both the dlPFC and rACC. In striking contrast, in placebo non-responders there were no significant changes in lPAG-coupling, nor changes in blood-oxygen level dependent (BOLD) signal intensity in either group within this stimulus-independent system (Supplementary tables 2,3).

In addition, we identified a stimulus-dependent network in which noxious-evoked connectivity changes were significantly greater during the placebo lidocaine-site versus control vaseline-site scans in placebo responders. Responders displayed significant increases in pain-related connectivity between the lPAG and the primary somatosensory cortex (S1), anterior insula (AI),

nucleus accumbens (NAc), supplementary motor area (SMA), rostral (rACC), dorsal (dACC), and mid (MCC) cingulate cortices during noxious stimulation of the placebo lidocaine-site compared to the control vaseline-site (Figure 2B, Table 1). Apart from S1, non-responders displayed no significant lPAG-connectivity changes within this stimulus-dependant network, nor did either responders or non-responders display significant BOLD activity changes (Supplementary tables 4, 5).

To determine whether regions within the stimulus-independent and stimulus-dependent networks were working collectively, and to determine the direction of information flow, i.e. whether regions within each network were modulating the lPAG or *vice versa*, we performed a DCM analysis. Each anatomically possible connection, as well as inhibitory self-connections between all regions within each of the two networks were entered as a "full model" (Figure 3A, B). The timing of noxious stimuli was included in the stimulus-dependent DCM analysis. Model estimation was performed at 256 maximum iterations, after which a nested search identified the combination of anatomical connections which optimized model free energy (i.e. which time-series data best predicted other VOI time-series data in either a forward, or reverse direction). Individual participant parameter estimates were then extracted from each connection which survived the nested search and were inspected for differences between placebo responders and non-responders.

Within the stimulus-independent network, placebo responses were driven by descending inputs from the left and right PH and the rACC onto the lPAG. These findings are consistent with the idea that reduced drive from the hypothalamus to the lPAG is required for a placebo analgesia

to occur (Figure 3A, Table 3). In addition, a reduced lPAG inhibitory self-connection suggests that in responders, the lPAG is under less inhibitory regulation and thus more capable of being modulated by extrinsic connections [21,22]. Within the stimulus-dependant network, differences also occurred in the descending rACC-lPAG connection as well as the NAc-rACC connection (Figure 3B, Table 3). These data reveal that the rACC regulates the lPAG in both a stimulus-dependant and stimulus-independent manner. Furthermore, these analyses show that within the stimulus-independent and stimulus-independent networks, the NAc, rACC and PH are the main sites that determine whether an individual will express a placebo analgesic response.

To explore the effects of these stimulus-independent and stimulus-independent network sites on placebo-evoked lPAG signal intensity changes, a dual-path mediation analysis was performed. The rACC-lPAG PPI values and right PH-lPAG FC values were entered as potential mediators of placebo responses and lPAG signal changes. We found that rACC-lPAG stimulus-dependent connectivity completely mediated this relationship, whereas the PH-lPAG connectivity directly related to placebo responsivity (group assignment) but did not drive the changes in lPAG signal intensity (Figure 3C). These data support our hypothesis that (i) the stimulus-independent network, particularly the PH, sets the sensitivity of the lPAG, whereas (ii) the stimulus-dependent network, particularly the rACC-lPAG, ultimately drives the output in descending analgesic pathways.

Our results show that placebo analgesia responsivity is regulated by two brain networks, one which sets the sensitivity of the lPAG, and another which drives descending inputs onto the lPAG during noxious stimuli. We propose a stimulus-independent network comprised of the

rACC and PH that sets the gain of the lPAG and ultimately whether an individual expresses placebo analgesia. This pathway has previously been described in experimental animals, with prelimbic, hypothalamic and amygdala projections to the lPAG critical for coordinating autonomic and homeostatic processes [23]. An integral part of the active emotional coping behaviours mediated by the lPAG is an analgesia thought to aid an individual's ability to cope immediately with the source of pain [24]. While analgesia forms a critical part of this primitive behavioural response, it appears that higher brain regions recruit the lPAG pain modulatory circuitry in more abstract situations such as during placebo analgesia. Our results demonstrate that the descending modulatory pathway is at least partially preserved in humans, and that reduced PH-lPAG connectivity likely represents a weakening of PH regulatory grip over the lPAG and disrupted its excitatory-inhibitory balance. This then enables top-down noxious-stimulus evoked modulation of the PAG by regions within the stimulus-dependent network. Importantly, all individuals expect a pain intensity reduction during the placebo scan, however in only those that subsequently mount an analgesic response, do changes in rACC-lPAG and PH-lPAG connectivity occur. This suggests that some individuals are set to respond, and others are not, despite having similar expectations. Whether the ability of the PH and rACC to modulate the lPAG is "hard-wired" in an individual, or is shaped by prior experience, influenced by genetic factors, or varies from day to day or between various conditioning effects or environmental associations remains to be determined.

In addition, we reveal a noxious stimulus-dependent network that underpins both lPAG signal intensity changes and placebo responses. Whilst this network consisted of multiple higher order processing regions such as the dACC, MCC, and insula, the rACC appears to be critical in

mediating both placebo responsivity and lPAG signal intensity changes. Indeed, prior investigations have demonstrated that heightened rACC-PAG coupling underlies placebo responses in acute settings [5,25]. Additionally, reductions in analgesic phenomena in response to naloxone (opioid antagonist) administration have been consistently tied with reductions in rACC-PAG coupling [7,26]. The NAc was also identified as part of our stimulus-dependent network. Forming part of the ventral striatum and acting as a cortical dopaminergic hub, the NAc contacts the prefrontal cortex to drive reward-anticipation, decision-making, and error-predictions [27]. Correcting perception-anticipation differentials is a critical component in mounting placebo analgesic responses, and one which has been associated with activation and neurotransmission within the NAc and its cortical efferents [27,28]. Our data shows that during noxious stimulation, phasic coupling between the NAc and rACC are critical for lPAG ability to drive analgesic responses and match anticipated pain.

Although we utilized PPI, FC, and directed connectivity analyses to unveil the most integral cortical networks that regulate and drive PAG output during placebo analgesia – these networks were identified by considering the phenomena as dichotomous. That is, we delineated and investigated a responder and non-responder group. Performing these analyses allowed us first to identify which cortical connections with the PAG were significantly altered in those demonstrating a placebo response as determined through the 2SD band method, and then assess these connections in non-responders for statistical differences. There exists conflicting literature over the method of determining placebo responses (for example by using arbitrary VAS changes or permutation testing), as well as if placebo analgesia should be considered a continuous variable [29-31]. Whilst these approaches are well- documented, the former is limited

by not considering an individual's perceived pain intensity baseline variability, and the latter would only identify clusters where PAG connectivity shows a linear relationship with graded changes in perceived pain – including in those where pain either did not change between the control vaseline- and placebo lidocaine-sites or indeed in those who's pain increased when exposed to placebo. As such, in our analysis we can be confident that we have described a functional architecture underpinning significant placebo analgesia, and that these same connections are unchanged in non-responders.

Additionally, as we conducted our DCM and mediation analyses using clusters that were first revealed using the lPAG timeseries as a seed, our results are constrained to solely regions which likely receive information from or project directly to the lPAG. Whilst this does not allow assessment of regions which comprise alternate projection pathways which may be involved in the response, encoding more nuanced aspects of placebo analgesia such as cognitive evaluation or complex emotional processing, the results presented do offer valuable insight into the functional projections regulating and driving brainstem output in humans to produce an antinociceptive state.

It is of note that despite receiving an identical response conditioning protocol, over half our sample did not demonstrate a significant placebo response. Whilst the focus of this investigation was to identify the functional networks of placebo responders, it would also be of interest to better understand the driving factors influencing why certain individuals fail to generate significant analgesic responses to placebo. Two leading theories – the Bayesian brain hypothesis [32] and an individual's underlying biological substrates [18] both centre on error-

prediction signalling and dopaminergic neurotransmission, which encompass roles of the NAc. Since we identified this region as feeding into the rACC-PAG pathway in a stimulus-dependent manner, it may be that this cortical site is a key delineating factor between an individual forming accurate stimulus-response relationships and generating placebo responses via response conditioning. Future studies could compliment this work by assessing the role of the NAc in the conditioning phases of placebo analgesia, and indeed whether NAc activation or neurotransmission during these phases could be a potential biomarker of placebo responsiveness.

In conclusion, we provide evidence for two brain networks responsible for altering descending brainstem pathways during placebo analgesia. Whilst this investigation utilized a specific protocol consisting of short lasting thermal stimuli and response conditioning to induce analgesic effects, regions in the network we describe have previously been tied with analgesia elicited from longer lasting stimuli or chronic conditions [7,33]. Recently, brainstem projections from the hypothalamus have been linked to pain anticipation in Fibromyalgia patients [34], and the cingulate cortex – specifically it's anterior division - has been proposed as a neurosurgical target to treat intractable pain due to its role in emotional and attentional processing during painful events [35]. Additionally, the same regions we identify from placebo analgesia generated by response conditioning appear to be involved in alternative placebo substances such as social observation which includes the amygdala and PAG [36], and pharmacological conditioning which include the rACC and PAG [37]. These data are consistent with frontotemporal and limbic structures playing a generalized role in recruiting brainstem pain-modulatory circuits to drive analgesia, emphasising the role of "mind set" and emotion in influencing our responses to pain.

Indeed, it remains to be seen whether these specific connections between cortical, subcortical, and brainstem sites are compromised in individuals with chronic pain or underpin alternative endogenous pain modulatory phenomena.

Figures

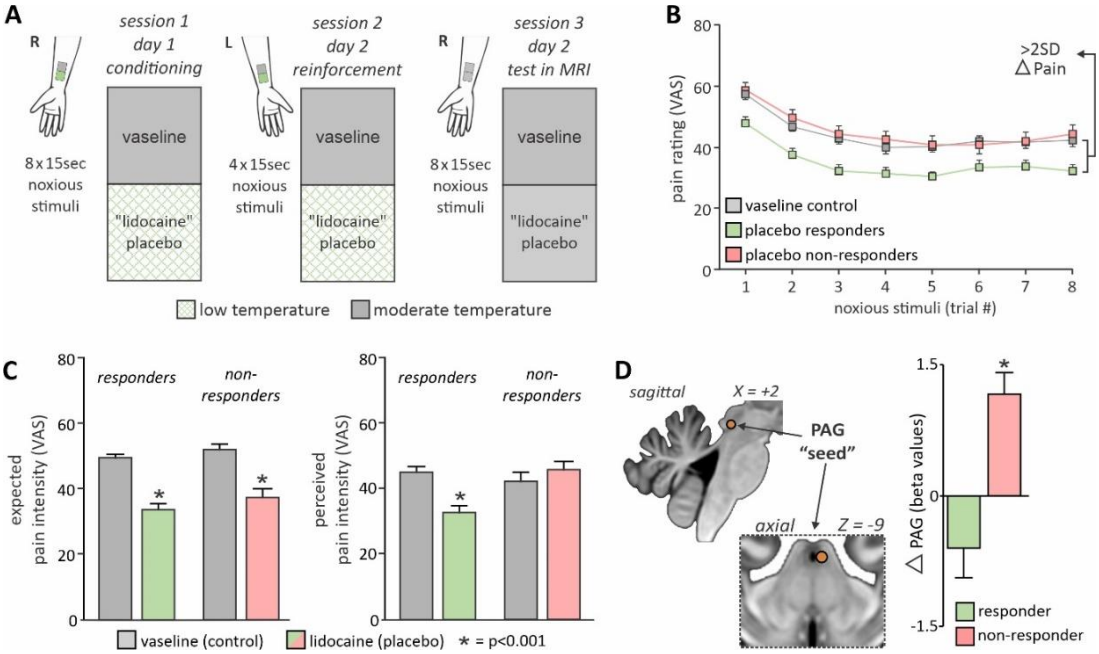

Fig 1: Experimental protocol, placebo-related activity, and connectivity functional maps. A) Placebo induction. Conditioning was performed by applying low intensity noxious stimuli to the lidocaine-site and moderate intensity to the Vaseline-site; crucially, during this phase participants believed stimuli of moderate intensity were being applied to both sites. On the following day, a reinforcement phase was conducted using the low and moderate temperatures on the opposite forearm. Then, after a washout period, two independent functional magnetic resonance imaging (fMRI) series were collected where we applied identical moderate intensity noxious stimuli to the control vaseline (scan 1), and placebo 'lidocaine' cream (scan 2) sites sequentially. During these two series, participants rated their expected and perceived pain on an MR-compatible visual analogue scale (*0 = no pain, 100 = worst pain imaginable*). **B) Perceived pain intensities during noxious stimuli.** Mean (±SEM) pain intensity ratings during the placebo lidocaine-site scan in placebo responder (n=23; green) and non-responder (n=24; pink) groups, relative to the average pain ratings from all 47 participants during the control vaseline-site scan (grey). **C) Expected and perceived pain intensities.** The difference in expected and reported pain directly prior and during the two series in placebo responder and non-responder groups. * p<0.001. **D) Midbrain periaqueductal gray matter (PAG) signal intensity changes.** Brainstem maps representing differences in noxious-stimulus evoked signal intensity changes during the placebo lidocaine- and control vaseline-site scans were entered into a 2-sample group analysis which compared placebo responder and non-responder groups. A significant cluster with a peak within the lateral PAG emerged. Beta values were extracted from a 1mm diameter sphere at the peak of this cluster and plotted. This sphere was used as the "seed" for subsequent analyses.

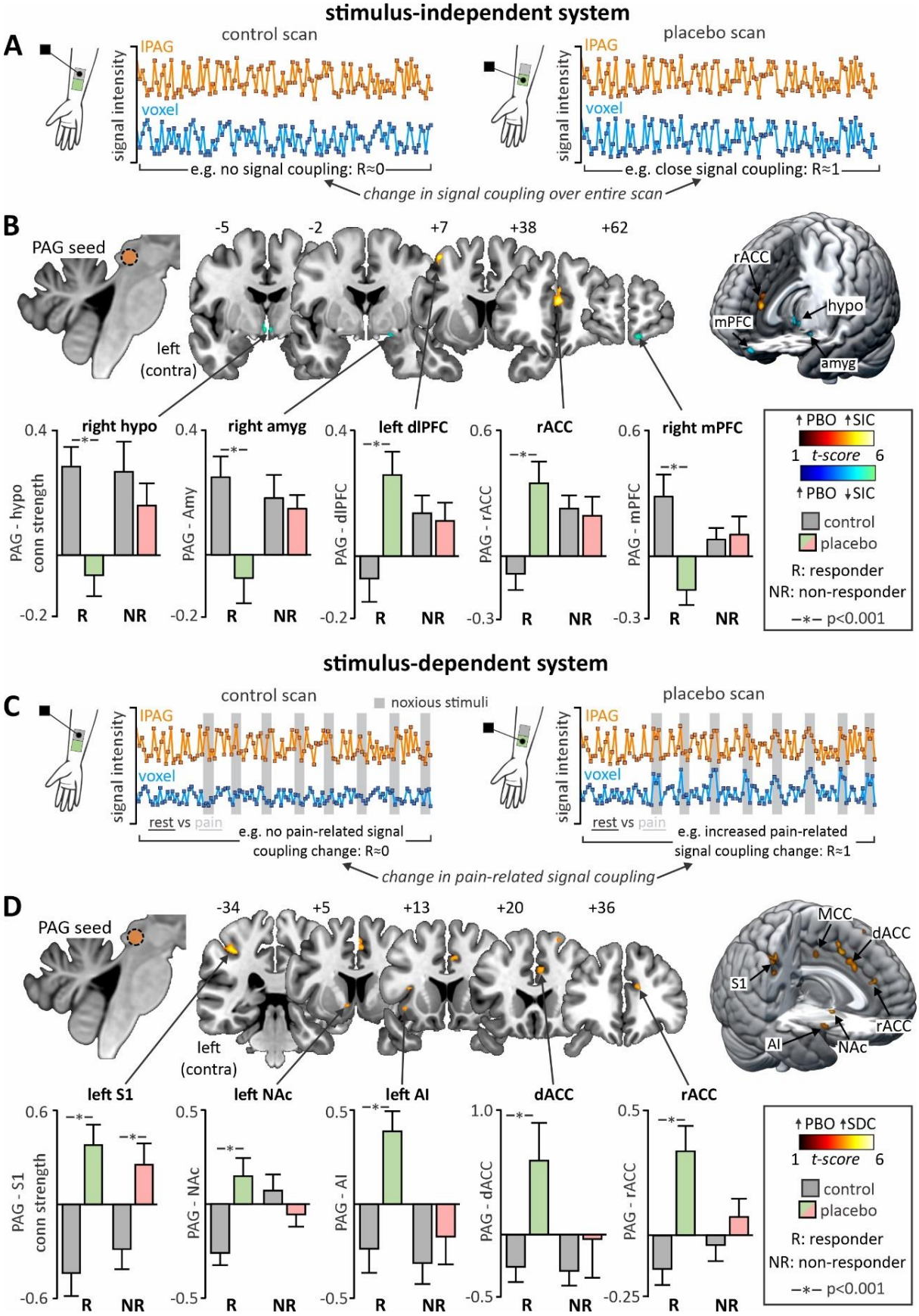
stimulus-independent system
A
control scan
placebo scan
IPAG
voxel
signal intensity
e.g. no signal coupling: R≈0
e.g. close signal coupling: R≈1
change in signal coupling over entire scan
B
PAG seed
left (contra)
-5
-2
+7
+38
+62
rACC
hypo
mPFC
amyg
right hypo
right amyg
left dlPFC
rACC
right mPFC
PAG - hypo conn strength
PAG - Amy
PAG - dlPFC
PAG - rACC
PAG - mPFC
R
NR
↑PBO ↑SIC
1 t-score 6
↑PBO ↓SIC
control
placebo
R: responder
NR: non-responder
–*– p<0.001
stimulus-dependent system
C
control scan
placebo scan
noxious stimuli
rest vs pain
e.g. no pain-related signal coupling change: R≈0
e.g. increased pain-related signal coupling change: R≈1
change in pain-related signal coupling
D
PAG seed
left (contra)
-34
+5
+13
+20
+36
MCC
dACC
S1
rACC
AI
NAc
left S1
left NAc
left AI
dACC
rACC
PAG - S1 conn strength
PAG - NAc
PAG - AI
PAG - dACC
PAG - rACC
↑PBO ↑SDC
1 t-score 6
control
placebo
R: responder
NR: non-responder
–*– p<0.001

Fig 2: Stimulus-independent and stimulus-dependent cortico-brainstem connectivity changes during placebo analgesia. A) Functional Connectivity (FC) Analysis. Functional connectivity determines areas which alter in coupling with a seed region across the entire scan. Positive values indicate a correlation between a seed and voxel timeseries, whereas a negative value indicates anticorrelation. Control vaseline- and placebo lidocaine-site functional scans were analyzed, allowing us to determine which brain areas altered their ongoing, stimulus-independent coupling with the lateral midbrain periaqueductal gray matter (lPAG) during placebo analgesia. B) Voxel by voxel FC analysis in placebo responders. Paired analysis (control vaseline versus placebo lidocaine-site scans) in placebo responders (n=23) revealed a pattern of stimulus independent connectivity changes. Relative to the control vaseline-site, connectivity decreased during the stimulation of the placebo lidocaine-site between the lPAG and the left and right posterior hypothalamus (hypo), right medial nucleus of the amygdala (amyg), and right medial prefrontal cortex (mPFC), and increased with both the left dorsolateral prefrontal cortex (dlPFC) and rostral anterior cingulate cortex (rACC). Non-responders (n=24) displayed no significant connectivity changes between the two functional series in these same brain regions. C) Psychophysiological Interaction (PPI) Analysis). PPI connectivity analysis considers the timeseries of an elected region (seed) activity during time-specific events. During the control vaseline- and placebo lidocaine-site scans, eight noxious stimuli were delivered at 8 time points, allowing us to determine which brain regions altered their connectivity's in a stimulus-dependent manner. D) Voxel by voxel PPI analysis in placebo responders. Paired analysis (control vaseline-versus placebo lidocaine-site scans) in placebo responders revealed significant increases in pain-related connectivity between the lPAG and the contralateral primary somatosensory cortex (S1), anterior insula cortex (AI), nucleus accumbens (NAc), rACC, dorsal ACC (dACC), and mid cingulate cortices (MCC). Non-responders displayed no significant pain-related connectivity changes in all of these brain regions, apart from S1 in which both responders and non-responders displayed connectivity increases during placebo lidocaine- compared with control vaseline-site scans.

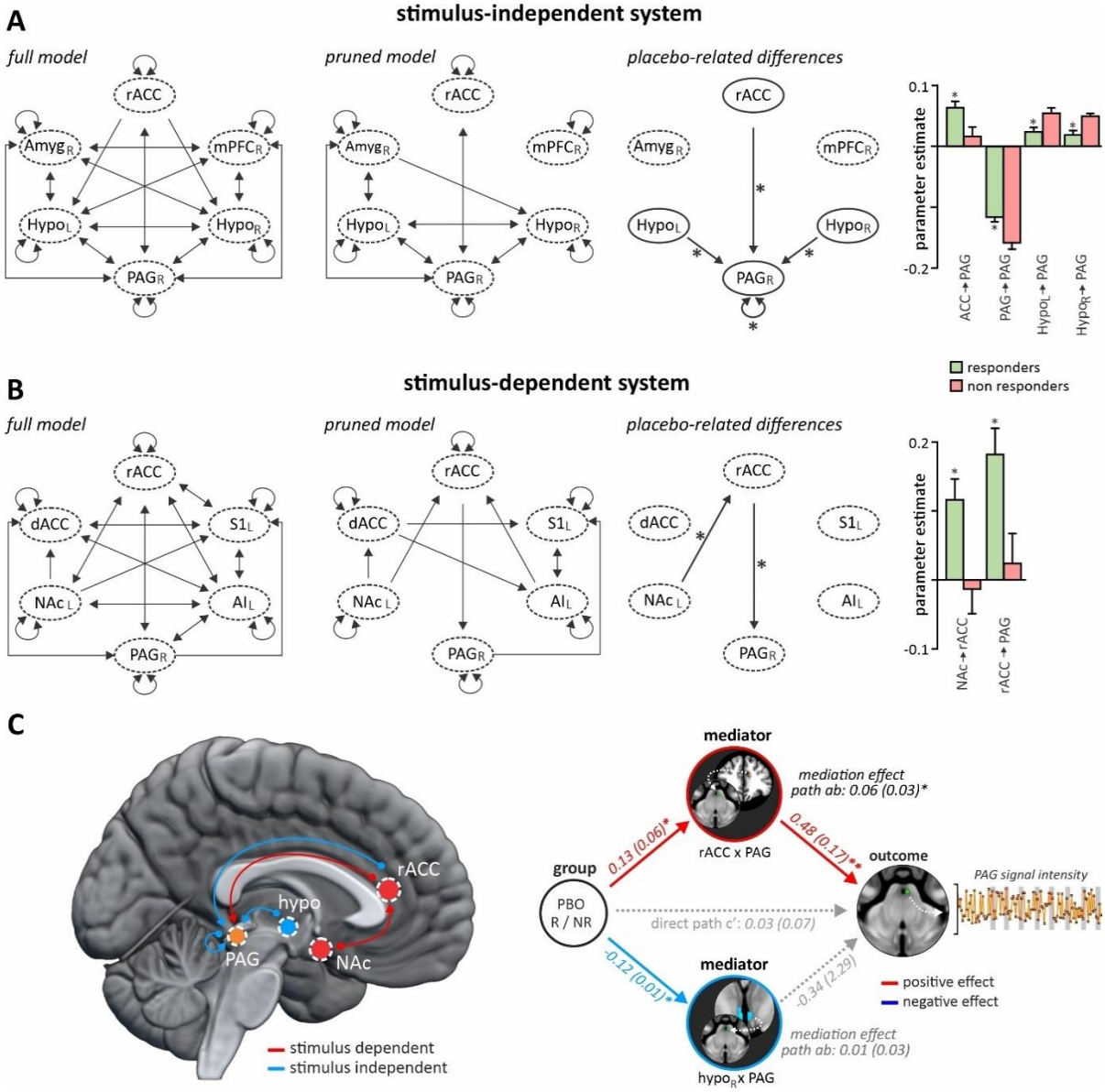

Fig 3: Defining a stimulus-dependent and -independent network of brainstem connectivity. Dynamic Causal Modelling (DCM) was conducted by entering the timeseries of each significant cluster revealed by stimulus dependent (PPI) and -independent (FC) analyses during the stimulation of the placebo lidocaine-site into two, separate, full model designs. Each anatomically possible connection was turned on to create a full model, and the timing of stimuli was added to the PPI DCM to account for the stimulus dependency of these connections. For both models, DCM was conducted as a bilinear model, one state per region, centred inputs, with stochastic effects off. After specifying and estimating these models, a nested search was conducted revealing connections between clusters whose timeseries significantly added to optimal model evidence. The pruned models displayed in the upper and lower central panels were threshold at p>0.99. Each first-level model was then inspected for each participant, and individual connection parameter estimates were extracted. Two-sample t-tests were conducted

comparing mean parameter estimates between responders and non-responders to identify cluster connections that significantly differed between placebo responders and non-responders. **A) Stimulus-independent system:** Significant differences were identified from the rostral anterior cingulate cortex (rACC), left and right posterior hypothalamus (hypo) to the midbrain periaqueductal gray matter (PAG), as well as in the PAG-PAG self-connection. **B) Stimulus-dependent system:** Significant differences were identified from the nucleus accumbens (NAc) to the rACC, and from the rACC to the PAG. **C) View of significant connections in both the stimulus-dependent (red circles and lines), and stimulus-independent (blue circles and lines) networks** displaying differences in PAG connectivity between placebo responders and non-responders. Mediation testing revealed that the stimulus-dependent connectivity between the rACC-PAG significantly and completely mediated the relationship between placebo responsivity (placebo responder or non-responder group assignment), and PAG signal intensity change. Stimulus independent connectivity between the right hypothalamus-PAG related directly to group assignment, however, did not act as a mediator in the signal change of the PAG.

Methods:

Ethics: All experimental procedures were approved by the University of Sydney Human Research Ethics Committee and were consistent with the Declaration of Helsinki. Written informed consent was obtained from participants at the commencement of the study. Participants were also provided with an emergency buzzer while inside the scanner so that they could stop the experiment at any time. At the conclusion of testing, participants were informed both verbally and through a written statement of the necessary deception and true methodology of the experiment and were invited to seek clarification of what they had just experienced.

Participants: Forty-seven healthy control participants were recruited for the study (25 male, 22 female; mean age, 24.0±0.5 years [± SEM]; range 19–37 years). In order to evaluate the necessary number of participants required for this study, an *apriori* power analysis was performed using results from a previous imaging study investigating cortico-brainstem connectivity during placebo analgesia [7]. This revealed a total sample size of 40 would be necessary to detect similar effect sizes with 95% power (d = 0.31, α = 0.05, power = 0.95). Before beginning the study, participants completed a data sheet recording current medication(s), and any alcohol or caffeine ingested in the 24 hours prior to testing.

Experimental Design: The study included three sessions occurring on two successive days: a conditioning session on day 1, and a reinforcement and MRI scanning session on day 2 (Figure 1A). Throughout the study, noxious stimuli were administered to the volar surfaces of participants' left and right forearms using a 3x3cm MR-compatible Peltier element thermode, which delivered a heat stimulus at a pre-programmed temperature via a Thermal Sensory Analyzer (TSA-II) (Medoc LTD Advanced Medical Systems, Rimat Yishai, Israel). Each stimulus lasted 15 seconds, including a ramp-up period (four degrees per second), a plateau period at a

noxious temperature and a ramp-down period (four degrees per second). Each stimulus was separated by a 15 second inter-stimulus-interval (ISI) at a non-painful baseline temperature of 32°C. Throughout conditioning, participants rated their pain on-line using a horizontal 10 cm visual analogue scale (VAS) ranging between 0 and 100, where 0 was described as "no pain" and 100 as "the worst pain imaginable". During scanning, participants used an MR-compatible button box to continuously report their pain perception. The VAS scale was shown on a reflected digital screen at the end of the magnet bore, and participants controlled the position of a slider to report their pain continuously by holding the left (moved slider towards zero) or right (moved slider towards ten) buttons with their left middle and index finger.

Conditioning: Session 1 was conducted outside the MRI and consisted of two rounds of a conditioning protocol. Participants were first informed both verbally and via a written statement that the study was designed to investigate the modulatory effects of a topical anaesthetic containing lidocaine, which had been shown to provide pain relief in some individuals. A second control cream was stated to be purely Vaseline and was stated as being necessary to evaluate typical pain responses. In reality, both creams contained vaseline and only differed in colour and their described properties. We calculated individual low and moderate pain responses by applying a series of randomised stimuli to the left forearm ranging from 44-48.5 degrees, asking participants to rate their perceived pain during each stimulus. Participants were informed that we were only recording a temperature which elicited a moderate subjective pain response (40–50 VAS rating), and that this temperature would be used throughout the remainder of the experiment. However, using the ratings provided during this process, we recorded two different temperatures: one which was rated between 20-30 on the VAS (low temperature); and one which was rated between 40-50 (moderate temperature). These two temperatures were then

deceptively applied to the "lidocaine" and Vaseline cream sites throughout the conditioning and reinforcement experimental phases.

Creams were then applied to two adjacent 3x3 cm squares on the volar surface of the participants' right forearm. To enhance the believability that the "lidocaine" cream contained an active analgesic, a false label was attached to the cream bottle and green food colouring was added. The positions of the "lidocaine" and vaseline creams were counterbalanced between proximal and distal sites on the volar right forearm between participants to reduce potential confounders of local sensitivity, however we ensured both creams always occupied the C6 dermatome. Ten minutes following cream application, we conducted two rounds of conditioning. Participants believed they would receive eight identical moderate thermal stimuli and were instructed to report their perceived pain intensity using the VAS. Participants were also asked prior to each set of stimuli for an average expectation of the pain they would experience, which acted both to measure belief that lidocaine was working to modulate their subjective pain, and to reinforce the pain relieving quality of the cream. During the two conditioning rounds we deceptively applied a moderate temperature to the control vaseline-site, and a low temperature to the placebo lidocaine-site.

Reinforcement and Test: At approximately the same time on the following day, sessions 2 and 3 were conducted with participants inside the MRI scanner and consisted of a reinforcement protocol (session 2) and a test protocol (session 3). The creams were applied to the volar surface of both left and right forearms, in the same order and locations as session 1, and participants were reminded of the "lidocaine's" pain-relieving qualities. To ensure that the protocol for conditioning was consistent between subsequent days, and the change in immediate environment (inside the MRI), reinforcement was conducted by applying four noxious

stimuli at the same low and moderate temperatures used throughout session 1 to participants' left volar forearm. This was performed on the opposite forearm to prevent sensitization of the testing area (the right volar forearm).

Following reinforcement, we waited 15 minutes for residual pain and sensitivity to dissipate from the left arm before beginning the test protocol. During this 15-minute period, structural brain scans were collected. Dissimilar to conditioning and reinforcement, during the test phase we applied *identical moderate temperature stimuli* to both the control vaseline- and placebo lidocaine-sites (Figure 1). We asked each participant for an average expectation of pain intensity directly prior to each stimulation series and instructed them to report the pain intensity continuously throughout the duration of the scan using the button box and the projected digital VAS. VAS responses were recorded every 0.5 seconds, and values during each pain period were averaged providing a pain intensity for each noxious stimulus period. Each participant received two consecutive series of eight stimuli, with a separate functional series collected during each series of stimuli. Each fMRI series began with a 90-second baseline period prior to the eight stimuli presentations. The control vaseline-site was always stimulated during the first series, and the placebo lidocaine-site was stimulated during the second series, so that we generated a "pre" and "post" condition, or, functional brain images encoding typical and placebo pain responses, respectively.

MRI data acquisition and preprocessing: Brain images were acquired using a whole body Siemens MAGNETOM 7 Tesla (7T) MRI system (Siemens Healthcare, Erlangen, Germany) with a combined single-channel transmit and 32-channel receive head coil (Nova Medical, Wilmington MA, USA). Participants were positioned supine with their head in the coil and sponges supporting the head laterally to minimise movement. A T1-weighted anatomical image set

covering the whole brain was collected (repetition time=5000 ms, echo time=3.1ms, raw voxel size=0.73x0.73x0.73mm, 224 sagittal slices, scan time=7mins). The two fMRI acquisitions each consisted of a series of 134 gradient echo echo-planar measurements using blood oxygen level dependant (BOLD) contrast covering the entire brain. Images were acquired in an interleaved collection pattern with a multi-band factor of four and an acceleration factor of three (repetition time=2500ms, echo time=26ms; raw voxel size=1.0x1.0x1.2mm, 124 axial slices, scan time=5:35mins).

Image preprocessing and statistical analyses were performed using SPM12 [38] and custom software. The first five volumes of each scan were removed from the model due to excessive signal saturation from the scanner. The remaining 129 functional images were slice-time and motion corrected and the resulting 6 directional movement parameters were inspected to ensure that all fMRI scans had no greater than 1mm of linear movement or 0.5 degrees of rotation movement in any direction. In no single participant in either the placebo lidocaine- or control vaseline-site scans did motion parameters exceed our elected threshold. Images were then linearly detrended to remove global signal changes, physiological noise relating to cardiac (frequency band of 60-120 beats per minute +1 harmonic) and respiratory (frequency band of 8-25 breaths per minute +1 harmonic) frequency was removed using the DRIFTER toolbox [39], and the 6-parameter movement related signal changes were modelled and removed using a linear modelling of realignment parameters (LMRP) procedure. Each individual's fMRI image sets were then coregistered to their own T1-weighted anatomical, the T1 was then spatially normalized to the DARTEL template in Montreal Neurological Institute (MNI) space and the parameters applied to the fMRI image sets. The normalized fMRI images were then spatially smoothed using a 6mm full-width at half maximum Gaussian filter.

Dichotomizing placebo responder and non-responder groups: Participants were grouped as either a responder or non-responder to placebo analgesia based on the two-standard deviation (SD) method described previously [19]. Briefly, for the 8 noxious stimuli delivered during the test phase to the control vaseline-site, the SD of the 8 pain intensity ratings was calculated. During the stimulation of the placebo lidocaine-site, the average pain intensity rating was calculated, and if this average rating was 2 SD lower than the control vaseline-site, the participant was considered a responder. If not, they were considered a non-responder. Significant differences between groups with respect to expected changes in pain intensities immediately prior to testing were determined using paired t-tests (two-tailed, $p<0.05$). Since participants were grouped into either responder or non-responder categories based on their perceived pain intensities during the fMRI scans (session 3), we did not assess significant differences between groups for the perceived pain intensity changes. A single factor ANOVA ($p<0.05$) was used to determine if there were differences in the temperature applied or pain intensity ratings reported between responder and non-responder groups during the control stimulated series to ensure any reported placebo effects did not relate to baseline thermal sensitivity.

PAG Region-of-interest generation: Previously, we identified a region of the caudal lateral PAG (lPAG) ipsilateral to the side of stimulation as primarily responsible for placebo analgesia [8]. We began by running a two-sample difference map between control vaseline and placebo lidocaine-site scans between placebo responder and non-responder groups and confirmed that the greatest change in placebo-related activity occurred at the same lPAG location as that reported earlier. We generated a 1mm radius spherical volume of interest mask (VOI) at this lPAG site and used this VOI throughout subsequent connectivity analyses to assess changes in stimulus-

dependent and -independent cortical coupling with the PAG during significant placebo responses (Fig 1D).

fMRI statistical analysis: To determine significant changes in signal intensity during each noxious thermal period, a repeating boxcar model convolved with a canonical hemodynamic response function was applied to each of the fMRI series. Within this model, scanning volumes overlying stimulus plateau periods were assigned a value of 1, and inter-stimulus-intervals and the initial 90-second baseline period were assigned a value of 0. The contrast images generated for each functional image series were then used in group analyses. We conducted four separate analyses to determine the cortical constituents of placebo analgesia and brainstem engagement.

Analysis 1: cortico-PAG stimulus independent connectivity changes in responders and non-responders were assessed by conducting a functional connectivity (FC) analysis. This analysis generates contrast images with includes the timeseries of the PAG seed as a regressor, independent to the timing of noxious stimuli applied (Fig 2A). As such, this analysis reveals cortical regions contacting the PAG during the entire scan period including the baseline anticipation, pain, ramp, and inter-stimulus-interval periods. Using these contrast images, a random-effects paired, voxel-by-voxel analysis was conducted in placebo responders comparing the control vaseline- and placebo lidocaine-site series. From resulting clusters, eigenvariates representing stimulus-independent connectivity with the PAG in each series were extracted from both placebo responder and non-responder contrast images and significance was determined in both groups using paired t-tests (Figure 2B,C).

Analysis 2: cortico-PAG stimulus dependent connectivity changes in responders and non-responders were assessed by conducting a psychophysiological interaction (PPI) analysis. This analysis involves extracting the timeseries of the PAG from each subject's control vaseline- and

placebo lidocaine-site scans and convolving it with the repeating boxcar model which isolates scan periods in which a noxious stimulus was applied. This generates a new stimulus*PAG timeseries regressor which is then applied to functional series to create new contrast images of stimulus-dependent PAG connectivity (Fig 2C). Using these contrast images, a random-effects paired, voxel-by-voxel analysis was conducted in placebo responders comparing the control vaseline- and placebo lidocaine-site scans. From resulting clusters, eigenvariates representing stimulus-dependent connectivity with the PAG in each series were extracted from both placebo responder and non-responder contrast images and significance was determined in both groups using paired t-tests (Figure 2E, F).

Analysis 3: network properties and directed connectivity in PPI and FC clusters were compared by conducting two separate Dynamic Causal Modelling (DCM) analyses. DCM is a technique whereby cluster timeseries are compared to consider is a region's activity over time can be used to predict the activity in a second, connected region. After entering an appropriate "full model" which includes all anatomically possible connections as well as inhibitory self-connections between each entered cluster, a nested search step can be performed which sequentially tests combinations of connections to produce the most likely "reduced model", or, the combination of connections which maximises free energy (Figure 3A) [22]. We conducted our two DCM's using the following parameters: slice timing = 1.25s (modelled to the centre slice of acquisition), echo time = 0.026s, bilinear modulatory effects, one state per region, stochastic effects off, centred inputs on, and a timeseries fit. The timings of noxious stimuli were modelled specifically in the PPI DCM and added as potential contributors to all extrinsic and intrinsic connections of the full model due to the inherent stimulus-dependency of these clusters. After identifying the optimal reduced model through nested search, individual participant parameter estimates for

each resulting between-cluster and self-connection were extracted and effect sizes and 95% confidence intervals calculated by Cohen's d to identify directed connections with medium to large effects between placebo responders and non-responders (Cohens d>0.5).

Analysis 4: potential cortical mediators of placebo responsiveness and PAG activation were investigated by entering the most pronounced connection elucidated from the two DCM analyses into a multiple mediation analysis performed using the Canlab Mediation Toolbox in Matlab R2022b [40]. Mediation analyses are routinely used to investigate if the relationship between two variables is direct, or reliant on a third, contingent variable. In our investigation, we entered "placebo responsiveness" as the input variable (X), and PAG signal intensity change as the output variable (Y). Connectivity between the rACC-PAG in the PPI analysis, and right hypothalamus-PAG in the FC analysis were entered as potential mediators (M1 and M2, respectively).

Analyses 1 and 2 were initially visualized at a threshold of $p<0.001$ uncorrected with a cluster extent threshold of 20 contiguous voxels. We then applied small volume correction ($p<0.05$) to reduce the likelihood of type II errors. The VOI used to perform these small volume corrections were derived from parcels in the extended human connectome project atlas (HCPex) which includes subcortical areas such as the amygdala and hypothalamus [41]. The locations of significant clusters in MNI space were tabulated and beta-values extracted to determine the directions of signal and PAG-connectivity change. For display purposes, significant clusters were overlaid onto a mean T1 weighted anatomical of all 47 participants. For Analysis 3, posterior probabilities of the reduced model after nested search were thresholded at $p>0.99$, and effect sizes of parameter estimate differences between responders and non-responders were

discerned using Cohen's d tests. Analysis 4 was performed at a false discovery rate correction of $p<0.05$, bootstrapped to 10,000 samples.

Data Availability:

All de-identified single participant functional data, as well as activation and connectivity contrast maps are available from the corresponding author upon reasonable request.

Code Availability:

The analysis methods and software used in this article are all either open source or enabled in SPM12's standard installation. No new methods or algorithms have been generated.

References

1 Kirsch, I. *et al.* Expectancy and Conditioning in Placebo Analgesia: Separate or Connected Processes? *Psychol Conscious (Wash D C)* **1**, 51-59, doi:10.1037/cns0000007 (2014).

2 Medoff, Z. M. & Colloca, L. Placebo analgesia: understanding the mechanisms. *Pain management* **5**, 89-96, doi:10.2217/pmt.15.3 (2015).

3 Tracey, I. & Mantyh, P. W. The cerebral signature for pain perception and its modulation. *Neuron* **55**, 377-391, doi:10.1016/j.neuron.2007.07.012 (2007).

4 Petrovic, P., Kalso, E., Petersson, K. M. & Ingvar, M. Placebo and Opioid Analgesia--Imaging a Shared Neuronal Network. *Science* **295**, 1737, doi:10.1126/science.1067176 (2002).

5 Schafer, S. M., Geuter, S. & Wager, T. D. Mechanisms of placebo analgesia: A dual-process model informed by insights from cross-species comparisons. *Prog Neurobiol* **160**, 101-122, doi:10.1016/j.pneurobio.2017.10.008 (2018).

6 Wager, T. D. *et al.* Placebo-induced changes in FMRI in the anticipation and experience of pain. *Science* **303**, 1162-1167, doi:10.1126/science.1093065 (2004).

7 Eippert, F. *et al.* Activation of the Opioidergic Descending Pain Control System Underlies Placebo Analgesia. *Neuron* **63**, 533-543, doi:10.1016/j.neuron.2009.07.014 (2009).

8 Crawford, L. S. *et al.* Brainstem Mechanisms of Pain Modulation: A within-Subjects 7T fMRI Study of Placebo Analgesic and Nocebo Hyperalgesic Responses. *The Journal of Neuroscience* **41**, 9794-9806, doi:10.1523/jneurosci.0806-21.2021 (2021).

9 Mokhtar, M. & Singh, P. Neuroanatomy, Periaqueductal Gray. *StatPearls [Internet]* (2020).

10 Ellison, G. D. & Flynn, J. P. Organized aggressive behavior in cats after surgical isolation of the hypothalamus. *Arch Ital Biol* **106**, 1-20 (1968).

11 Eclancher, F. & Schmitt, P. [Effect of early lesions of the amygdala and median hypothalamus on the development of interspecific aggression behavior in rats]. *J Physiol (Paris)* **65**, Suppl:231A (1972).

12 Huang, Y. H. & Flynn, J. P. Unit activities in the hypothalamus and midbrain during stimulation of hypothalamic attack sites. *Brain Res* **93**, 419-440, doi:10.1016/0006-8993(75)90181-x (1975).

13 Wang, W. *et al.* Coordination of escape and spatial navigation circuits orchestrates versatile flight from threats. *Neuron* **109**, 1848-1860 e1848, doi:10.1016/j.neuron.2021.03.033 (2021).

14 Stroman, P. W., Ioachim, G., Powers, J. M., Staud, R. & Pukall, C. Pain processing in the human brainstem and spinal cord before, during, and after the application of noxious heat stimuli. *Pain* **159**, 2012-2020, doi:10.1097/j.pain.0000000000001302 (2018).

15 Craig, A. D. Interoception: the sense of the physiological condition of the body. *Curr Opin Neurobiol* **13**, 500-505, doi:10.1016/s0959-4388(03)00090-4 (2003).

16 Eippert, F., Finsterbusch, J., Bingel, U. & Büchel, C. Direct evidence for spinal cord involvement in placebo analgesia. *Science* **326**, 404, doi:10.1126/science.1180142 (2009).

17 Wager, T. D., Scott, D. J. & Zubieta, J. K. Placebo effects on human mu-opioid activity during pain. *Proc Natl Acad Sci U S A* **104**, 11056-11061, doi:10.1073/pnas.0702413104 (2007).

18 Scott, D. J. *et al.* Placebo and nocebo effects are defined by opposite opioid and dopaminergic responses. *Arch Gen Psychiatry* **65**, 220-231, doi:10.1001/archgenpsychiatry.2007.34 (2008).

19 Nourbakhsh, M. R. & Ottenbacher, K. J. The Statistical Analysis of Single-Subject Data: A Comparative Examination. *Physical Therapy* **74**, 768-776, doi:10.1093/ptj/74.8.768 (1994).

20 Necka, E. A. *et al.* Applications of dynamic functional connectivity to pain and its modulation. *PAIN Reports* **4** (2019).

21 Snyder, A. D., Ma, L., Steinberg, J. L., Woisard, K. & Moeller, F. G. Dynamic Causal Modeling Self-Connectivity Findings in the Functional Magnetic Resonance Imaging Neuropsychiatric Literature. *Frontiers in Neuroscience* **15**, doi:10.3389/fnins.2021.636273 (2021).

22 Friston, K. J., Harrison, L. & Penny, W. Dynamic causal modelling. *NeuroImage* **19**, 1273-1302, doi:https://doi.org/10.1016/S1053-8119(03)00202-7 (2003).

23 Ongür, D., An, X. & Price, J. L. Prefrontal cortical projections to the hypothalamus in macaque monkeys. *J Comp Neurol* **401**, 480-505 (1998).

24 Bandler, R. & Shipley, M. T. Columnar organization in the midbrain periaqueductal gray: modules for emotional expression? *Trends Neurosci* **17**, 379-389, doi:10.1016/0166-2236(94)90047-7 (1994).

25 Bingel, U., Lorenz, J., Schoell, E., Weiller, C. & Buchel, C. Mechanisms of placebo analgesia: rACC recruitment of a subcortical antinociceptive network. *Pain* **120**, 8-15, doi:10.1016/j.pain.2005.08.027 (2006).

26 Oliva, V. *et al.* Parallel cortical-brainstem pathways to attentional analgesia. *NeuroImage* **226**, 117548, doi:https://doi.org/10.1016/j.neuroimage.2020.117548 (2021).

27 Benedetti, F. & Amanzio, M. Mechanisms of the placebo response. *Pulmonary Pharmacology & Therapeutics* **26**, 520-523, doi:https://doi.org/10.1016/j.pupt.2013.01.006 (2013).

28 Scott, D. J. *et al.* Individual differences in reward responding explain placebo-induced expectations and effects. *Neuron* **55**, 325-336, doi:10.1016/j.neuron.2007.06.028 (2007).

29 Jensen, K. B. *et al.* A Neural Mechanism for Nonconscious Activation of Conditioned Placebo and Nocebo Responses. *Cerebral cortex (New York, N.Y. : 1991)* **25**, 3903-3910, doi:10.1093/cercor/bhu275 (2015).

30 Kaptchuk, T. J. *et al.* Do "placebo responders" exist? *Contemp Clin Trials* **29**, 587-595, doi:10.1016/j.cct.2008.02.002 (2008).

31 Olsen, M. F. *et al.* Minimum clinically important differences in chronic pain vary considerably by baseline pain and methodological factors: systematic review of empirical studies. *J Clin Epidemiol* **101**, 87-106.e102, doi:10.1016/j.jclinepi.2018.05.007 (2018).

32 Pagnini, F. *et al.* Placebo and Nocebo Effects as Bayesian-Brain Phenomena: The Overlooked Role of Likelihood and Attention. *Perspect Psychol Sci*, 17456916221141383, doi:10.1177/17456916221141383 (2023).

33 Schmid, J. *et al.* Placebo analgesia in patients with functional and organic abdominal pain: a fMRI study in IBS, UC and healthy volunteers. *Gut* **64**, 418-427, doi:10.1136/gutjnl-2013-306648 (2015).

34 Ioachim, G. *et al.* Altered Pain in the Brainstem and Spinal Cord of Fibromyalgia Patients During the Anticipation and Experience of Experimental Pain. *Front Neurol* **13**, 862976, doi:10.3389/fneur.2022.862976 (2022).

35 Peyron, R., Quesada, C. & Fauchon, C. in *Handbook of Clinical Neurology* Vol. 166 (ed Brent A. Vogt) 317-326 (Elsevier, 2019).

36 Schenk, L. A. & Colloca, L. The neural processes of acquiring placebo effects through observation. *Neuroimage*, 116510, doi:10.1016/j.neuroimage.2019.116510 (2019).

37 Vachon-Presseau, E. *et al.* Brain and psychological determinants of placebo pill response in chronic pain patients. *Nat Commun* **9**, 3397, doi:10.1038/s41467-018-05859-1 (2018).

38 Penny, W. D., Friston, K. J., Ashburner, J. T., Kiebel, S. J. & Nichols, T. E. *Statistical parametric mapping: the analysis of functional brain images*. (Elsevier, 2011).

39 Särkkä, S. *et al.* Dynamic retrospective filtering of physiological noise in BOLD fMRI: DRIFTER. *NeuroImage* **60**, 1517-1527, doi:10.1016/j.neuroimage.2012.01.067 (2012).

40 Wager, T. D. *et al.* Brain mediators of cardiovascular responses to social threat, part II: Prefrontal-subcortical pathways and relationship with anxiety. *Neuroimage* **47**, 836-851, doi:10.1016/j.neuroimage.2009.05.044 (2009).

41 Huang, C. C., Rolls, E. T., Feng, J. & Lin, C. P. An extended Human Connectome Project multimodal parcellation atlas of the human cortex and subcortical areas. *Brain Struct Funct* **227**, 763-778, doi:10.1007/s00429-021-02421-6 (2022).

Table 1. Location, level of significance, and cluster size of regions altering in connectivity with the right lateral PAG specifically during stimulus application in the placebo responder group. Co-ordinates are in Montreal Neurological Institute (MNI) space. Cluster sizes are derived from resliced 1mm isotropic image series. "ipsilateral" = right. *NAcc = nucleus accumbens; rACC = rostral anterior cingulate cortex; dACC = dorsal anterior cingulate cortex; MCC = mid cingulate cortex; SMA = supplementary motor area; S1 = primary somatosensory cortex.*

	MNI coordinates			t-value	cluster size	PAG Stimulus-dependent connectivity change (mean ± SEM)	
	X	Y	Z			control scan	lidocaine scan
Psychophysiological Interaction (PPI) *PBO>control*							
Contralateral Anterior	-37	12	-7	72	3.64	-0.26±0.12	0.40±0.14
Insula	-33	13	11	31	3.65	-0.23±0.13	0.40±0.11
Contralateral NAcc	-7	5	-5	64	4.27	-0.26±0.06	0.16±0.10
Ipsilateral rACC	12	36	20	82	3.84	-0.14±0.06	0.34±0.10
Ipsilateral dACC	6	15	37	271	3.71	-0.26±0.13	0.59±0.20
Ipsilateral MCC	4	-16	40	50	3.62	-0.36±0.09	0.32±0.20
Ipsilateral SMA	4	8	45	180	4.31	-0.47±0.17	0.34±0.19
Contralateral S1	-38	-34	46	367	4.10	-0.45±0.17	0.39±0.14

Table 2. Location, level of significance, and cluster size of regions altering in connectivity with the right lateral PAG across the entire scan timecourse in the placebo responder group. Co-ordinates are in Montreal Neurological Institute (MNI) space. Cluster sizes are derived from resliced 1mm isotropic image series. "ipsilateral" = right. *rACC = rostral anterior cingulate cortex; dlPFC = dorsolateral prefrontal cortex; mPFC = medial prefrontal cortex; MeA = medial nucleus of the amygdala.*

	MNI coordinates			t-value	cluster size	PAG Whole scan connectivity change (mean ± SEM)	
	X	Y	Z			control scan	lidocaine scan
Functional Connectivity (FC)							
PBO>Control							
Ipsilateral rACC	3	40	17	5.10	311	-0.009±0.008	0.035±0.006
Contralateral dlPFC	-29	25	27	4.16	98	-0.007±0.007	0.025±0.008
PBO>Control							
Ipsilateral mPFC	11	60	-18	4.58	203	0.029±0.011	-0.016±0.007
Ipsilateral MeA	21	-2	-13	3.90	44	0.025±0.007	-0.008±0.008
Ipsilateral Posterior Hypothalamus	3	-5	-8	4.64	40	0.023±0.006	-0.007±0.006
Contralateral Posterior Hypothalamus	-1	-4	-7	5.63	83	0.020±0.005	-0.012±0.006

Table 3. Significant modulatory parameter estimates mean and standard error as determined by nested search dynamic causal modelling (DCM). Effect sizes were calculated by Cohen's D.

Connection	Mean (±SEM) Responder	Mean (±SEM) Nonresponder	Cohen's D (effect size) 95% CI
Stimulus Dependent System			
NAcc → rACC	0.11±0.03	-0.01±0.04	0.76 [0.16 – 1.37]
rACC → PAG	0.18±0.04	0.02±0.05	0.81 [0.20 – 1.42]
Stimulus Independent System			
Right Hypo → PAG	0.02±0.01	0.05±0.01	0.92 [0.31 – 1.54]
Left Hypo → PAG	0.02±0.01	0.05±0.01	0.73 [0.12 – 1.33]
rACC → PAG	0.06±0.01	0.01±0.02	0.68 [0.09 – 1.29]
PAG → PAG	-0.10±0.01	-0.16±0.01	0.76 [0.16 – 1.37]

Supplementary Information:

Table 1. Temperatures applied throughout conditioning and test phases in placebo responder and nonresponder groups.

	Responder	Nonresponder	P-value
Moderate Temperature (degrees±SEM)	46.8±0.17	46.7±0.20	0.94
Low Temperature (degrees±SEM)	45.8±0.17	45.6±0.22	0.57

Table 2. Placebo non responder functional connectivity values in significant clusters of the stimulus independent network.

	PAG Whole scan connectivity change (mean ± SEM)		
	control scan	lidocaine scan	P-value
Functional Connectivity (FC) *PBO>Control*			
Ipsilateral rACC	0.023±0.010	0.019±0.001	0.84
Contralateral dlPFC	0.014±0.005	0.011±0.006	0.14
PBO>Control			
Ipsilateral mPFC	0.008±0.006	0.010±0.009	0.84
Ipsilateral MeA	0.017±0.007	0.014±0.005	0.68
Ipsilateral Posterior Hypothalamus	0.026±0.010	0.015±0.007	0.30
Contralateral Posterior Hypothalamus	0.024±0.010	0.009±0.006	0.14

Table 3. Placebo responder and non-responder signal intensity change values in significant clusters of the stimulus independent network.

	Responder Signal Intensity Change (mean ± SEM)			Non-responder Signal Intensity Change (mean ± SEM)		
	control scan	lidocaine scan	P-value	control scan	lidocaine scan	P-value
Functional Connectivity (FC) *PBO>Control*						
Ipsilateral rACC	-0.12±0.34	-0.08±0.34	0.86	-0.16±0.18	-0.19±0.19	0.88
Contralateral dlPFC	0.13±0.10	0.19±0.09	0.47	0.10±0.09	0.05±0.09	0.46
PBO>Control						
Ipsilateral mPFC	-0.11±0.09	-0.15±0.09	0.74	-0.09±0.10	-0.09±0.06	0.99
Ipsilateral MeA	0.22±0.12	0.32±0.14	0.51	0.05±0.12	0.16±0.07	0.36
Ipsilateral Posterior Hypothalamus	-0.24±0.22	-0.08±0.28	0.57	-0.08±0.15	-0.08±0.13	0.99
Contralateral Posterior Hypothalamus	0.08±0.21	0.42±0.27	0.22	0.32±0.11	0.14±0.15	0.36

Table 4. Placebo non responder psycho-physiological interaction values in significant clusters of the stimulus dependent network.

	PAG Stimulus-dependent connectivity change (mean ± SEM)		
	control scan	lidocaine scan	P-value
Psychophysiological Interaction (PPI) *PBO>control*			
Contralateral Anterior Insula	-0.31±0.11	-0.17±0.14	0.40
Contralateral NAcc	0.07±0.09	-0.05±0.06	0.35
Ipsilateral rACC	-0.04±0.06	0.07±0.07	0.22
Ipsilateral dACC	-0.29±0.12	-0.03±0.07	0.22
Ipsilateral MCC	-0.25±0.12	-0.04±0.16	0.52
Ipsilateral SMA	-0.24±0.14	0.05±0.17	0.11
Contralateral S1	-0.28±0.13	0.38±0.13	0.004

Table 5. Placebo responder and non-responder signal intensity change values in significant clusters of the stimulus dependent network.

	Responder Signal Intensity Change (mean ± SEM)			Non-responder Signal Intensity Change (mean ± SEM)		
	control scan	lidocaine scan	P-value	control scan	lidocaine scan	P-value
Psychophysiological Interaction (PPI) *PBO>control*						
Contralateral Anterior Insula	1.28±0.22	1.29±0.23	0.93	1.07±0.26	1.25±0.22	0.54
Contralateral NAcc	0.05±0.10	0.19±0.10	0.36	-0.01±0.07	-0.02±0.08	0.92
Ipsilateral rACC	0.09±0.15	0.18±0.16	0.34	-0.10±0.11	-0.01±0.12	0.38
Ipsilateral dACC	1.85±0.34	1.73±0.34	0.65	1.47±0.18	1.47±0.20	0.99
Ipsilateral MCC	0.05±0.27	-0.06±0.27	0.64	0.22±0.21	0.19±0.13	0.89
Ipsilateral SMA	2.79±0.39	2.42±0.35	0.29	1.45±0.18	1.45±0.20	0.99
Contralateral S1	0.58±0.20	0.40±0.21	0.43	0.34±0.38	0.16±0.27	0.70

Table 6. Demographic data of each participant's age, gender, and group assignment for placebo responders and non-responders.

Responders n=23			Non-responders n=24		
Subject ID	Age	Gender	Subject ID	Age	Gender
2013	20	M	2009	20	M
2015	19	F	2010	20	F
2022	20	F	2011	20	M
2025	22	F	2014	21	M
2028	24	F	2016	19	F
2035	24	F	2021	24	F
2036	21	M	2023	22	M
2037	21	M	2024	21	M
2039	32	F	2026	33	M
2049	23	M	2027	24	M
2056	24	M	2029	27	F
2078	24	F	2030	23	F
2079	25	M	2032	20	F
2082	29	M	2034	23	F
2083	21	F	2040	26	M
2084	25	M	2041	22	M
2085	24	M	2077	23	F
2086	32	F	2080	24	F
2087	25	M	2081	31	M
2088	25	M	2091	23	M
2089	25	F	2095	27	F
2090	24	M	2097	23	F
2094	23	M	2098	23	M
			2099	37	F
Mean±SEM	24.0±0.68	13M :10F		24.0±0.84	12M :12F

Chapter 3: Summary

This chapter presented a novel experiment combining numerous imaging-based analytical techniques to define a cortical architecture present in the development and manifestation of placebo analgesia. Supporting prior research and chapter 2, we first demonstrated it was possible to elicit placebo analgesia in response to acute thermal stimuli within a proportion of individuals, and that this responsiveness was associated with changes in signal intensity within the lateral PAG column.

In this chapter we also introduced connectivity and mediation analyses, as well as DCM to directly test our hypotheses of two distinct cortical networks that regulate the output of the lPAG and underpin greater analgesic responses. In line with our hypotheses, functional connectivity (FC) and psycho-physiological interaction (PPI) revealed these networks: the first stimulus-independent and comprised of limbic structures like the posterior hypothalamic nuclei (PH) and medial amygdaloid nucleus (MeA), and the second stimulus-dependent and comprised of the rostral anterior cingulate cortex (rACC) and nucleus accumbens (NAc), among other regions respectively. DCM was then used to specifically pinpoint which anatomical connections between sites behaved differently between placebo responders and nonresponders, as well as their directionality – that is, whether two regions were changing in their coupling in a top-down or bottom-up pattern.

The major discovery and novelty of this study was that brainstem regulatory circuits described in experimental animals over 50 years ago appeared to be partially preserved in humans and underpinned the ability to mount the phenomenon of placebo analgesia. Specifically, we found differences in how efferent projections from the PH to the lPAG behaved between placebo responders and nonresponders. Whilst these connections did not mediate the relationship between placebo responsivity and lPAG signal intensity change (i.e. activation), they related directly to group placement, suggesting that the PH-lPAG projection was critical in establishing an excitatory tone of the lPAG which could then be recruited by other projections during noxious stimuli. Indeed, the stimulus-dependent rACC-lPAG projection also demonstrated differences between groups in the DCM analysis, and this connection completely mediated the relationship between placebo responsiveness and lPAG signal.

Overall, our findings indicated the presence of two distinct cortical networks which contacted descending pain modulatory nuclei during the context of placebo separate to during the application of noxious stimuli. The results support the idea of placebo analgesia as a complex

phenomenon, with the neural signature of the response incorporating information extending far beyond sensory input alone.

An interesting finding which arose in this study, and one which motivated the experiment and manuscript presented in Chapter 4 was that we found no interaction between dorsolateral prefrontal cortex (dlPFC) connectivity and placebo analgesia. With this node within the frontal lobe previously being defined as crucial in the development of placebo responses, our attention turned to what role it played in this analgesic phenomenon. Inspired by the work outlined in Appendix A, our focus turned away from pain modulation itself, and rather towards an individual's ability to form accurate expectations of pain-relief during the administration of a placebo.

Chapter 4:

Matching expectation to experience: biochemistry and function of the dlPFC in placebo analgesia

"The problem is not the problem.
The problem is your attitude
about the problem."
– Jack Sparrow, 2007

Chapter 4: Overview

This chapter contains the following publication: Crawford LS, Mills EP, Peek A, Macefield VG, & Henderson LA. (2023). *Function and biochemistry of the dorsolateral prefrontal cortex during placebo analgesia: How the certainty of prior experiences shapes endogenous pain relief*, which was submitted to Human Brain Mapping on 6 April 2023 and is currently under review. The submitted manuscript, in its entirety, is reproduced in this chapter.

Where up to this point we had been concerned with defining function within cortical and brainstem sites relating to pain modulation, we were unable to make any inferences on underlying differences in biochemistry which may have been contributing to the phenomena. This manuscript took a different approach in order to directly assess Aim 3 of this thesis: combining information on biochemical concentration (as revealed through ^{1}H-MRS) within a pre-defined node, the right dlPFC, with functional changes contributing to placebo analgesia. Specifically, we had a strong hypothesis that altered excitatory-inhibitory balance within this cortical site would influence the consistency of pain rating patterns in the conditioning phase of placebo, which in turn would contribute to the development of stronger placebo effects. This hypothesis was informed by both our own work (Appendix A), as well as recent developments in the field highlighting a Bayesian model of placebo analgesia: that prior information is integrated within discrete sites of the cortex and brainstem to inform an individual's future responses to an inert treatment.

38 participants received an identical conditioning-based placebo design as described in chapter 3, however a ^{1}H-MRS scan directly prior to the test phase of the experiment. At ultra-high field, ^{1}H-MRS has the capacity to resolve both Glutamate and gamma-aminobutyric acid (GABA) – the brain's primary excitatory and inhibitory neurochemicals, respectively. The inclusion of this scan enabled us to investigate if any specific metabolite concentration within the dlPFC contributed to either rating variability or placebo responses. Additionally, we conducted connectivity analyses using a significant cluster within the dlPFC which showed activation differences between placebo responders and nonresponders as a main effect of placebo.

We hypothesized that placebo responders would be more consistent in rating their pain throughout conditioning relative to non-responders, and that placebo-related reductions in pain would relate to specific functional and biochemical differences within the dlPFC.

Title: Function and biochemistry of the dorsolateral prefrontal cortex during placebo analgesia: How the certainty of prior experiences shapes endogenous pain relief.

Authors: L.S. Crawford[1], E.P. Mills[1], A Peek[1], V.G. Macefield[2], K.A. Keay[1], and L.A. Henderson[1].

[1] School of Medical Sciences (Neuroscience), Brain and Mind Centre, University of Sydney, Australia; [2]Department of Neuroscience, Monash University, Victoria, Australia

Correspondence: Luke A. Henderson, Department of Anatomy and Histology, F13, University of Sydney, Australia. lukeh@anatomy.usyd.edu.au (email); +612 9351 7063 (Tel) +612 9351 6556 (Fax)

Running title: Prior experiences and placebo analgesia

Conflict of interest: The authors declare no conflict of interest.

Abstract: Prior experiences, conditioning cues, and expectations of improvement are essential for placebo analgesia expression. The dorsolateral prefrontal cortex (dlPFC) is considered a key region for converting these factors into a placebo response. Since dlPFC neuromodulation can either attenuate or amplify placebo, we sought to investigate dlPFC biochemistry and function in 38 healthy individuals during placebo analgesia. After deceptively conditioning participants to expect pain relief from a placebo "lidocaine" cream, we collected baseline magnetic resonance spectroscopy (^{1}H-MRS) at 7 Tesla over the right dlPFC. Following this, functional magnetic resonance imaging (fMRI) scans were collected during which identical noxious heat stimuli were delivered to the control and placebo-treated forearm sites. There was no significant difference in concentration of pain-related metabolites at the level of the right dlPFC between placebo responders and non-responders. However, we identified a significant inverse relationship between the excitatory neurotransmitter glutamate and pain rating variability during conditioning. Moreover, we found placebo-related activation within the right dlPFC and altered fMRI coupling between the dlPFC and the midbrain periaqueductal gray which also correlated with dlPFC glutamate. These data suggest that the dlPFC formulates stimulus-response relationships during conditioning which are then translated to altered cortico-brainstem functional relationships and placebo analgesia expression.

Keywords: placebo analgesia, dorsolateral prefrontal cortex, acute pain, conditioning, variability.

Introduction:

Placebo effects, which occur when an inactive treatment or visual cue leads to a significant physiological benefit, are grounded in both an individual's previous experiences and their expectations of improvement (Amanzio and Benedetti 1999; Büchel et al. 2014; Medoff and Colloca 2015; Ashar et al. 2017). In the context of pain, the phenomenon of placebo analgesia has been extensively studied, and associated with a number of factors including conditioning effectiveness, participant-experimenter interactions, and value statements (Colloca and Benedetti 2006; Kaptchuk et al. 2009; Kong et al. 2009; Colloca et al. 2010; Colloca 2019). Together, these factors inform a participant's expectations of how strongly they believe a placebo will work to reduce their pain – that is, precision.

It has been hypothesized that the midbrain periaqueductal gray (PAG) – rostral ventromedial medulla (RVM) – dorsal horn brainstem circuit mediates placebo analgesia and we recently used ultra-high resolution functional magnetic resonance imaging (fMRI) to show that placebo analgesic responses are indeed associated with activity changes in this circuit (Crawford et al. 2021). Given that placebo analgesia requires complex cognitive function to integrate prior experiences and expectations with incoming nociceptive events, it is likely that PAG activity and ultimately placebo analgesia is driven by descending inputs from higher-order cortical regions. Consistent with this idea, human brain imaging studies have revealed that activity changes in the dorsolateral prefrontal cortex (dlPFC) are associated with placebo analgesia in addition to expectations, decision making and error-prediction (Wager et al. 2004; Rosenbloom et al. 2012; Schenk and Colloca 2019; Hibi et al. 2020).

Furthermore, experimental manipulation of dlPFC function can either attenuate or amplify placebo analgesia. For instance, Krummenacher et al. (2010) demonstrated in an expectation-based placebo pain paradigm, that transient disruption of the dlPFC through repetitive transcranial magnetic stimulation (rTMS) blocked the generation of placebo analgesia. This same intervention, however, had no effect on the sensory experience of pain itself. Conversely, Tu et al. (2021) used transient direct current stimulation (tDCS) to demonstrate that compared to sham stimulation, cathodal tDCS applied to the right dlPFC increased placebo analgesic response magnitudes and altered dlPFC functional connectivity. Given these findings, it is likely that the PAG is driven by inputs from the dlPFC either directly or via a relay in the rostral anterior cingulate cortex (rACC) (Eippert et al. 2009; Sevel et al. 2015).

It has also been recently proposed that brainstem pain modulatory circuits are governed by a Bayesian system in which prior experiences are integrated with incoming nociceptive information to form the pain percept and subsequent placebo analgesia. Grahl et al. (2018) applied a Bayesian model over two groups of participants. One group experienced a highly precise conditioning paradigm whereby they received identical low noxious stimuli (to generate consistent expectations of analgesia), and the other group experienced a low precision conditioning paradigm in which they received highly variable noxious stimuli (to generate variable expectations of analgesia). High conditioning precision (leading to consistent expectations of low pain) was associated with greater placebo analgesia, and changes in PAG activation reflected how effectively a participant was able to combine their current pain experience with their prior experiences and expectations for pain reduction to elicit placebo analgesia.

The current study aimed to investigate i) whether low variability in pain intensity ratings during conditioning phase (high conditioning precision) is associated with stronger placebo responses; and ii) whether dlPFC biochemistry, function, and functional connectivity are associated with placebo responsiveness and conditioning precision. Previously we demonstrated that dlPFC signal intensity changes were strongly associated with variability of perceived pain intensity during a set of identical noxious stimuli (Crawford et al. 2022). Given this, we propose that decreased pain intensity variability during identical noxious stimuli (conditioning precision) would be associated with increased magnitude of placebo responses as well as altered function of the dlPFC and its connections to the PAG. Additionally, we hypothesized that placebo responders would demonstrate greater consistency in pain rating responses during conditioning and that this consistency, as well as reported reductions in pain (placebo responses), would relate directly to both functional connectivity differences and biochemistry in the dlPFC.

Methods:

Ethics:

All experimental procedures were approved by the University of Sydney Human Research and Ethics Committee and were consistent with the declaration of Helsinki. Participants provided written informed consent prior to experimental proceedings. Whilst inside the MRI scanner participants were supplied with an emergency button and instructed to squeeze the button to stop the experiment at any time. After all testing, participants were informed verbally and through a written statement of the necessary deception within the experiment and invited to seek any additional clarification of what they had experienced. The data were collected as part of a larger study, some of which has already been published (Crawford *et al.* 2021).

Participants:

Thirty-eight healthy control participants were recruited for the study (18 female; mean±SEM age 25.0±0.8 years; range 20-37 years). An a priori power analysis using a previous brain imaging study investigating interactions between positive expectations and pain relief (Grahl et al. 2018) revealed that a total sample size of 34 would be necessary to detect similar neural effects with 90% power (***d*** = 0.46, **α** = 0.9, *power* = 0.90) (Faul et al. 2007).

Experimental Design:

The study involved three independent sessions conducted over two successive days: *Conditioning (day 1)*, *Reinforcement (day 2)*, and *Test (day 2)* (Fig 1A.). Throughout all three sessions, noxious thermal stimuli were administered using a 3x3 cm Peltier-element thermode (TSA-II, Medoc) applied to the left or right arms, onto sites where two different creams were applied, a *control* (vaseline) or *placebo* (lidocaine) cream. Each stimulus lasted a total of 15 seconds, including a ramp up from baseline (32°C), a plateau at the designated noxious temperature (low or moderate, depending on cream site), and a ramp down to baseline. Each stimulus period was separated by a 15-second inter-trial-interval at the baseline 32°C temperature. Outside the scanner (sessions 1 and 2) participants rated their pain continuously using a computerized visual analogue scale (VAS). Inside the scanner (session 3) the VAS was replicated onto a digital screen overhead and participants continually reported their pain throughout scanning by controlling a slider on this screen using a two-button button box with their left index finger (100 = worst pain imaginable; 0 = no pain).

Day 1 - conditioning:

Prior to conditioning, thermal thresholds were assessed and participants were presented with the control and placebo creams. A determination of moderate pain protocol was conducted where 0.5°C interval temperatures between 44°C and 48.5°C were randomly applied sequentially to participant's left volar forearm. Participants were informed that one temperature was being recorded: a moderate temperature which elicited a VAS pain rating between 50-60, which would then be applied to both cream sites throughout the remainder of the experiment. In reality, we recorded two temperatures: a low temperature (one which elicited a 20-30 VAS rating), as well as the moderate temperature (50-60 VAS).

The two creams were then applied to participant's right forearm. Despite both creams being identical, the verbal description given, and physical appearance of the control and placebo creams differed to elicit initial expectations of analgesic properties. The control cream bottle appeared white, with a label stating it was a Vaseline solution and no colouring additives were added to this cream. In contrast, the placebo cream bottle appeared green, with a label stating it was a "Lidocaine" solution. Green food colouring was also added to this cream and it was described to hold analgesic properties which could reduce the thermal sensitivity in a localized region. The two creams were then applied in a counterbalanced fashion to proximal and distal sites on volar aspect of the forearm overlapping with the C6 dermatome.

Following this procedure, two rounds of conditioning were conducted on the respective cream sites. Each round of conditioning involved a series of eight noxious stimuli being applied to each of the cream sites. Participants were informed that both creams were receiving *identical* noxious

stimuli at the temperature previously eliciting a moderate pain intensity during the determination procedure. In reality, we applied the *low* temperature to the placebo "Lidocaine" cream site, and the *moderate* temperature only to the control Vaseline site. Prior to each series of stimuli, participants were asked to report their average expected pain across the eight stimuli and reported their pain throughout using the computerized VAS. After a total of 16 stimuli had been applied to each cream site, participants were asked to return the following day at an identical time to conduct the scanning component of the experiment.

Day 2 – reinforcement and test:

Upon return, both the control vaseline and placebo "lidocaine" creams were applied in the same counterbalanced locations to participant's left and right volar forearms. Reinforcement was conducted inside the MRI scanner room with participants laying supine on the scanner bed, and noxious stimuli were applied to the left volar forearm to reduce the likelihood of sensitization effects on the right forearm. Reinforcement involved a series of four noxious stimuli being applied to both cream sites at the same moderate and low temperatures as in conditioning to ensure that despite the change of day and immediate environment, participants continued to both expect and experience different pain responses between the control and placebo creams, respectively (Fig 1A.).

Following reinforcement, structural brain scans including a T1-weighted anatomical and magnetic resonance spectroscopy (^{1}H-MRS) scan were collected prior to the test phase. Unlike conditioning and reinforcement, the test phase involved identical moderate intensity stimuli being applied to *both* the control vaseline and placebo "lidocaine" cream sites on the right volar

forearm. Each cream site received eight noxious stimuli, during which functional brain scans (fMRI) was collected. During both the reinforcement and test phases, participants continued to report their average expected pain prior to each series of noxious stimuli, as well as rate their pain continuously using the VAS systems.

Imaging protocol:

Brain images were collected with a whole-body Siemens MAGNETOM 7T MRI system with a combined single-channel transmit and 32-channel receive head coil (Nova Medical). Participants were positioned supine on the scanner bed with support to minimize head movement. Prior to the test protocol, a T1-weighted anatomical image set covering the whole brain was acquired (repetition time = 5000ms, echo time = 3.1ms, flip angle 1 = 4°, flip angle 2 = 5°, raw voxel size = 0.73x0.73x0.73 mm, 224 sagittal slices, scan time = 7 min). Immediately following this acquisition, the siemens standard automated shimming procedure (repetition time = 8500ms, echo time = 6ms, flip angle = 90°) and a single voxel ^{1}H-MRS was acquired using a Stimulated Echo Acquisition Mode (STEAM) sequence (32 averages, spectral width = 6000Hz, water suppression = standard, reference amplitude = 215V, mixing time = 32ms, scan time = 5 min 06 sec) encompassing a 20x20x20mm voxel overlying the right dorsolateral prefrontal cortex (dlPFC) (Mylius et al. 2013). In all participants, the dlPFC MRS cube was bordered laterally by the skull wall, superiorly by the superior frontal sulcus, and inferiorly by the inferior frontal sulcus (Fig 2B). The two fMRI sequences each consisted of a series of 134 gradient-echo echo-planar, using blood oxygen level-dependent (BOLD) contrast covering the entire brain. Images were acquired interleaved with a multiband factor of four and an acceleration factor of three

(repetition time = 2500ms, echo time = 26ms, raw voxel size = 1.0x1.0x1.2 mm, 124 axial slices, scan time = 6 min 25 sec).

Imaging preprocessing and statistical analyses were performed using SPM12 (Penny et al. 2011) and custom software. Each participant's raw dlPFC ^{1}H-MRS spectroscopy was first inspected to ensure data quality and maximal CRLB values for GABA, glutamate, mINS, and NAA were 19%, 2%, 3%, and 2% respectively. Additionally, the FWHM of the spectra across all 36 participants did not exceed the established rejection cutoff of 21 Hz (mean±SEM FWHM 9.63±0.25 Hz). ^{1}H-MRS metabolite concentrations were quantified using LCModel version 6.3-1N (Provencher 1993) operated within the custom GUI provided by Osprey version 2.0.0 (Oeltzschner et al. 2020). A raw 7-T STEAM basis set was loaded and parsed into LCModel to create outputs of estimated concentration (mmol.L^{-1}) and Cramer-Rao lower bounds (CRLB); standard deviations expressed as a percent of the estimated concentrations. The Gannet Co-Register and Gannet Segment modules were utilized within Osprey, calling SPM12 to determine the tissue volume fractions of grey matter, white matter and cerebrospinal fluid. Gannet Quantify then returns relative metabolite levels in institutional units (IU), corrected for effects of tissue water content and relaxation effects (Harris et al. 2015). Metabolites of interest included Glutamate and gamma-aminobutyric acid (GABA): the central nervous system's primary excitatory and inhibitory neurotransmitters, which play a broad role in emotional and cognitive processing of pain, as well as signaling memory formation and retrieval (Hassel and Dingledine 2012; Peek et al. 2020). Additionally, due to their role in pain processing, we also assessed Myo-inositol (mINS) and N-acetylaspartate (NAA) for group-level differences. Reduced concentrations of both mINS and NAA have been associated with chronic pain

disorders and suggested to contribute to deficiencies in pain processing (Grachev et al. 2000; Grachev et al. 2002; Gussew et al. 2011).

Functional image series were slice-timing corrected, motion corrected, and the resulting six directional movement parameters inspected to ensure that neither 1mm of linear movement nor 0.5° of rotational movement was exceeded in any direction. Images were then linearly detrended to remove global signal changes, and the DRIFTER toolbox was used to remove physiological noise associated with cardiac and respiratory frequencies and harmonics (Särkkä et al. 2012). Signal change relating to the six movement parameters previously extracted were modelled and removed using a linear modelling of realignment parameters (LMRP) procedure. Each individual's two fMRI image series were then co-registered to their own T1-weighted anatomical image, and the T1 was spatially normalized to the DARTEL template in Montreal Neurological Institute (MNI) space. These normalization parameters were then applied to the fMRI images to ensure each image series between participants occupied an identical template space. Normalized fMRI images were then spatially smoothed using a 6mm full width at half maximum (FWHM) Gaussian filter.

After revealing a cluster within the midbrain PAG in initial analyses, the spatially unbiased infratentorial template (SUIT) toolbox image segmentation and normalization pipelines were conducted, resulting in the brainstem and cerebellum of each participant being isolated in T1- and fMRI image series (Diedrichsen 2006). During this process, raw images were resliced to 0.5mm isotropic voxels, and spatially smoothed using a 1mm FWHM Gaussian filter to enable better spatial localization and parameter estimate extraction from this specific cluster.

Determining placebo responses:

Each participant was classified as either a placebo responder or non-responder based on the two standard deviation band technique employed previously in investigating functional brain changes related to pain modulatory phenomena (Youssef et al. 2016; Crawford et al. 2021). During the test phase, the mean and standard deviation of perceived pain intensities during each stimulation period were calculated for both the control vaseline cream series and the placebo "lidocaine" cream series. If the mean pain intensity during the placebo series was more than two standard deviations lower than the mean control pain intensity, the participant was considered to be a placebo responder.

Statistical Analyses:

Perceived pain intensity magnitude changes and variability during both the conditioning and test phases, as well as metabolite concentrations within the right dlPFC, specifically GABA, Glutamate, mINS, and NAA, were compared between placebo responders and non-responders using two-sample t-tests (two tailed, $p<0.05$). Additionally, linear regression analyses were conducted between pain intensity changes, pain intensity variability, and the concentrations of each of these pain-related metabolites (Pearsons correlations, $p<0.05$ corrected for multiple comparisons).

Changes in signal intensity during the fMRI scans were determined using a repeated boxcar model convolved with a hemodynamic delay function where "1" was entered for the noxious stimulation periods and "0" for the baseline and inter-stimulus interval periods. The resultant brain contrast maps were then entered into second level, random effects analyses to determine

the main effect of pain (pain>baseline / pain<baseline) and the main effect of placebo (placebo>pain / placebo<pain). A threshold of $p<0.05$, family wise error corrected for multiple comparisons were applied to both analyses. Since no voxels survived this stringent threshold for the main effect of placebo analysis, the threshold was set at $p<0.001$ uncorrected for multiple comparisons. To reduce the likelihood of type II errors, a cluster extent threshold of 20 contiguous voxels was applied to both functional analyses and small volume correction was performed on each cluster (Woo et al. 2014).

In addition, a significant cluster within the right dlPFC resulting from the main effect of placebo analysis was used as a seed region for conducting a whole brain, voxel-by-voxel functional connectivity analysis over each of the fMRI scans. The location of this dlPFC seed overlapped with the dlPFC ^{1}H-MRS voxel. These whole scan functional connectivity contrast images were then entered into second level random effects analysis and a main effect of placebo assessed ($p<0.001$, uncorrected for multiple comparisons). Connectivity values were extracted from significant clusters and differences between "vaseline" and "lidocaine" scan connectivities determined in the placebo responder and non-responder groups (paired t-tests, $p<0.05$). A brainstem-specific functional connectivity analysis was also conducted using the time series of the dlPFC cluster and a mask of the PAG to determine in which discrete longitudinal column, dlPFC coupling was altered during placebo analgesia. Finally, after extracting parameter estimates from SUIT images representing dlPFC-PAG connectivity, linear relationships between dlPFC connectivity strength and both pain intensity ratings and dlPFC metabolite concentrations were determined (Pearsons correlations, $p<0.05$).

For both the functional activation and connectivity analyses, the location of significant clusters in MNI space were tabulated and labelled consistent to Mai et al "Atlas of the Human Brain" (2015). For display purposes, significant clusters were overlaid onto a mean T1-weighted anatomical image of all participants.

Results:

Psychophysics:

Two of the participants MRI scans were excluded due to technical or data quality issues resulting in 36 participants remaining for further analysis. Twenty of the remaining 36 participants were classified as placebo responders (mean±SEM pain intensity: control 45.82±1.54, lidocaine 34.02±1.91, $p<0.001$), and the remaining 16 non-responders (mean±SEM pain intensity control 41.22±3.31, lidocaine 44.69±3.07, $p=0.07$). Despite differences in placebo responsivity, both responder and non-responder groups alike *expected* a significant pain reduction during stimulation of the placebo "lidocaine" cream site (mean±SEM pain intensity: responder control 49.35±0.77, lidocaine 33.48±1.55; non-responder; control 51.67±1.76, lidocaine 37.14±2.47; both $p<0.001$) (Fig 1B). Additionally, conditioning pain intensity ratings on both the control vaseline and placebo lidocaine sites did not differ between placebo responders and non-responders (mean±SEM pain intensity: control responder 46.52±2.37, nonr-esponder 42.35±3.85, $p=0.37$; lidocaine responder 28.58±2.75, non-responder 27.45, $p=0.82$).

Consistent with our hypothesis, placebo responders displayed greater pain percept precision (low variability to identical noxious stimuli) than non-responders during the conditioning phase when low and moderate temperatures were applied to the placebo "lidocaine" and control

vaseline cream sites, respectively, as well as when moderate intensity stimuli were applied to the control site during the test phase (pain intensity SD±SEM: conditioning control site; responders 6.66±0.54, non-responders 10.55±1.30, p=0.008; conditioning "lidocaine" site; responders 8.34±0.64, non-responders 11.98±1.42, p=0.02; test control site: responders 3.27±0.47, non-responders 6.36±1.01, p=0.008; test "lidocaine" site: responders 4.08±0.42, non-responders 5.10±0.62, p=0.19) (Fig 1C).

In all participants, a significant positive relationship was found between pain percept precision in both the conditioning and test phases during stimulation of the placebo "lidocaine" site ($r=0.59$, $p<0.001$) (Fig 1D). In addition, there was a significant negative relationship between the magnitude of placebo analgesia and pain percept precision during stimulation of the placebo "lidocaine" site during the conditioning phase ($r=-0.53$, $p<0.001$) (Fig 1E). That is, greater percept precision during conditioning was associated with greater precision during the test phase on the following day, and greater percept precision during the conditioning phase was associated with greater placebo analgesia.

Spectroscopy:

A representation 1H-MRS spectra and the location of the dlPFC sampling region are shown in Figures 2A and B. The mean tissue fraction within the dlPFC volume collected consisted of 46% gray matter, 53% white matter, and 1% CSF – and each participant's relative tissue fraction was then utilized in calculating tissue-corrected concentrations of metabolites of interest. Fractions of each tissue type and mean spectra FWHM did not demonstrate significant group-level differences (mean±SEM gray matter: responder 0.47±0.01, non-responder 0.44±0.02,

p=0.16; white matter: responder 0.52±0.01, non-responder 0.55±0.02, p=0.19; CSF: responder 0.01±0.01, non-responder 0.01±0.01, p=0.84; FWHM: responder 9.38±0.37, non-responder 9.94±0.29, p=0.27).

In contrast to our hypothesis, we identified no significant group-level differences in tissue-corrected concentration in any metabolite of interest between placebo responders and non-responders (mean±SEM tissue-corrected concentration GABA: responders = 0.99±0.09, non-responders = 0.94±0.11; glutamate: responders = 13.62±0.19, non-responders = 13.24±0.24; NAA: responders = 14.60±0.29, non-responders = 14.25±0.27; mINS: responders = 9.57±0.16, non-responders = 9.46±0.24) (two-sample t test, all p>0.05) (Fig 2C).

Linear regression was then conducted using the tissue-corrected concentration of each metabolite of interest and pain percept precision during conditioning to assess whether, instead of being involved with the placebo response itself, altered dlPFC biochemistry related to an individual's ability to acquire accurate placebo associations. No significant interaction was observed in GABA, mINS, or NAA. However, a robust inverse correlation was identified between the tissue-corrected concentration of glutamate and conditioning pain percept precision on the placebo-treated site (R=-0.41, p=0.01) (Fig 2D). That is, less variable pain rating responses during placebo-site conditioning were associated with greater concentration of dlPFC glutamate.

Brain activation changes associated with pain and placebo:

During pain, across all participants, signal intensity increased in the bilateral primary somatosensory cortex, bilateral insula, anterior cingulate cortex, ipsilateral thalamus and ipsilateral dlPFC (Figure 3A, Table 1). In addition, significant signal intensity decreases occurred in areas of the default mode network such as in the bilateral angular gyrus, bilateral posterior cingulate cortex, bilateral medial prefrontal cortex extending into the subgenual anterior cingulate cortex, contralateral orbitofrontal cortex and bilateral angular gyrus.

Comparison of signal intensity change during stimulation of the placebo-treated relative to control-treated sites revealed altered activation in several discrete brain regions (Figure 3B, Table 2). These regions included the mid- and posterior cingulate cortices, contralateral ventrolateral prefrontal cortex, ipsilateral superior parietal lobule, and ipsilateral dlPFC. In each of these regions, signal intensity increased during placebo but did not change significantly from baseline during control-site stimulation. In two regions, the posterior cingulate cortex and ipsilateral ventrolateral prefrontal cortex, we observed a decrease in signal during the stimulation of the control-site, which did not change significantly from baseline during placebo-site stimulation. Uniquely, in the rostral anterior cingulate cortex, signal decreased during stimulation of the control-site, and increased during stimulation of the placebo-site.

We further inspected the placebo-related signal intensity increase in the dlPFC region and identified a significant group-level difference between stimulation of the control- and placebo-treated sites. Specifically, whilst both groups followed similar directions of signal change, after controlling for group, only placebo responders demonstrated a significant signal intensity

increase in this region (mean±SEM activation responder: control -0.04±0.12, placebo 0.22±0.11, p=0.003; non-responder: control -0.02±0.11, placebo 0.17±0.10, p=0.15) (Fig 3C).

Altered cortico-cortical and cortico-subcortical coupling underpin placebo responses

Functional connectivity analysis revealed a number of brain regions in which dlPFC connectivity over the entire scan was significantly different during stimulation of the vaseline (control) compared with stimulation of the lidocaine (placebo) sites (Figure 4A, Table 3). Significantly greater dlPFC connectivity strengths during the lidocaine compared with the vaseline cream stimulations occurred in the left dorsal anterior cingulate cortex, right superior parietal lobule, right putamen and the right PAG. In all of these clusters, extraction of dlPFC connectivity values revealed that it was the responders driving these connectivity changes, i.e., connectivity strength values were significantly different between the two scans in the responder group only. Significantly lower dlPFC connectivity strengths during the lidocaine compared with the vaseline cream stimulations occurred in the medial prefrontal cortex and left and right amygdala and again these connectivity differences were driven by the responder group.

Given the well -described role of the PAG in placebo and the brain's descending pain control system, we further investigated the functional coupling between the dlPFC and PAG to determine its specific localization, and whether connectivity changes were related to the overall magnitude of placebo analgesia, pain percept precision during the conditioning phase, or dlPFC biochemistry (Figure 4B). Brainstem specific analysis revealed that the significant PAG cluster resided primarily within the caudal lateral PAG column (lPAG) and spreading dorsally into the dorsolateral column. Further, a robust positive correlation was identified across all participants

between the change in dlPFC-lPAG coupling and the intensity of placebo analgesia (R=0.43, p=0.009). This indicates that this connection not only demarcates a placebo responder, but also shows a graded response in the magnitude of pain relief experienced across all participants. Change in dlPFC-lPAG coupling also positively correlated with dlPFC tissue-corrected glutamate (R=0.38, p=0.02). In contrast, no significant interaction was observed between pain percept precision during conditioning phase stimulation of the placebo-treated site and dlPFC-lPAG coupling (R=0.03, p>0.05). That is, whilst greater dlPFC-lPAG connectivity was associated with both the magnitude of placebo responses and underlying dlPFC biochemistry, the strength of this connection was not directly associated with more precise pain rating responses during conditioning phases.

Discussion:

This investigation provides a multimodal assessment of the dorsolateral prefrontal cortex and its role in conditioning-based placebo analgesia. Specifically, we suggest this role encompasses acquiring strong stimulus-response relationships in conditioning required to translate positive expectations into pain relief via placebo. We first demonstrate that a more precise pain percept during conditioning is correlated with an individual's ability to express placebo analgesia, i.e., the greater the pain percept precision the greater the subsequent placebo analgesia. Importantly, conditioning pain ratings did not differ between placebo responders and non-responders, suggesting that this precision was unrelated to sensory processing or thresholds. Furthermore, we demonstrate that dlPFC biochemistry is linearly related to this pain percept precision, i.e., the greater the pain percept precision the greater the dlPFC glutamate levels, although not directly to the magnitude of placebo analgesia. We extended this finding to show that dlPFC

activity significantly changed only in those individuals who displayed a robust placebo analgesia response. Finally, we showed that dlPFC-lPAG connectivity strength was correlated with both placebo analgesia magnitude and dlPFC glutamate levels, supporting a critical role for the dlPFC to lPAG connection in placebo analgesia. Overall, our results show that there is a complex relationship between an individual's pain percept precision, placebo analgesia, and dlPFC biochemistry, connectivity, and function.

Despite expectations of pain reduction on the placebo-treated site in all participants, only 20 of our 36 participants (55%) demonstrated significant pain modulatory responses. This proportion is consistent within the literature describing analgesic phenomena as being highly variable, only presenting in 30-50% of individuals across a population (Benedetti 1996; Petrovic et al. 2002; Youssef et al. 2016). Whilst expectations of pain reduction did not differ between groups, there were significant differences in pain percept precision during the series of identical noxious stimuli applied to the placebo "lidocaine" cream during the conditioning and test phases. This pain percept precision displayed intra-individual consistency, suggesting that an individual's precision can be carried over between experimental phases despite the change in temperature of noxious stimulation (conditioning - low stimulus temperature, test - moderate stimulus temperature), day of testing (conditioning – day 1, test – day 2), and surrounding environment (conditioning - outside scanner, test - inside scanner).

Consistent with our hypothesis, we found a significant relationship between pain percept precision and placebo analgesia magnitude, i.e. the greater the precision the greater the placebo analgesia magnitude. Interestingly, whilst we found no significant relationship between any

recorded dlPFC metabolite and placebo responsivity, there was a significant linear relationship between dlPFC glutamate levels and pain percept precision. Of course, the glutamate concentration was collected at rest and represents a relatively static measurement. It may be the case that if one could measure dynamic changes in dlPFC glutamate concentration, then changes in glutamate concentration during noxious stimulation may indeed display a significant relationship with placebo analgesia magnitude. Moreover, our results suggest that resting dlPFC excitatory neurotransmission may underpin the variability of sensory percept (precision) and may in turn promote placebo analgesic responses. Indeed, previous investigations have identified a role of dlPFC glutamate in driving cognitive control and efficient neural processing during task performance. In both stroop colour-word conflict and two-back counting tasks, heightened dlPFC glutamate is associated with greater task performance, i.e., guiding appropriate response selection (Woodcock et al. 2018; Morgenroth et al. 2019; Woodcock et al. 2019) and it is possible that pain percept precision is driven by this same process. It is conceivable that this dlPFC-driven precision strengthens the relationship during conditioning between the conditioned stimulus and reductions in perceived pain, thereby generating greater conditioned responses in subsequent experimental phases, i.e., greater placebo analgesia.

Indeed, intracerebral glutamate injections to both the hippocampus and dorsolateral striatum – sites which reciprocally project with the dlPFC in humans – accelerate response learning in experimental animals (Goodman 2020), suggesting that in humans greater resting excitatory neurotransmission may play a similar role in forming strong stimulus-response associations. Moreover, since conditioning and test phases occurred on subsequent days, and pain precision variability between these two phases were linearly related, our results provide evidence that

resting excitatory tone of the dlPFC may encode an individual's ability to consistently perceive and report pain responses to identical noxious stimuli.

Alongside informing response selection, the dlPFC is also known to play a critical role in maintaining and updating internal representations of goals and expectations (Cohen and Servan-Schreiber 1992; Miller and Cohen 2001). Alongside other cortical areas such as the rACC and ventrolateral prefrontal cortex, the dlPFC is part of the brain's executive-attentional network, directing our attention during the experience of sensory stimuli (Kane and Engle 2002; Curtis and D'Esposito 2003; Lorenz et al. 2003). Consistent with this, we found that placebo analgesia was associated with noxious-stimulus evoked signal changes in these three brain regions. When our current experience does not match with our goals or expectations, it is thought that the dlPFC modulates regional brain activity in an attempt to match expected-experienced differentials (Roy et al. 2014; Alexander and Brown 2018; Pagnini et al. 2023).

Our analysis revealed that, during placebo, the dlPFC altered its connectivity strength with areas of the descending modulatory control network, including with the amygdala and PAG, possibly to produce this error-prediction. Preclinical and more recent human studies have shown that the PAG can produce both pro- and anti-nociceptive effects (Eippert et al. 2009; Yoshida et al. 2013; Crawford et al. 2021). Specifically, as revealed through our brainstem-specific analysis, it was the lateral and dorsolateral columns of the PAG demonstrating significant coupling changes with the dlPFC. Stimulation of these two columns in experimental animals produces hyper-reactivity and active defensive behaviours such as flight and fight, as well as an opioid-insensitive analgesia. Additionally, we have previously demonstrated that these two columns are

responsible for producing placebo analgesia when elicited via a response conditioning model utilizing short-lasting thermal stimuli (Crawford *et al.* 2021). The co-ordinates in the present investigation are remarkably similar to where we observed the greatest correlation with placebo magnitude previously, suggesting that the dlPFC may be indeed tapping into this same core brainstem circuitry. Indeed, in addition to a relationship between dlPFC glutamate and precision, we found that altered dlPFC-lPAG coupling was significantly correlated to both placebo analgesia and dlPFC glutamate levels. The greater the dlPFC-lPAG coupling, the greater the excitatory tone of the dlPFC and the greater the placebo analgesia. Together, these findings support a role of the dlPFC in integrating stimulus-response associations learned through conditioning with current experience, driving error-predictive signals to match associations of pain relief by recruiting areas of the subcortex and brainstem.

Together our results indicate that whilst expectation of relief alone are insufficient in activating descending pain-modulatory pathways to produce analgesia, associations learned through conditioning play a strong role in producing this phenomenon, with more precise associations between the conditioning stimulus and conditioned response leading to greater placebo analgesia that are in line with treatment expectations. Precision, or how confident a participant feels a treatment will produce an expected outcome, has previously been tied with mounting placebo analgesia (Grahl et al. 2018), as well as treatment response in clinical scenarios (Doyle et al. 2013; Bombard et al. 2018). It appears that the dlPFC, and in particular its connection with the PAG, plays a critical role in mediating this form of placebo analgesia.

It is important to note some limitations. First, despite the location of control Vaseline and placebo “lidocaine” creams being counterbalanced between participants, it was not possible to completely counterbalance the design such that the control cream site was always stimulated first and the placebo lidocaine second. This ordering effect could have introduced sensitization and or habituation effects, although given only approximately half of the participants displayed an analgesic response, we suggest that a significant ordering effect is unlikely. Second, our connectivity analysis involved determining dlPFC connectivity strengths over the entire scanning period which included periods of noxious stimulation. Whilst signal coupling may have been influenced by changes in overall signal intensity, we suggest that given the stimulus periods made up less than 25% of the total scan, that the effects would have been minimal, if there was any influence of stimulation at all. Third, functional analyses were threshold at $p<0.001$, uncorrected for multiple comparisons. To reduce the chances of Type II errors, we implemented a minimum cluster threshold of 20 contiguous voxels in the wholebrain and 10 contiguous voxels in the brainstem-specific analysis. Furthermore, dlPFC placebo-related activation survived family wise error correction for multiple comparisons. Finally, our investigation included relatively young healthy adults (mean age 25 years; range 20-37 years) and there is evidence that brain regions such as the ACC display glutamate concentration decreases with age and this decrease may be sex-related (Hädel et al. 2013). Whilst we do not know if similar changes occur in the dlPFC, it would be of interest in future investigations to widen the age range considerably and assess the potential interactions between age, dlPFC metabolite concentration, pain percept precision and placebo analgesia responsivity.

Conclusions:

By combining ^{1}H-MRS and fMRI, this investigation demonstrates how dlPFC biochemical composition and functional connectivity may provide a route for translating associations learned through conditioning to endogenous pain relief. We provide evidence that conditioning pain percept precision plays a profound role in generating greater analgesic effects, and further provide evidence that this precision may be underpinned by basal excitatory neurotransmission in the dlPFC.

Acknowledgements: The authors acknowledge the facilities, scientific and technical assistance from the National Imaging Facility, a National Collaborative Research Infrastructure Strategy capability, at the Melbourne Brain Centre Imaging Unit, The University of Melbourne. This work was supported by a research collaboration agreement with Siemens Healthineers. The MRS sequence was developed by Edward J Auerbach and Malgorzata Marjanska and provided by the University of Minnesota under a C2P agreement. This work was supported by funding from the Australian National Health and Medical Research Council (Grant ID 1130280).

Data Availability:

All de-identified single participant functional data, as well as activation and connectivity contrast maps are available from the corresponding author upon reasonable request.

Code Availability:

The analysis methods and software used in this article are all either open source or enabled in SPM12's standard installation. No new methods or algorithms have been

References:

Alexander WH, Brown JW. 2018. Frontal cortex function as derived from hierarchical predictive coding. Scientific Reports. 8:3843.

Amanzio M, Benedetti F. 1999. Neuropharmacological dissection of placebo analgesia: expectation-activated opioid systems versus conditioning-activated specific subsystems. J Neurosci. 19:484-494.

Ashar YK, Chang LJ, Wager TD. 2017. Brain Mechanisms of the Placebo Effect: An Affective Appraisal Account. Annu Rev Clin Psychol. 13:73-98.

Benedetti F. 1996. The opposite effects of the opiate antagonist naloxone and the cholecystokinin antagonist proglumide on placebo analgesia. Pain. 64:535-543.

Bombard Y, Baker GR, Orlando E, Fancott C, Bhatia P, Casalino S, Onate K, Denis J-L, Pomey M-P. 2018. Engaging patients to improve quality of care: a systematic review. Implementation Science. 13:98.

Büchel C, Geuter S, Sprenger C, Eippert F. 2014. Placebo Analgesia: A Predictive Coding Perspective. Neuron. 81:1223-1239.

Cohen JD, Servan-Schreiber D. 1992. Context, cortex, and dopamine: a connectionist approach to behavior and biology in schizophrenia. Psychol Rev. 99:45-77.

Colloca DL, Petrovic DP, Wager DT, Ingvar DM, Benedetti DF. 2010. How the number of learning trials affects placebo and nocebo responses. Pain. 151:430-439.

Colloca L. 2019. How do placebo effects and patient-clinician relationships influence behaviors and clinical outcomes? Pain Rep. 4:e758.

Colloca L, Benedetti F. 2006. How prior experience shapes placebo analgesia. Pain. 124:126-133.

Crawford L, Mills E, Meylakh N, Macey PM, Macefield VG, Henderson LA. 2022. Brain activity changes associated with pain perception variability. Cerebral Cortex.

Crawford LS, Mills EP, Hanson T, Macey PM, Glarin R, Macefield VG, Keay KA, Henderson LA. 2021. Brainstem Mechanisms of Pain Modulation: A within-Subjects 7T fMRI Study of Placebo Analgesic and Nocebo Hyperalgesic Responses. The Journal of Neuroscience. 41:9794-9806.

Curtis CE, D'Esposito M. 2003. Persistent activity in the prefrontal cortex during working memory. Trends Cogn Sci. 7:415-423.

Diedrichsen J. 2006. A spatially unbiased atlas template of the human cerebellum. Neuroimage. 33:127-138.

Doyle C, Lennox L, Bell D. 2013. A systematic review of evidence on the links between patient experience and clinical safety and effectiveness. BMJ Open. 3:e001570.

Eippert F, Bingel U, Schoell ED, Yacubian J, Klinger R, Lorenz J, Büchel C. 2009. Activation of the Opioidergic Descending Pain Control System Underlies Placebo Analgesia. Neuron. 63:533-543.

Faul F, Erdfelder E, Lang AG, Buchner A. 2007. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods. 39:175-191.

Goodman J. 2020. Place vs. Response Learning: History, Controversy, and Neurobiology. Front Behav Neurosci. 14:598570.

Grachev ID, Fredrickson BE, Apkarian VA. 2000. Abnormal brain chemistry in chronic back pain: an in vivo proton magnetic resonance spectroscopy study. Pain. 89:7-18.

Grachev ID, Thomas PS, Ramachandran TS. 2002. Decreased levels of N-acetylaspartate in dorsolateral prefrontal cortex in a case of intractable severe sympathetically mediated chronic pain (complex regional pain syndrome, type I). Brain Cogn. 49:102-113.

Grahl A, Onat S, Büchel C. 2018. The periaqueductal gray and Bayesian integration in placebo analgesia. Elife. 7.

Gussew A, Rzanny R, Güllmar D, Scholle HC, Reichenbach JR. 2011. 1H-MR spectroscopic detection of metabolic changes in pain processing brain regions in the presence of non-specific chronic low back pain. Neuroimage. 54:1315-1323.

Hädel S, Wirth C, Rapp M, Gallinat J, Schubert F. 2013. Effects of age and sex on the concentrations of glutamate and glutamine in the human brain. J Magn Reson Imaging. 38:1480-1487.

Harris AD, Puts NAJ, Barker PB, Edden RAE. 2015. Spectral-editing measurements of GABA in the human brain with and without macromolecule suppression. Magnetic resonance in medicine. 74:1523-1529.

Hassel B, Dingledine R. 2012. Chapter 17 - Glutamate and Glutamate Receptors. In: Brady ST, Siegel GJ, Albers RW, Price DL, editors. Basic Neurochemistry (Eighth Edition) New York: Academic Press p 342-366.

Hibi D, Takamoto K, Iwama Y, Ebina S, Nishimaru H, Matsumoto J, Takamura Y, Yamazaki M, Nishijo H. 2020. Impaired hemodynamic activity in the right dorsolateral prefrontal cortex is associated with impairment of placebo analgesia and clinical symptoms in postherpetic neuralgia. IBRO Rep. 8:56-64.

Kane MJ, Engle RW. 2002. The role of prefrontal cortex in working-memory capacity, executive attention, and general fluid intelligence: an individual-differences perspective. Psychon Bull Rev. 9:637-671.

Kaptchuk TJ, Shaw J, Kerr CE, Conboy LA, Kelley JM, Csordas TJ, Lembo AJ, Jacobson EE. 2009. "Maybe I made up the whole thing": placebos and patients' experiences in a randomized controlled trial. Cult Med Psychiatry. 33:382-411.

Kong J, Kaptchuk TJ, Polich G, Kirsch I, Vangel M, Zyloney C, Rosen B, Gollub R. 2009. Expectancy and treatment interactions: A dissociation between acupuncture analgesia and expectancy evoked placebo analgesia. NeuroImage. 45:940-949.

Krummenacher P, Candia V, Folkers G, Schedlowski M, Schönbächler G. 2010. Prefrontal cortex modulates placebo analgesia. PAIN. 148:368-374.

Lorenz J, Minoshima S, Casey KL. 2003. Keeping pain out of mind: the role of the dorsolateral prefrontal cortex in pain modulation. Brain. 126:1079-1091.

Mai JK, Majtanik M, Paxinos G. 2015. Atlas of the human brain: Academic Press.

Medoff ZM, Colloca L. 2015. Placebo analgesia: understanding the mechanisms. Pain management. 5:89-96.

Miller EK, Cohen JD. 2001. An Integrative Theory of Prefrontal Cortex Function. Annual Review of Neuroscience. 24:167-202.

Morgenroth E, Orlov N, Lythgoe DJ, Stone JM, Barker H, Munro J, Eysenck M, Allen P. 2019. Altered relationship between prefrontal glutamate and activation during cognitive control in people with high trait anxiety. Cortex. 117:53-63.

Mylius V, Ayache SS, Ahdab R, Farhat WH, Zouari HG, Belke M, Brugières P, Wehrmann E, Krakow K, Timmesfeld N, Schmidt S, Oertel WH, Knake S, Lefaucheur JP. 2013. Definition of DLPFC and M1 according to anatomical landmarks for navigated brain stimulation: Inter-rater reliability, accuracy, and influence of gender and age. NeuroImage. 78:224-232.

Oeltzschner G, Zöllner HJ, Hui SCN, Mikkelsen M, Saleh MG, Tapper S, Edden RAE. 2020. Osprey: Open-source processing, reconstruction & estimation of magnetic resonance spectroscopy data. J Neurosci Methods. 343:108827.

Pagnini F, Barbiani D, Cavalera C, Volpato E, Grosso F, Minazzi GA, Vailati Riboni F, Graziano F, Di Tella S, Manzoni GM, Silveri MC, Riva G, Phillips D. 2023. Placebo and Nocebo Effects as Bayesian-Brain Phenomena: The Overlooked Role of Likelihood and Attention. Perspect Psychol Sci.17456916221141383.

Peek AL, Rebbeck T, Puts NAJ, Watson J, Aguila M-ER, Leaver AM. 2020. Brain GABA and glutamate levels across pain conditions: A systematic literature review and meta-analysis of 1H-MRS studies using the MRS-Q quality assessment tool. NeuroImage. 210:116532.

Petrovic P, Kalso E, Petersson KM, Ingvar M. 2002. Placebo and Opioid Analgesia-- Imaging a Shared Neuronal Network. Science. 295:1737.

Provencher SW. 1993. Estimation of metabolite concentrations from localized in vivo proton NMR spectra. Magn Reson Med. 30:672-679.

Rosenbloom MH, Schmahmann JD, Price BH. 2012. The functional neuroanatomy of decision-making. J Neuropsychiatry Clin Neurosci. 24:266-277.

Roy M, Shohamy D, Daw N, Jepma M, Wimmer GE, Wager TD. 2014. Representation of aversive prediction errors in the human periaqueductal gray. Nature neuroscience. 17:1607-1612.

Särkkä S, Solin A, Nummenmaa A, Vehtari A, Auranen T, Vanni S, Lin F-H. 2012. Dynamic retrospective filtering of physiological noise in BOLD fMRI: DRIFTER. NeuroImage. 60:1517-1527.

Schenk LA, Colloca L. 2019. The neural processes of acquiring placebo effects through observation. Neuroimage.116510.

Sevel LS, Craggs JG, Price DD, Staud R, Robinson ME. 2015. Placebo Analgesia Enhances Descending Pain-Related Effective Connectivity: A Dynamic Causal Modeling Study of Endogenous Pain Modulation. The Journal of Pain. 16:760-768.

Tu Y, Wilson G, Camprodon J, Dougherty DD, Vangel M, Benedetti F, Kaptchuk TJ, Gollub RL, Kong J. 2021. Manipulating placebo analgesia and nocebo hyperalgesia by changing brain excitability. Proc Natl Acad Sci U S A. 118.

Wager TD, Rilling JK, Smith EE, Sokolik A, Casey KL, Davidson RJ, Kosslyn SM, Rose RM, Cohen JD. 2004. Placebo-induced changes in FMRI in the anticipation and experience of pain. Science. 303:1162-1167.

Woo C-W, Krishnan A, Wager TD. 2014. Cluster-extent based thresholding in fMRI analyses: pitfalls and recommendations. NeuroImage. 91:412-419.

Woodcock EA, Anand C, Khatib D, Diwadkar VA, Stanley JA. 2018. Working Memory Modulates Glutamate Levels in the Dorsolateral Prefrontal Cortex during (1)H fMRS. Front Psychiatry. 9:66.

Woodcock EA, Greenwald MK, Khatib D, Diwadkar VA, Stanley JA. 2019. Pharmacological stress impairs working memory performance and attenuates dorsolateral prefrontal cortex glutamate modulation. Neuroimage. 186:437-445.

Yoshida W, Seymour B, Koltzenburg M, Dolan RJ. 2013. Uncertainty increases pain: evidence for a novel mechanism of pain modulation involving the periaqueductal gray. The Journal of neuroscience : the official journal of the Society for Neuroscience. 33:5638-5646.

Youssef AM, Macefield VG, Henderson LA. 2016. Cortical influences on brainstem circuitry responsible for conditioned pain modulation in humans. Hum Brain Mapp. 37:2630-2644.

Tables

Table 1. Main effect of pain. Cortical regions displaying significant signal intensity changes during periods of pain relative to baseline during stimulation of the vaseline control cream site. contra: contralateral; dACC: dorsal anterior cingulate cortex; dlPFC: dorsolateral prefrontal cortex; ipsi: ipsilateral; mPFC: medial prefrontal cortex; OFC: orbitofrontal cortex; PCC: posterior cingulate cortex; S1: primary somatosensory cortex.

	MNI Coordinates						
Region Name	*X*	*Y*	*Z*	cluster Size	t value	Z-score	Signal change (±SEM)
Pain > Baseline							
dACC	7	6	48	6220	11.57	7.48	1.72±0.18
ipsi S1	40	-4	50	16132	9.82	6.85	2.62±0.26
contra S1	-54	-24	23	924	7.96	6.04	1.17±0.15
ipsi dlPFC	38	39	28	2160	8.44	6.26	1.71±0.20
ipsi insula	50	8	15	12714	12.64	7.81	1.71±0.15
contra insula	-43	10	-4	3231	9.04	6.53	1.41±0.15
ipsi thalamus	12	-19	9	479	8.16	6.13	0.60±0.07
Pain < Baseline							
PCC	-6	-39	37	5207	9.2	6.59	-1.51±0.19
ipsi angular gyrus	-42	-71	24	3460	7.65	5.89	-1.75±0.22
contra angular gyrus	55	-61	23	936	7.90	6.01	-1.17±0.14
ipsi mPFC	6	53	-8	3300	7.94	6.03	-0.94±0.11
contra OFC	-35	29	-20	698	8.61	6.34	-0.58±0.07

Table 2. Main effect of placebo. Location in Montreal Neurological Institute Space (MNI), cluster size, t value and signal intensity changes of brain region displaying significant signal intensity changes during noxious stimulation of the placebo "lidocaine" cream site relative to the control vaseline cream site. contra: contralateral; dlPFC: dorsolateral prefrontal cortex; ipsi: ipsilateral; MCC: mid-cingulate cortex; OFC: orbitofrontal cortex; PCC: posterior cingulate cortex; rACC: rostral anterior cingulate cortex; SPL: superior parietal lobule; vlPFC: ventrolateral prefrontal cortex.

	MNI Coordinates						
Region Name	*X*	*Y*	*Z*	cluster size	t value	control signal change (±SEM)	placebo signal change (±SEM)
placebo > control							
ipsi SPL	16	-58	29	304	4.17	0.31±0.21	1.04±0.23
ipsi dlPFC	28	26	28	63	4.01	-0.03±0.08	0.21±0.07
MCC	5	-24	26	38	3.59	0.15±0.15	0.59±0.16
PCC	5	-44	17	285	5.21	-0.56±0.04	0.04±0.12
rACC	8	33	12	73	3.92	-0.19±0.11	0.15±0.11
ipsi vlPFC	-23	59	6	134	4.16	-0.67±0.24	-0.03±0.21
contra vlPFC	19	56	-8	103	3.81	-0.11±0.12	0.31±0.11
placebo < control							
contra OFC	-32	35	-11	21	3.27	-0.33±0.06	-0.51±0.07
ipsi amygdala	28	-15	-14	32	3.99	-0.25±0.08	-0.52±0.08

Table 3. Right dorsolateral prefrontal cortex (dlPFC) coupling. Location in Montreal Neurological Institute Space (MNI), cluster size, t value and for clusters that displayed significantly different dorsolateral prefrontal cortex (dlPFC) connectivity strength values between vaseline and lidocaine scans. contra: contralateral; dACC: dorsal anterior cingulate cortex; ipsi: ipsilateral; mPFC: medial prefrontal cortex; lPAG: lateral midbrain periaqueductal gray matter; SPL: superior parietal lobule.

	MNI Coordinates					placebo responders dlPFC connectivity strength (mean±SEM x10^{-2})		placebo non-responders dlPFC connectivity strength (mean±SEM x10^{-2})	
region	*X*	*Y*	*Z*	cluster Size	t value	control scan	placebo scan	control scan	placebo scan
placebo > control									
ipsi SPL	32	-60	49	409	4.49	1.3±1.9	7.8±1.4	4.8±1.4	3.5±1.7
ipsi dACC	-8	-5	48	187	4.66	-0.9±1.1	2.6±0.8	0.5±1.2	2.4±0.7
ipsi putamen	21	9	-10	110	4.08	-1.5±0.1	1.6±0.7	0.8±0.4	0.4±0.5
contra putamen	-17	10	-4	138	4.13	-0.5±0.6	2.3±0.6	1.5±0.5	1.5±0.6
ipsilateral lPAG	4	-30	-9	31	4.13	-0.4±0.6	1.8±0.5	0.3±0.8	0.6±0.7
placebo > control (SUIT)									
ipsi lPAG	2.5	-32.5	-9.5	10	5.67	0.3±0.7	2.2±0.6	0.9±0.6	0.8±0.5
placebo < control									
contra mPFC	-4	47	-12	34	3.51	2.3±1.2	-2.3±1.1	-0.2±1.1	-0.1±1.2
ipsi amygdala	20	-7	-21	114	4.24	1.5±0.8	-1.3±0.6	0.3±0.8	0.6±0.7
contra amygdala	-31	-7	-30	131	5.25	1.8±0.7	-1.3±0.7	0.2±1.3	-0.1±1.3

Figures:

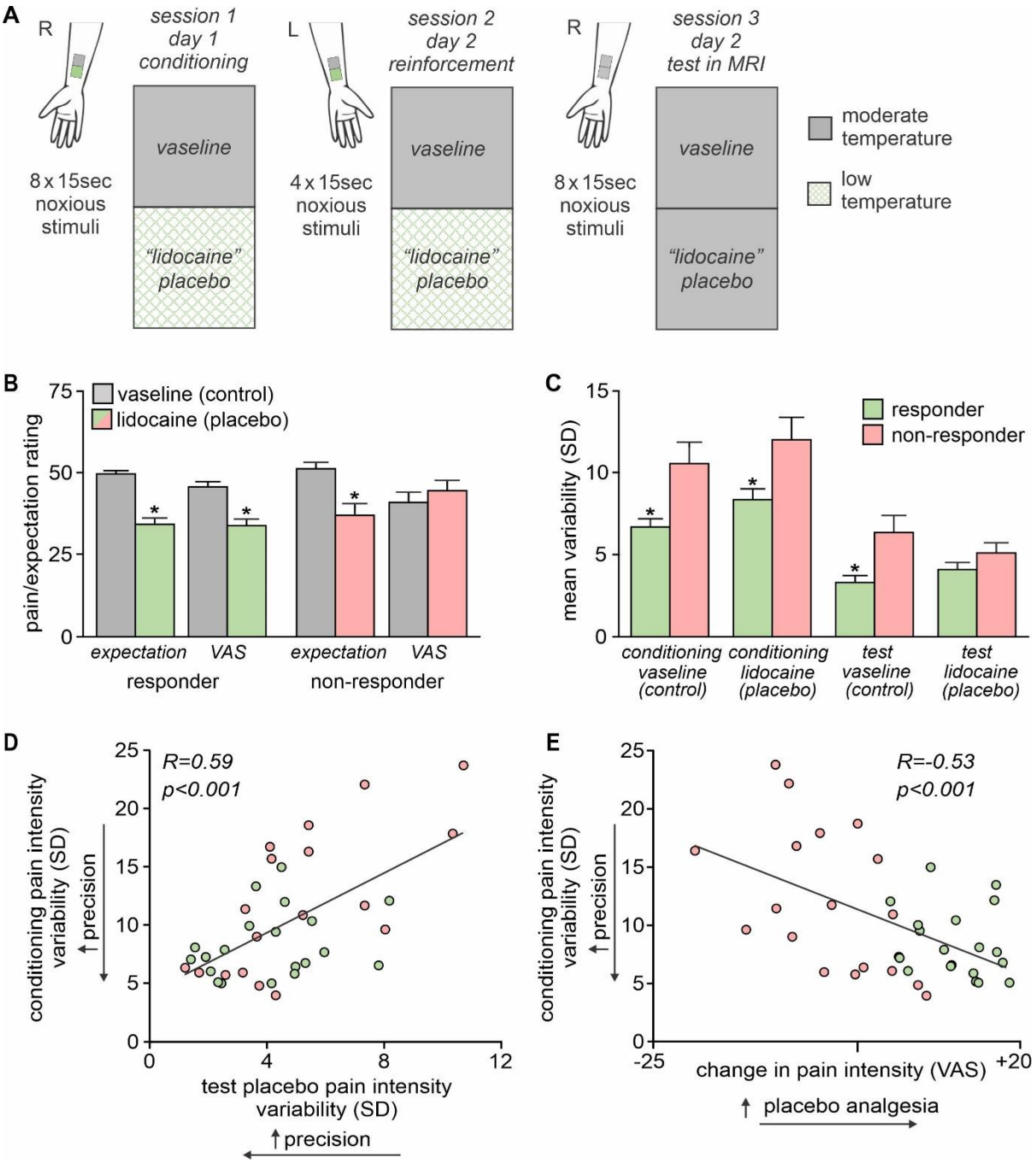
A
R
session 1
day 1
conditioning
vaseline
"lidocaine"
placebo
8 x 15sec
noxious
stimuli
L
session 2
day 2
reinforcement
vaseline
"lidocaine"
placebo
4 x 15sec
noxious
stimuli
R
session 3
day 2
test in MRI
vaseline
"lidocaine"
placebo
8 x 15sec
noxious
stimuli
moderate
temperature
low
temperature
B
vaseline (control)
lidocaine (placebo)
pain/expectation rating
75
50
25
0
expectation
VAS
expectation
VAS
responder
non-responder
C
responder
non-responder
mean variability (SD)
15
10
5
0
conditioning
vaseline
(control)
conditioning
lidocaine
(placebo)
test
vaseline
(control)
test
lidocaine
(placebo)
D
R=0.59
p<0.001
conditioning pain intensity
variability (SD)
precision
25
20
15
10
5
0
0
4
8
12
test placebo pain intensity
variability (SD)
precision
E
R=-0.53
p<0.001
conditioning pain intensity
variability (SD)
precision
25
20
15
10
5
0
-25
change in pain intensity (VAS)
+20
placebo analgesia

Figure 1. Experimental methodology and psychophysics. **A)** During conditioning and reinforcement phases, lower intensity noxious thermal stimuli were deceptively applied to the placebo "lidocaine" cream site relative to a control Vaseline cream. In the test phase, whilst collecting functional MRI, the two cream sites received identical moderate intensity noxious stimuli sequentially (i.e., scan 1 = stimulation of the Vaseline control site, scan 2 = stimulation of the placebo "lidocaine" site). **B)** Plots of mean±SEM expected and actual pain intensity ratings in responder and non-responder groups. Despite not demonstrating a placebo response, non-responders expected significant pain relief via administration of the placebo "lidocaine" cream directly prior to the application of test phase stimuli. *p<0.001 **C)** Plots of mean±SEM pain intensity variability (standard deviation: SD) during conditioning and test. Placebo non-responders demonstrated significantly greater variability in their pain ratings during conditioning, which continued during the test phase inside the scanner when the control vaseline cream site was stimulated. * p<0.001 **D)** Plot of pain rating variability during the stimulation of the placebo "lidocaine" cream during the test phase against variability during stimulation of the placebo "lidocaine" cream during the preceding conditioning phase. Note that pain percept precision displays intra-individual consistency between experimental phases occurring over two subsequent days. **E)** Plot of pain rating variability during the conditioning phase stimulation of the placebo "lidocaine" site against the magnitude of placebo analgesia generated in the test phase. That is, the lower the variability in participant's pain ratings during placebo conditioning, the greater a placebo analgesic response they generated on the following day.

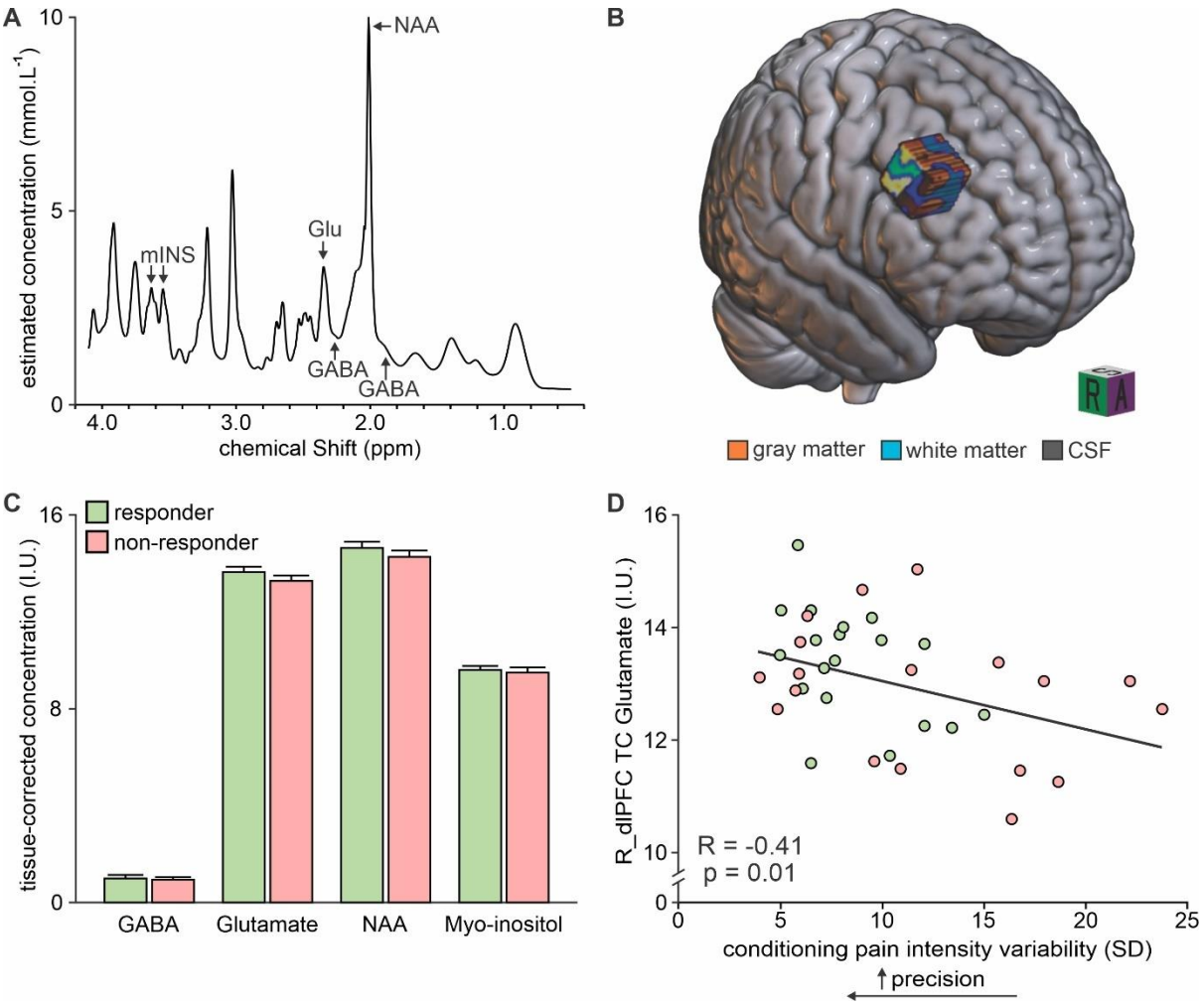

Figure 2. Biochemical metabolite concentrations within the right dorsolateral prefrontal cortex (dlPFC). A: An example trace of metabolite concentrations as resolved through the LCModel spectroscopy processing pipeline; B: Location of the mean cube placed over the right dlPFC overlaid onto a rendered view of a T1-weighted anatomical image. Different colors indicate tissue composition; C: Mean tissue-corrected concentration of key metabolites within the right dlPFC in responders and non-responders. We identified no group-level differences in gamma-aminobutyric acid (GABA), glutamate (Glu), N-acetylaspartate (NAA), or myo-inositol (mINS); D: Plot of tissue-corrected concentration of Glu and pain intensity rating variability (precision) during application of low intensity noxious stimuli to the placebo-treated site during conditioning. CSF: cerebrospinal fluid; TC: tissue corrected.

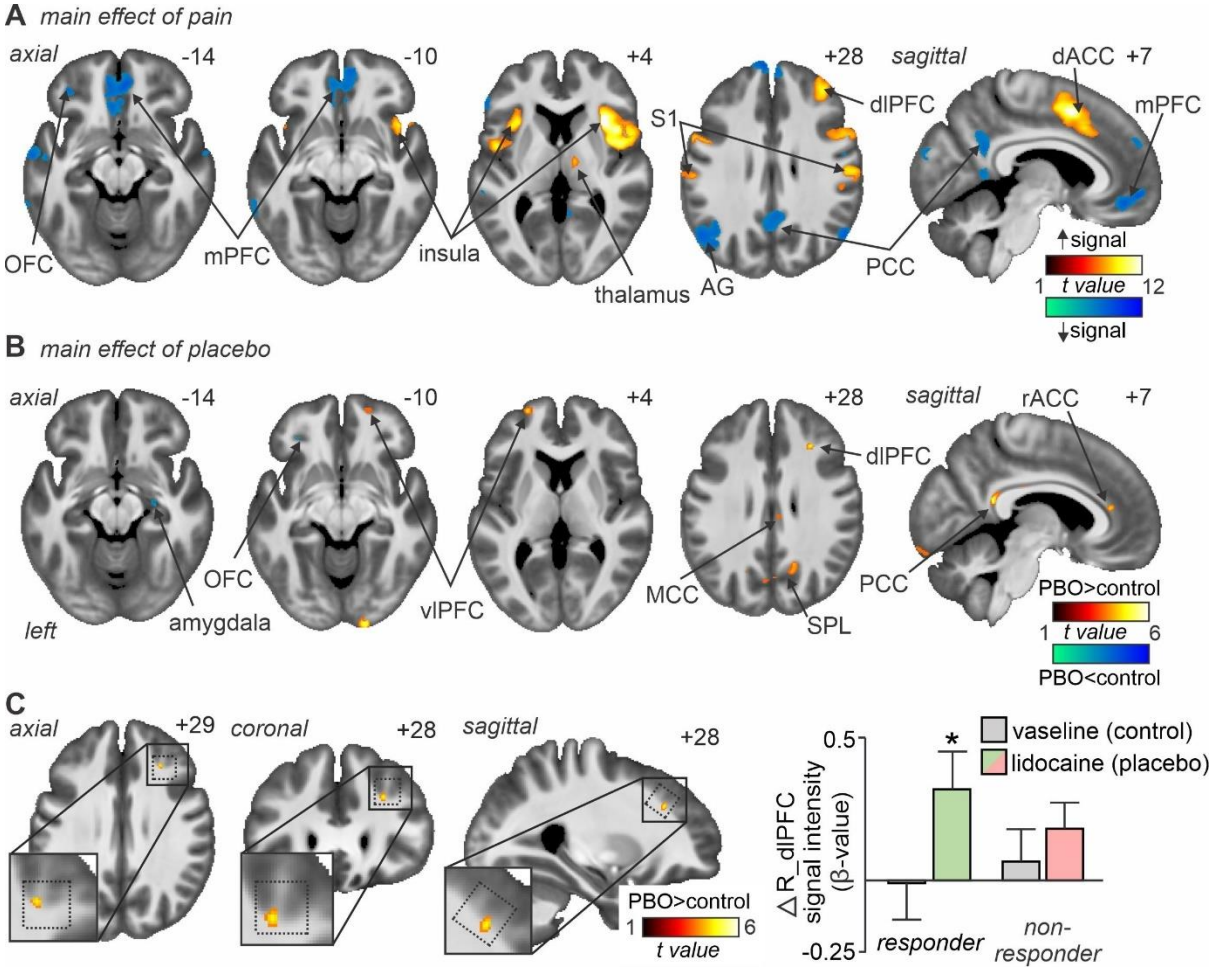

Figure 3. Main effects of pain and placebo, biochemical and functional overlap within the right dlPFC. A: Areas in which signal intensity increased and decreased during noxious stimulation of the control vaseline cream site. B: Areas in which signal intensity increased during noxious stimulation of the "lidocaine" cream site were significantly greater to those during noxious stimulation of the control vaseline cream site. C) Converting the mean spectroscopy cube to a volume of interest mask and applying it to the placebo (PBO) main effect analysis, revealed that the significant activation observed within the ipsilateral dorsolateral prefrontal cortex (dlPFC) was both within the spectroscopy volume collected, and additionally, survived multiple comparisons correction. To the right are plots of right dlPFC signal intensity change differences during noxious stimuli delivered to the control versus "lidocaine" cream sites in responder and non-responder groups. All clusters are overlaid onto a series of slices of a T1-weighted anatomical image set. Slice location in Montreal Neurological Institute space are indicated at the top of each slice. AG: angular gyrus; dACC: dorsal anterior cingulate cortex; rACC: rostral anterior cingulate cortex; MCC: mid-cingulate cortex; mPFC: medial prefrontal; cortex; OFC: orbitofrontal cortex; PCC: posterior cingulate cortex; SPL: superior parietal lobule; vlPFC: ventrolateral prefrontal cortex.

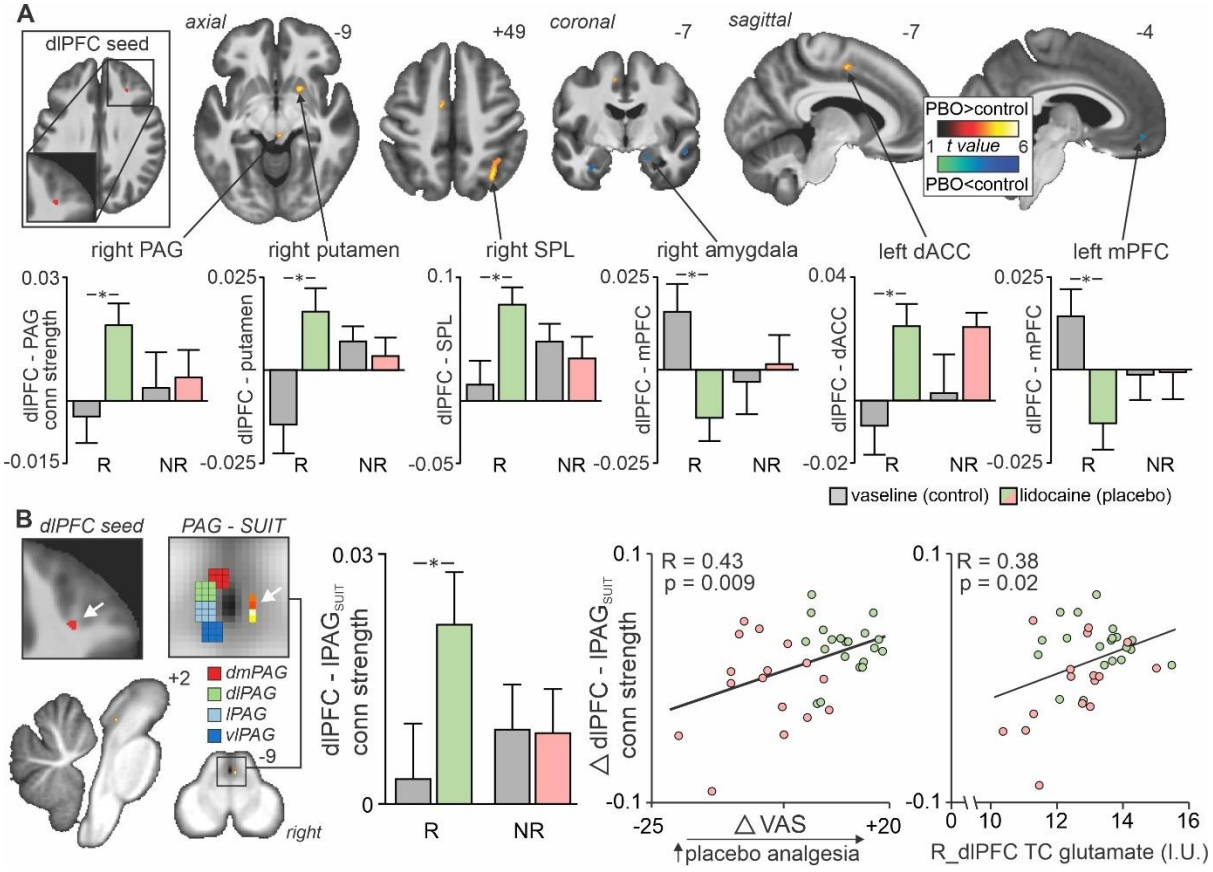

Figure 4. Changes in functional coupling with the right dorsolateral prefrontal cortex (dlPFC). **A:** Brain regions in which functional coupling with the right dlPFC was significantly different between stimulation of the placebo "lidocaine" treated site relative to stimulation of the control vaseline site. Clusters are overlaid onto an individual T1-weighted anatomical image. Slice locations in Montreal Neurological Institute space are indicated at the top of each slice. Below are plots of mean (±SEM) dlPFC connectivity strengths during stimulation of Vaseline and lidocaine creams and

split between responder (R) and non-responder groups (NR). *p<0.05. **B:** Plots of connectivity strengths between the dlPFC and midbrain periaqueductal grey matter (PAG) against placebo analgesia, pain percept variability (precision) during the stimulation of the placebo-treated site during the conditioning phase, and dlPFC glutamate concentration. dACC: dorsal anterior cingulate cortex; mPFC: medial prefrontal cortex; SPL: superior parietal lobule; TC: tissue corrected.

Chapter 4: Summary

The data presented throughout this chapter indicate that a Bayesian framework of placebo analgesia is indeed likely, and that information processing at the level of the dlPFC is involved in the process of converting accurate representations of pain reduction learned throughout conditioning procedures into placebo effects. In support of our initial hypothesis, we identified that placebo responders demonstrated significantly more precise pain rating responses through the conditioning phase of the experiment relative to nonresponders. Pain rating variability additionally correlated with the magnitude of placebo analgesia, suggesting a less binary influence and more graded interaction between how variable an individual perceives pain and their ability to mount endogenous analgesic responses.

Interestingly, whilst we were unable to detect any significant difference in metabolite concentration at the level of the dlPFC between placebo responders and nonresponders, the concentration of dlPFC Glutamate showed a significant and inverse interaction with conditioning pain rating variability – such that more precise pain responses were associated with greater levels of Glutamate within the dlPFC. This result led to the discussion that whilst biochemical composition of the dlPFC may not directly relate to the manifestation of pain-modulatory effects, it *may* relate to adjacent variables which do – such as precision in pain perception. Functional analyses revealed greater dlPFC-PAG coupling in responders relative to nonresponders, and that this connection correlated with both pain rating variability during conditioning as well as the magnitude of placebo analgesia. Together, these findings provide a potential route that associations between a placebo and pain reduction learned throughout conditioning could interact with descending pain modulatory circuits to aid in matching expectations to perception. Additionally, placebo-related changes in signal intensity were observed in the amygdala and posterior cingulate cortices (PCC) – two major nodes in the Default Mode Network (DMN) which is responsible for memory retrieval and associative learning. Combined, we suggest that during placebo analgesia, active integration of prior experiences occurs, and if these priors are precise, an prediction-error signal led by the dlPFC and constituents of the DMN contact the PAG to drive modulatory responses.

Chapter 5:

General Discussion: limitations, conclusions, and future directions

"Yesterday is history, tomorrow,
is a mystery, but today is a gift.
That's why it's called the present"
– Master Oogway, 2008

5.1 Summary of findings

The onset of this thesis presented three overarching aims, each of which was explored experimentally throughout chapters 2-4. Aim 1 was to establish the functional role of known and presumed pain modulatory nuclei of the brainstem during the manifestation of placebo and nocebo responses; Aim 2 sought to identify the cortical projection patterns to the midbrain PAG during placebo analgesia, and how these connections work to drive perceived pain relief; and Aim 3 investigated whether a detectable biochemical substrate within the cortex underpinned an individual's ability to mount pain modulatory responses.

Chapter 2 presented our findings of brainstem involvement in pain modulatory phenomena. We utilized a well-described within-subjects response conditioning model for invoking placebo analgesia and nocebo hyperalgesia (Voudouris et al., 1990, Freeman et al., 2015, Schienle et al., 2018, Egorova et al., 2019). Building on this model, we leveraged 7T-fMRI and the SUIT imaging toolbox to isolate BOLD signal within discrete brainstem nuclei and compared their activation patterns between individuals that successfully and failed to mount either placebo or nocebo responses. The responder to non-responder ratio was reasonable and matched previous investigations, with 36% and 56% of participants classified as placebo and nocebo responders, respectively (Beecher, 1955, Levine et al., 1979, Benedetti, 1996, Schmid et al., 2015). Investigation of demographic and expectancy data revealed no interaction between age, gender, or expectations of pain relief or enhancement, suggesting we had triggered primarily conditioning-based endogenous pain modulation.

A voxel-by-voxel group analysis revealed largely that activation in responder groups occurred within known pain modulatory nuclei shown throughout preclinical studies: the PAG, RVM, LC, and PB. Additionally, in a number of these nuclei we found significant interactions with the magnitude of pain modulation elicited – although largely in opposite directions between the two phenomena. For instance, the activation within the PAG-RVM system was paralleled depending on whether placebo analgesia or nocebo hyperalgesia was elicited. Interestingly, the rostral ventrolateral medulla (RVLM) and subnucleus reticularis dorsalis (SRD) - which plays a key role in the manifestation of Conditioned Pain Modulation (CPM) - only demonstrated activation during placebo analgesia. This suggested a more complex brainstem involvement underpinned conditioning-based endogenous pain relief, centred around a medullary system identified as an interconnected "triad" by Martins and Tavares (2017). This system collectively plays a role in experimental animals balancing anti- and pro-nociceptive responses to face threatening events, and receives extensive projections not only from the PAG, but from several higher brain

structures such as the hypothalamus and amygdala - which we also demonstrate play a key role in this phenomenon throughout chapter 3. A final discovery from this investigation was the pronounced and similar increased activation within the SN, shared between both placebo analgesia and nocebo hyperalgesia. For the first time we demonstrated brainstem involvement of the dopaminergic system underlying both pro- and anti-nociceptive endogenous pain modulation. We concluded these activations likely reflected increased cortical supply of this neurotransmitter, driving processes such as learning signals, reward, aversion, and error-prediction – all of which are key to establishing a brain state necessary to mount these two phenomena (Matsumoto and Hikosaka, 2009, Pauli et al., 2015, Henderson et al., 2020).

In Chapter 3, instead of considering activation, we instead extended our previous work by determining which cortical areas changed in the way they communicated with the PAG during placebo analgesia. Since the effectiveness of the conditioning paradigm was the only discernible factor revealed in Chapter 2 underlying greater analgesic and hyperalgesic expression, we hypothesised that differences in the recruitment of descending analgesic pathways - which originate in the PAG – would provide another characteristic which differed between placebo responders and non-responders. We extended our dataset for this investigation, and surprisingly elicited a greater response rate (48%) in our 47 healthy control participants. By combining connectivity and DCM analyses, we identified two distinct neural systems contacting the PAG during placebo analgesia: the first stimulus dependent and consisting of frontotemporal brain regions responsible for the cognitive-evaluative component of pain perception, and the second stimulus independent, consisting of limbic subregions long believed to exert regulatory control over brainstem modulatory output. Our results provided a unique perspective on how descending analgesic pathways are recruited during conditioning-based placebo analgesia. Whilst the well-established descending projection from the rACC to PAG indeed mediated the classification of an individual as a responder or non-responder to placebo, we demonstrated that a broad cortical system is at play in the lead up and when faced with noxious events, leading to endogenously manifested inhibitory effects on pain.

Finally, in Chapter 4, we aimed to identify if any underlying differences in biochemistry related to an individual's ability to mount a pain modulatory response. Since the right dlPFC specifically has been the elected region of neuromodulatory efforts to alter the expression of placebo analgesia and nocebo hyperalgesia, we focussed our analysis on this region (Krummenacher et al., 2010, Tu et al., 2021). By combining both ^{1}H-MRS and fMRI, we conducted a multimodal assessment of the dlPFC's role in the expression of placebo analgesia. We originally considered

a null result since no biochemical differences could be identified between placebo responders and non-responders. However, upon investigating the lead-in phase of the experiment (i.e. conditioning), we found a unique interaction between dlPFC glutamate, pain rating variability, and analgesic expression – such that individuals that were more consistent in rating their pain during this phase had both greater dlPFC Glutamate and placebo responses. Functional interactions were also identified between several structures identified in chapters 2 and 3, including the PAG, rACC, and amygdala – suggesting that a supporting role of the dlPFC in mounting strong stimulus-response relationships tied in with circuits we identify as driving pain modulatory effects.

Together, data presented throughout chapters 2-4 outline a number of key advancements to the field of pain modulatory phenomena and provoke a number of questions – each of which are discussed in the following sections.

(1) How important is it to correctly dissect responder and non-responder groups in experimental designs?
(2) What are the core brainstem circuits underpinning these responses, and how could we assess their specific roles?
(3) What is the mechanism by which conditioning cues become pain modulatory phenomena?
(4) Does there exist a neural model which can be used to assess an individual's likelihood of demonstrating conditioning-based pain modulation?

5.2 The importance of determining responders in experimental pain modulation

We have known since Henry Beecher's seminal meta-analysis that the ability to mount pain modulatory phenomena is not ubiquitous, and as described in section 1.3 of this thesis, their rate of expression varies between experimental designs, environmental settings, and the nature of noxious stimuli being presented. Despite countless reports describing the distinct representations of placebo and nocebo responses between individuals, to date there exists no consensus on how to statistically delineate responder groups to these phenomena. Experimental pain studies in the past have utilized several methods – including median splits and arbitrary cut offs in reported pain (Zubieta et al., 2005, Hashmi et al., 2014). These methods collectively suffer from two striking problems. First and foremost - median splits cannot be effectively

utilized if less than 50% of participants report a reduction in pain. Since positive placebo effects are found in 30-50% of participants in clinical placebo-controlled trials (Price et al., 2008), the decision to implement a median split based on changes in pain ratings could mean the classification of a responder with minimal reduction if not an increase in reported pain. Second, whilst arbitrary pain intensity cut-offs may appear well supported throughout the literature, the values used differ greatly between studies. Where Todd et al. (1996) found that a 13 point reduction on an 100 point VAS scale constituted a clinically relevant change in acute pain, this cut-off appears to vary dependent on the environmental setting and nature of pain being investigated (Jensen et al., 2015, Olsen et al., 2018). What neither of these methods consider however is the influence of baseline pain processing in the generation of placebo or nocebo responses. Indeed, the variability of pain processing can influence stimulus-response relationships (Grahl et al., 2018, Zaman et al., 2021), and, as demonstrated in Appendix B, pain rating variability directly relates to altered neural activation within the same frontotemporal regions that principally encodes pain modulatory phenomena – the dlPFC.

All three of our investigations instead utilized a novel approach for delineating responders from non-responders which both conformed with statistical cornerstones and accounted for an individuals' inherent variability in pain processing. The two-standard deviation band method, as described by Nourbakhsh and Ottenbacher (1994), is a method for generating the normally-distributed gaussian curve for statistical significance using single participant, multi-trial data. By calculating the mean and standard deviation across a baseline series (i.e. stimulation of the control cream site), and then comparing the average pain response in a subsequent experimental series (i.e. stimulation of the placebo / nocebo cream sites), this method can determine whether or not a participant has fallen more or less than two standard deviations away (equivalent to $p<0.05$) from their nociceptive baseline through intervention with a placebo or nocebo treatment (Figure 5.1). As previously reported by Cragg et al. (2016), in chronic pain patients increased baseline pain variability relates to a reduced placebo response across a breadth of conditions. As such, leveraging pain processing variability to determine responders from non-responders provides additional benefit by bridging experimental designs into a clinically relevant landscape.

We additionally employed individualized thermal sensitivity calibration prior to experimental proceedings, as well as a custom pain rating procedure inside the MRI which allowed participants to rate their ongoing pain level throughout the entire scanning protocol. Together, these methodological decisions bolstered our ability to accurately assess each individual's pain

experience and modulatory ability with high specificity and sensitivity. Indeed, there is not one placebo (or nocebo) response, but many, and the circuits described in the following sections represent only some of many potential neural systems an individual may be calling upon to drive an endogenous modulation of pain. However, in order to provide clear hypotheses and avenues for future testing, it is critical that these responses are accurately assessed, and that a distinction is made to be able to correctly compare those that successfully and fail to mount pain modulatory phenomena.

Our findings from chapters 2 and 3 show that both placebo and nocebo responders engage a different brainstem and cortical circuitry than is observed in non-responders – with altered activation in core structures such as the PAG and rACC that broadly support our classification of individuals based off changes in pain relative to baseline variability. Chapter 4 provides evidence that baseline pain percept variability directly influences an individual's ability to mount pain modulatory phenomena. Given that we likely triggered a conditioning-based analgesia and hyperalgesia, it is unsurprising that pain variability played such a considerable role, as forming strong associations between the conditioning stimulus and associated responses sits at the crux of the classical conditioning design. The repetition of our conditioning trials and two-day design likely also favoured conditioning-based pain modulation, as both the number of learning trials and temporal proximity of the stimulus-response relationship has shown critical in the formation of conditioned responses (Colloca et al., 2010, Eelen, 2018).

The findings from all three of our experimental chapters offer additional insight into the interplay between expectation and conditioning for the manifestation of placebo and nocebo responses. In all cases, regardless of whether a participant was classified a responder or non-responder, they maintained a strong expectation that their pain would be significantly modulated by the application of placebo and nocebo creams. Modern explanations of pain modulatory phenomena interpret their manifestation as a Bayesian-brain phenomena, such that sensory inputs (likelihood) are updated in line with expectations and learned responses (priors) to produce a perceived change in pain (posterior). As Pagnini et al. (2023) describes, two general processes can occur in a placebo trial: "perceptual" and "active" inference – that is, an individual either updates their expectations to match incoming sensory information (i.e. a non-responder) or recruits neural systems to modify incoming sensory information in-line with expectations (i.e. a responder). Our data align with this theory in two ways: first, we suggest that active inference more easily occurs when strong associations are made between conditioning stimulus and conditioned response (less baseline pain variability), and it is not necessarily bound to

expectations alone. Second, echoing the seminal study from Krummenacher et al. (2010), we suggest a trait-like quality of mounting active inference – which can aid in successfully mounting pain modulatory phenomena, driven by the dlPFC in concert with cognitive-associative cortical sites and pain modulatory nuclei of the brainstem.

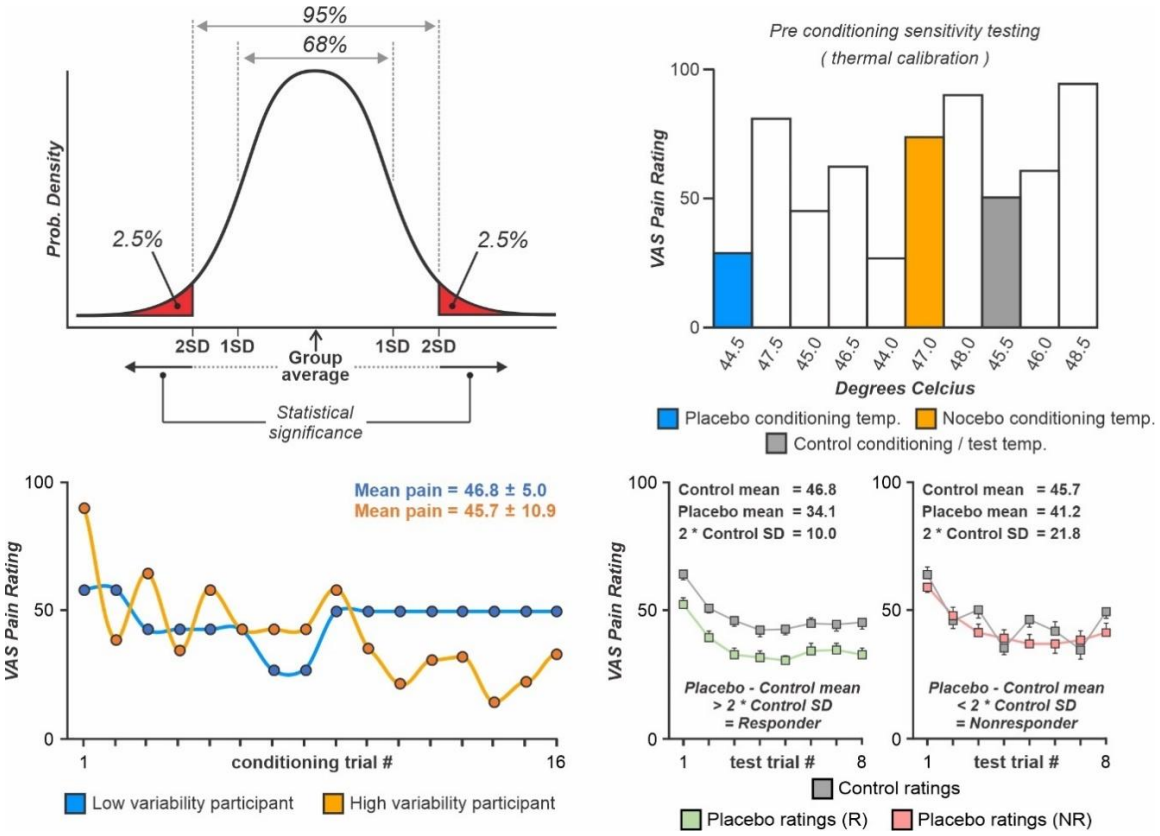

Figure 5.1 Delineation of responder and non-responder groups. The normally distributed gaussian bell curve has become a statistical mainstay, capable of detecting beyond change the likelihood of a significant effect in group-level data. Borrowing from this notion, our experimental design employed thermal sensitivity testing and an ongoing rating system to determine accurately an individual's pain responses as well as inherent variability across the multi-trial design. Indeed, by solely asking for a mean pain rating two individuals may report a similar pain experience but show disparate fluctuations in this experience across time. A low and high variability participant data across the conditioning phase is shown in the lower left plot – clearly demonstrating that despite having similar mean pain levels, the variance in these reports can greatly differ. During testing a two standard deviation band method is employed, effectively recreating the gaussian curve in single participant data, and enabling an accurate assessment of

in an individual has indeed demonstrated a greater than statistical chance change in their pain report (i.e. demonstrated a significant placebo or nocebo response). *VAS = visual analogue scale, SD = standard deviation, R = responder, NR = non-responder.*

5.3 A cortico-brainstem schema for top-down pain modulation

5.3.1 Brainstem involvement in anti- and pro-nociceptive contexts

As discussed in Chapter 1, the role of the PAG-RVM system in facilitating anti- and pro-nociceptive states via direct innervation of the DH has long been described in experimental animals and in humans (Mantyh, 1983, Lovick, 1993, Helmchen et al., 1995, Heinricher et al., 2009). Indeed, experimental evidence also existed when beginning this thesis that this was the primary pathway involved in the development of placebo analgesia and nocebo hyperalgesia (Bingel et al., 2006, Eippert et al., 2009a, Tinnermann et al., 2017, Makovac et al., 2021). What was less understood however, was the precise region of the PAG that was involved and the role of adjacent medullary, pontine, and midbrain sites which also contain the necessary neurochemicals to enable cognitive and emotional processes involved in the modulation of pain. Our results indicate that rather than relying on one single descending pathway to produce these phenomena, multiple brainstem sites are intrinsically involved in the production of pain modulatory effects – with four primary candidate systems emerging. These consist of dopaminergic and noradrenergic components, the parabrachial complex, and the lateral PAG-RVM axis. Inviting future discussion, below presents our interpretation of how these four systems coalesce to dynamically influence the pain percept during placebo analgesia and nocebo hyperalgesia.

The PAG-RVM axis: is undoubtedly the most well-documented and described descending pain modulatory pathway of the brainstem and has become a cornerstone in pain research since its first discovery in the late 1980's (Fields and Heinricher, 1985, Heinricher et al., 1989). The central aim of this thesis was not to demonstrate once again that this is the primary system via which placebo analgesia and nocebo hyperalgesia are potentiated - although we did successfully identify this - but rather indicate specifically which components of this system are driving these phenomena. Utilizing the enhanced spatial acuity offered by 7T-MRI, we were successfully able to discern BOLD signal change within each of the PAG's four functional columns – which contain the necessary substrate to produce profoundly different behavioural and modulatory effects in response to pain (Bandler and Keay, 1996, Keay and Bandler, 2001). For instance, the

ventrolateral PAG column produces freezing behaviours and an opioid-dependent analgesia; whereas the lateral and dorsolateral PAG columns initiate active-coping (fight or flight) responses, accompanied by an opioid-independent analgesia. Despite being neurochemically separate, both systems exert their pain modulatory influence via the RVM – although sparse direct-spinal projections exist within the PAG as well. The initial findings of chapter 2 strongly support the involvement of a non-opiate based system centred on the lateral PAG column for the generation of conditioning-based placebo analgesia and nocebo hyperalgesia.

By association, these results suggest that upstream sites including the rACC and hypothalamus are capable of selectively contacting specific PAG columns to trigger pain modulatory effects dependent on the required analgesic/hyperalgesic effect for any given stimulus. The likely mechanism of action producing these effects is endogenous cannabinoids – as non-opiate conditioning based analgesia has been shown to be sensitive to Rimonabant (a CB1 receptor antagonist), and a large concentration of CB1 receptors are isolated to the dorsal aspect of the PAG (Millan, 2002, Palazzo et al., 2010). Indeed, the antinociceptive action of endogenous cannabinoids have also been shown within the amygdala, and a large concentration of CB1 receptors are found in both the hypothalamus and ACC – with the action of CB1 in the latter contributing to analgesia via disinhibition of PAG output neurons (Martin et al., 1999, Iversen, 2003, Connell et al., 2006, Lau and Vaughan, 2014).

Whilst chapter 2 established the involvement of the PAG-RVM axis in both these phenomena – being recruited in opposing manners to produce pain relieving and enhancing effects, respectively, questions remained surrounding which cortical networks were selectively modulating the activity within this PAG subregion to elicit changes in perceived pain. Combining the results from Chapters 3 and 4 allowed us to determine a twin network cortical model of placebo analgesia, specifically demonstrating which connections were driving inhibitory effects on pain via PAG-dependent signalling. Whilst these investigations were only performed using a placebo substance, supporting literature allows us to postulate that these same connections may be recruited to drive hyperalgesic phenomena.

Substantia Nigra: Throughout conditioning, learned associations are made between the conditioning stimulus and behavioural response – be it appetitive (pain reduction) or aversive (pain enhancement) in nature. These associations must then be actively recalled, triggering the conditioned response (placebo or nocebo effects). It is possible that this recall is encoded by ascending dopaminergic connections from the SN to its upstream targets. Indeed, two of the primary recipients of brainstem-released dopamine are the ventral striatum – including the NAc,

and the prefrontal cortex (Büchel et al., 2014, Li et al., 2018, Harris and Peng, 2020, Islam et al., 2021). As revealed in chapters 3 and 4, not only are these two structures integral in the development of stronger placebo analgesia, but activity and biochemistry of the dlPFC specifically relates to an individual's ability to form stronger stimulus-response relationships. Scott et al. (2008) in discovering that ventral striatum dopaminergic responses differentially encoded placebo and nocebo effects suggested that this neurotransmitter was necessary to "trigger" downstream adaptive responses. The results across our investigations not only support but bolster this finding – reinforcing the role of the NAc in feeding into the rACC-PAG circuit, but also suggest that the supply of cortical dopamine producing these effects originates primarily within the SN.

Locus coeruleus: also known as the A7 adrenergic cell group, plays a fundamental role in cortical noradrenaline supply, and has also been shown to send direct spinal efferents which can modulate nociceptive processing and potentiate pain chronicity (Taylor and Westlund, 2017, Munn et al., 2021). Beyond pain processing, cortical noradrenaline has also been implicated in several cognitive processes including hypervigilance, learning, and executive-control – making a clear determination of the role in placebo analgesia and nocebo hyperalgesia difficult to disentangle. However, combining Hirschberg et al. (2017) recent preclinical findings with the observation that we observed *contralateral* LC activity relating to placebo and *ipsilateral* LC activity relating to nocebo allows us to speculate as to the nature of spinal- and cortical-influence exerted by the LC to potentiate these divergent pain modulatory effects.

A clear ipsilateral (to side of noxious input) predominance exists in spinally-projecting LC neuronal activity – suggesting that the change in activation we observe during nocebo hyperalgesia likely reflects reduced drive from the LC in selectively suppressing nociceptive excitation within the DH, known to occur via α2-adrenoreceptor binding within outer laminae (Llorca-Torralba et al., 2016). Dissimilarly, the LC sends efferent projections to several prefrontal sites responsible for threat processing and avoidance behaviours which are important for appropriate action selection during noxious events. The reduction in LC activation during placebo analgesia may indicate a reduction in global noradrenergic supply to these cortical sites, encoding the rewarding nature of experiencing a placebo response.

Whilst these lateralized hypotheses are plausible, an alternate explanation is tied with the role of cortical noradrenaline and its effects on extinction learning. Given that both responder and non-responder groups expected reduced/enhanced pain by administration of our inert substances, a "non-responder" to these phenomena could be considered to undergo a rapid

extinction of expected outcome (reduced or enhanced pain). Indeed, our primary results within the LC was that activity inversely correlated with the magnitude of both phenomena, meaning both non-responder groups demonstrated the greatest increase in LC activity. In experimental animals, adrenergic mPFC and infralimbic (rACC homologue) projections originating within the LC promote both aversive and appetitive extinction – the process of eliminating the tether between the conditioning stimulus and conditioned response (Mueller et al., 2008, Latagliata et al., 2016). In contrast, chemogenetic de-activation of this same projection pathway prologues the process of extinction (Uematsu et al., 2017). Whether the changes in LC activity we observe relates to different spinal- and cortical-projection pathways or indeed encodes a shared reduction in the rate of extinction tied with these phenomena would be an interesting route of future investigation.

The parabrachial complex: receives both ascending nociceptive signals from lamina I DH neurons via the spinoparabrachial tract and drives descending modulation via direct spinal projections and additional relay with the RVM (Hunt and Mantyh, 2001, Stroman et al., 2021). Its involvement in pain processing is well-described, relaying ascending nociceptive information to both dopaminergic sites of the midbrain to encode the initial response to pain (Coizet et al., 2010), as well as forming the start of a top-down circuit between the PB-hypothalamus-PAG which is believed to be involved in the emotional-affective dimension of pain processing, generating behavioural responses when presented with a potentially noxious event (Bester et al., 1999, Puopolo, 2019). An additional projection from the PB terminates within the amygdala, which has been shown in both humans and experimental animals to be critical in memory-formation, storing information about the nature of a noxious event which can persist beyond the administration of a stimulus (Kissiwaa and Bagley, 2018, Chen and Heinricher, 2019a).

In addition to ascending transmission, the PB holds modulatory influence directly over the RVM, and is capable of eliciting both pro- and anti-nociceptive states by directly modulating the net balance of activity across RVM ON and OFF cells, respectively (Chen et al., 2017). When beginning this thesis, it had been shown that brainstem circuitry including the PB was involved in mounting CPM responses in humans, however no studies had resolved its involvement in other modulatory phenomena despite its likely relevance (Youssef et al., 2016). In direct support of the PB's role in establishing a hyperalgesic state, we showed greater PB BOLD signal changes related to greater nocebo responses, which accompanied a net reduction in RVM activation during this phenomenon – suggesting a diminished OFF cell activation and producing a pro-nociceptive state (Chen and Heinricher, 2019b).

During placebo analgesia however, not only did we observe PB signal changes, but an additional modulation of the ascending circuitry which the PB projects to relative to the PAG. Both the hypothalamus and amygdala were shown in Chapter 3 to form part of our "stimulus independent system", suggesting that it may be via a modulation of ascending nociceptive transmission initiated by the PB that establishes this cortical circuitry, leading to a change in excitatory-inhibitory balance in the PAG and significant placebo analgesia. Indeed, by employing a multi-viral optogenetic model, Chiang et al. (2020) established that efferent projections from the PB to the amygdala, hypothalamus, and lateral PAG column encoded escape-like behaviours and aversive learning in a mechanical hypersensitivity model. This circuitry is remarkably similar to what we describe in Chapter 3, providing further evidence that the PB may be involved in the establishment of the stimulus independent system underlying placebo analgesia. Dissecting the relative involvement of ascending and descending connections of the PB during experimentally altered pain responses would be a crucial next step in better understanding how cortico-brainstem loops dynamically potentiate endogenous pain modulation.

5.3.2 A twin network model for placebo analgesia

Identifying the core pathways which trigger analgesic effects through the recruitment of brainstem pathways has influenced experimental investigations for decades. Their categorization not only offers insights into the human ability to modulate our own pain in threatening or painful settings, but also could allow for the development of novel treatments which exploit or leverage these mechanisms in acute or chronic pain states. Importantly, the circuits we reveal in Chapters 3 and 4 which contact the brainstem to drive placebo analgesia not only concord well with preclinical and human imaging studies investigating pain modulatory phenomena, but also shed light on novel mediating factors which subsist the inhibitory effects exerted by brainstem nuclei on the DH.

Stimulus-independent system: As pain perception is itself multifaceted, convolving cognitive and emotional factors with any true sensory input – it is not a long bow to draw that modulating perceived pain involves neural components outside the time window of applied sensory input. Anatomical tract-tracing performed in experimental animals throughout the late 1990's established the regulatory role of infralimbic and limbic subcomponents in maintaining the homeostatic and autonomic balance of the PAG (Ongür et al., 1998). Functionally, stimulation of the mPFC in animals causes significant shifts in threat and pain responses mediated by

monosynaptic pathways directly to the amygdala and hypothalamus, which in turn project to the PAG (Quirk et al., 2003, Enck et al., 2008, Taylor et al., 2019). Whilst this circuit includes reciprocal connections between the mPFC, hypothalamus, amygdala, and PAG – it appears that the crucial connection for maintaining nociceptive balance is the hypothalamic-PAG pathway. Indeed, ablation of posterior hypothalamic nuclei in rodents causes shifts in baseline pain responses, and disrupting hypothalamic function through opioid injection elicits behavioural analgesia (Millan, 2002, Holland and Goadsby, 2007).

These findings largely support that the hypothalamus plays a regulatory role in pain perception, allowing for the accurate assessment of pain to learn and adapt to our environment. By association, they suggest that the baseline state of this pathway is to tonically inhibit analgesic properties of brainstem nuclei, i.e. a stimulus independent system. This top-down circuit has additionally has been linked to pain in humans – since nocebo hyperalgesia is associated with an upregulation of adrenocorticotropic hormone and a hyperactivity in the hypothalamic-pituitary-adrenal (HPA) axis, and connectivity between the hypothalamus and PAG predicts pain rating responses in periods prior to noxious stimuli application (Frisaldi et al., 2015, Stroman et al., 2018). The identification of our stimulus-independent system supports that these limbic-brainstem pathways are present in humans as they are animals and proposes that they play an important role in setting the excitatory tone of brainstem nuclei to elicit pain modulation. Whilst this system included the mPFC, rACC, amygdala, hypothalamus, and PAG, DCM confirmed that it is the hypothalamus-PAG pathway that best delineated significant analgesic responses. With the gold standard of placebo research now turning to identifying underlying properties of placebo responders, our results suggest that baseline coupling between these two regions could be used to determine the likelihood of an individual exhibiting pain modulatory responses.

The dlPFC also emerged as altering its ongoing connectivity with the PAG – a finding which does not fit with the defined circuitry regulating autonomic and homeostatic processes in experimental animals. Whilst it is possible that in humans this circuitry extends to incorporate the dlPFC, Chapter 4 revealed a potential different role for the dlPFC-PAG connection that likely explains why this region was found in the stimulus independent system. In order to process and express perceived pain at any given time, it is integral that cognitive-associative areas like the dlPFC can tap into descending analgesic circuitry to maintain contextual information and attention to the environment, ultimately either matching expectation to experience or vice versa (Büchel et al., 2014). Since non-invasive disruption of the dlPFC blocks the expression of

placebo analgesia but holds no effect on the pain experience (Krummenacher et al., 2010), this connection likely runs parallel to the stimulus-independent system, and underpins an individual's ability to internalize and process their shifts in perceived pain experience on an ongoing basis – forming part of an "error-prediction" conditioning circuit.

Stimulus dependant system: With the error-predictive circuit and stimulus-independent systems serving roles in constant monitoring of brainstem systems and establishing the excitatory tone necessary for endogenous pain modulation, a set of regions is still required to drive the output in the brainstem to cause an effect across the DH. This role we propose is served by our stimulus dependent system – the collection of regions which altered in PAG coupling specifically during noxious stimulus application. It is widely regarded that the rACC plays an executive role in recruiting brainstem pathways emanating from the PAG, since the effects of naloxone on abolishing placebo analgesia are also associated with an attenuation of coupling between these two sites. rACC-PAG coupling however appears to be capable of dynamically altering perceived pain, since greater nocebo effects are also associated with this connection, as well as increased DH activity (Tinnermann et al., 2017).

Consistent with these findings, the results from Chapter 3 demonstrate that significant placebo responses relative to PAG activity are mediated by rACC-PAG coupling during noxious periods, and DCM analyses further confirmed the top-down directionality of this connection. Two other cingulate components: the MCC and dACC were also found to alter their PAG coupling during stimulus periods – but neither were found to directly contact the PAG in our DCM analyses. These findings align with the role of these cingulate subregions serving executive over pain-modulatory roles (Vogt, 2005, Cavanagh and Shackman, 2015). Specifically, it is believed that whilst their activation is sensitive to pain, their function during pain is to provide a behavioural alert during salient events, triggering cognitive processes and guiding action selection.

Network neuroscience has established the blueprint of the brain's salience network, which encompasses both the dACC and AI – another region demonstrating stimulus-dependent increases in PAG coupling (Seeley et al., 2007). Like the dlPFC's inclusion in the stimulus-independent system, we suggest that placebo-related increases in PAG connectivity observed in the dACC, MCC, and AI suggest that significant placebo analgesia is hallmarked by an upregulation in connectivity between major nodes of the salience network. Rather than directly signalling brainstem pathways, greater inter-network connectivity between salience network nodes indicates placebo responders' ability to integrate incoming noxious stimuli with their conditioned beliefs (Sikora et al., 2016). When these two do not match, the salience network

enlists the rACC to recruit the PAG and drive changes in perceived pain. The final region revealed in our stimulus-dependent system was the NAc, the cortex's dopaminergic hub that encodes reward-responsivity. In both preclinical and clinical models deep brain stimulation of the NAc has shown to elicit analgesia, and our findings suggest that this capability comes from direct modulation of the rACC-PAG pain modulatory connection (Segal and Sandberg, 1977, Mallory et al., 2012, Harris and Peng, 2020).

Overall, our major findings across the three publications included in this thesis provide coverage of the major brain systems recruited during placebo analgesia during acute thermal stimuli. These brain systems represent neural activation in the cortex and brainstem – with brainstem involvement also being shown during nocebo hyperalgesia, an error-prediction system, and both a stimulus dependent and independent system for recruiting brainstem pathways (Figure 5.2).

Admittedly, these systems collectively cover a substantial area of the cortex and brainstem, making it difficult to precisely identify the essential components responsible for inducing a change in perceived pain. What would be more beneficial is simplifying this schema, looking for overlap and interpreting which are the necessary connections that can be experimentally evaluated in the future for their involvement in endogenous pain modulation. To accomplish this objective, we introduce the core conditioning-based circuit of human pain modulation, which comprises a subset of these systems with the required components for producing endogenous pain modulation.

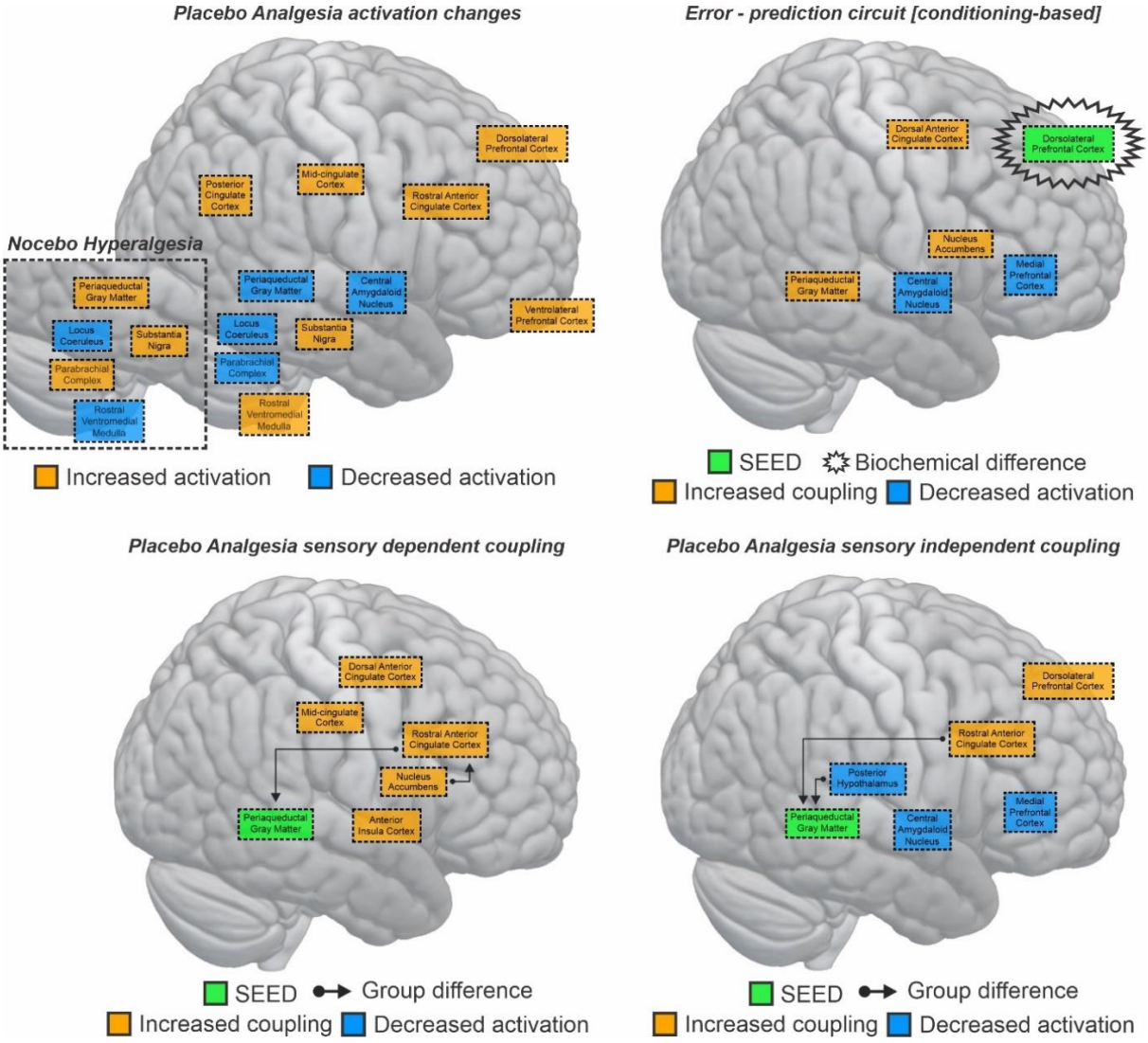

Figure 5.2 The basic neural circuitry of conditioning-based pain modulation. These renderings record each of the circuits unveiled within the three experimental chapter of this thesis. A strikingly similar group of brainstem nuclei were found to alter in activation during both analgesic and hyperalgesic pain modulation – centred on the PAG-RVM-DH pathway with contributing influence from neighbouring noradrenergic (LC) and dopaminergic (SN) sites. Within the cortex, successful elicitation of placebo analgesia related to increased activation within the prefrontal and cingulate cortices, and a reduction in activity within the central amygdaloid nucleus (CeA). Chapter 4 revealed a role of the dlPFC in encoding pain rating variability, which, via coupling with the brainstem and a cortical circuit similar to that described by Wager et al. (2008) responsible for emotional regulation (VS-mPFC-CeA), also relates to an individual's ability to produce placebo analgesia. The parallel top-down circuits that control brainstem output via the PAG were found to comprise of frontotemporal sites which were also

activated by placebo (stimulus dependent), as well as limbic sites (stimulus independent), such as the MeA and mPFC which when combined with activation and error-prediction maps now represent a triplet of roles: emotional processing, encoding stimulus-response relationships, and regulating the sensitivity of core brainstem centers.

5.4 Placebo and nocebo – a coin with two sides or just a common face?

5.4.1 The core conditioning-based circuit of pain modulation

Despite being elicited from strikingly similar experimental procedures (expectation generation and conditioning cues), prominent early literature disputed that placebo analgesia and nocebo hyperalgesia existed as "two sides of the same coin" (Freeman et al., 2015). Indeed, their neurobiological underpinnings (placebo = opioid/cannabinoid; nocebo = cholecystokinin) and associated neural activation patterns have been shown to be disparate, with recent meta analytic approaches confirming distinct neural circuits underpin the two phenomena (Fu et al., 2021, Zunhammer et al., 2021). However, whilst *cortical* activation patterns may differ between these phenomena, our primary finding of Chapter 2 demonstrated that common *brainstem* regions play a role in their generation. Within the PAG-RVM system and the PB we found opposing engagement during placebo analgesia and nocebo hyperalgesia – reflecting their opposite effects on pain processing. Additionally, whilst neural activity may differ between phenomena, a common descending connection between the rACC-PAG has shown to relate to both placebo analgesia and nocebo hyperalgesia (Eippert et al., 2009a, Tinnermann et al., 2017), and non-invasive stimulation of the dlPFC can modulate the intensity of both phenomenon (Krummenacher et al., 2010, Tu et al., 2021). Indeed, our results largely support these previous findings, with the novel discovery of a stimulus-independent circuit that sets the gain of the brainstem to a responsive state enabling top-down modulation. Combining the results within this thesis and drawing on supporting literature regarding cognitive processes involving the dlPFC-PAG reciprocal connection and functional connectivity underlying nocebo hyperalgesia – we would argue that these two phenomena may not be two sides of the same coin, yet share cortico-brainstem similarities, at least within the context of conditioning-based pain modulation in response to acute noxious stimuli. Pairing back the four systems revealed throughout this thesis to their most integral components, we propose that the core conditioning-based circuit for acute pain modulation consists of four components (Figure 5.3):

- Acute noxious input entering from the body is transmitted in both the spinothalamic tract (activating the cortical pain system) and spinoparabrachial tract – which establishes the tone of the stimulus independent system (amygdala and hypothalamus) to associate certain stimuli with pain relief.
- The affective-homeostatic pathway which includes the parabrachial complex, and ascending projections to the central amygdaloid and posterior hypothalamic nuclei. These regions encode the affective value of noxious stimuli learned during conditioning, and descending input from these limbic structures to the PAG sets the excitatory-inhibitory balance of brainstem pathways to respond to future exposures of the same conditioning cues. To initiate placebo analgesia, a weakening of the hypothalamus-PAG connection emerges as particularly important in shifting the baseline tone of brainstem pathways emerging from the PAG.
- The expectancy regulation pathway reciprocally connects the dlPFC to the PAG, with the dlPFC storing information regarding an individual's expectations of physiological benefit or detriment – during test phases, these expectations are relayed to the PAG, and updated on an ongoing basis dependent on whether perceptual or active inference predominates. This pathway allows an individual to consistently track their pain, and report positive or negative perceptual effects.
- The pain modulatory pathway encompassing the rACC-PAG-RVM circuit is triggered when a noxious stimulus is applied, with top-down projections from the rACC modulating the activity in spinal- and RVM-projecting neurons within the PAG to produce dynamic changes across the DH. During acute pain modulation, the lateral PAG column is involved, and this circuit appears reliant on the affective-homeostatic pathway first establishing a tone within the PAG to allow for top-down modulation.

The benefit of establishing a core-circuitry involved in both storing and recalling conditioning-based effects to cause shifts in perceived pain lies in its simplicity. With the known between-study heterogeneity that exist in models of experimental pain modulation (Zunhammer et al., 2021), identifying precise connections that can be experimentally evaluated focusses study designs and provides a starting point for researchers aiming to better understand the neural mechanisms of pain modulation. Importantly, this model is anatomically possible – with countless studies affirming the top-down innervation of the PAG-RVM pathway from both the dlPFC and rACC with both structural and functional techniques (Sevel et al., 2015, Lui et al., 2021); as well as the terminal projections of the spinoparabrachial tract being recognised in

both experimental animals and humans. As a model this core circuitry also aligns well with established literature on the influence of conditioning on perceived pain – since disrupting the expectancy regulation pathway would cause a loss in the ability to report placebo or nocebo effects – which account for the findings from non-invasive stimulation. Similarly, even if the affective-homeostatic pathway was active, without recruitment of the rACC – for instance during pharmacological blockade via naloxone administration, there would be no driving influence over the PAG-RVM-DH pathway – resulting in no change in DH neurotransmission and ascending nociceptive information.

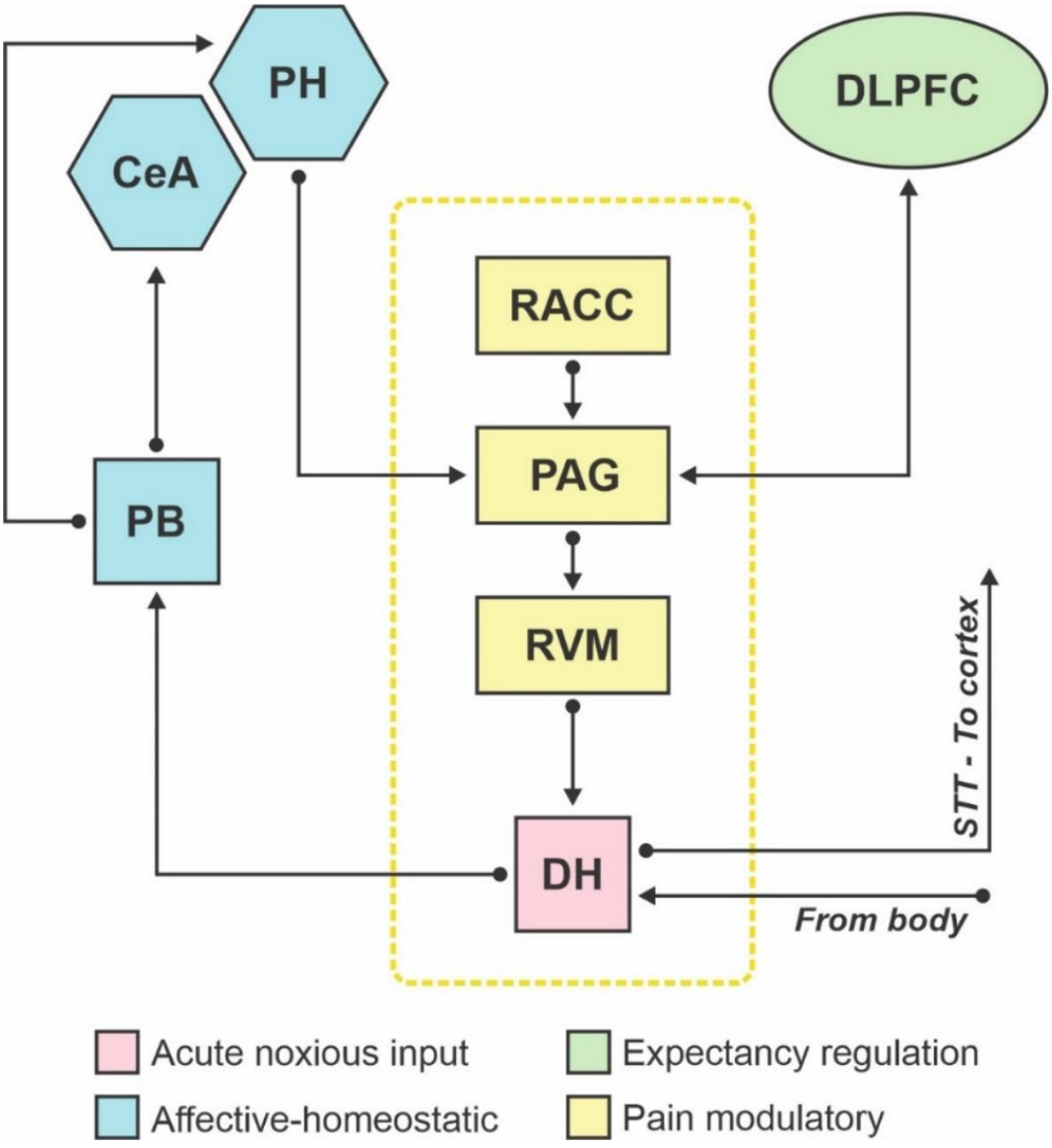

Figure 5.3 The core conditioning-based circuit for acute pain modulation. This circuit comprises of four components - each contributing to an individual's ability to call upon descending brainstem circuitry and dynamically modulate acute nociceptive transmission across the spinal cords dorsal horn. The first component is the noxious input itself, received by outer laminae and transmitted via either the spinothalamic tract (STT) – entering the cortex's sensory-discriminative or emotional-affective pain receptive regions, or via the spinoparabrachial tract, the origin of the affective-homeostatic component. This component, which includes the parabrachial complex (PB) and ascending connections to the central amygdaloid (CeA) and posterior hypothalamic (PH) nuclei encodes conditioning effects, representing an individual's past experiences with a "analgesic" or "hyperalgesic" substance, and sets the inhibitory tone of brainstem pathways to be modulated by the cortex via reduced innervation of the midbrain periaqueductal gray (PAG). The third component – the "expectancy regulation" pathway includes the dorsolateral prefrontal cortex (dlPFC), which responds to the expectancy of an individual's pain relief or enhancement, operating as a function of their certainty (variability) during conditioning. The dlPFC receives and sends input to the PAG to constantly update expectations towards noxious stimuli – with the participant either initiating perceptual or active inference, perceiving pain closer to their expected value or magnitude of actual incoming sensory information, respectively. Finally, the pain modulatory pathway consists of rACC-PAG-RVM-DH projections, with the cingulate contacting spinal- and medullary-projection neurons of the PAG to elicit adaptive changes in nociceptive transmission across the dorsal horn. Critically, we would argue, neither the action of the expectancy-regulation or pain modulatory components is achievable without the hypothalamus-PAG connection first relaxing the tone of brainstem analgesic pathways to establish a state optimal for pain modulatory effects to occur.

5.4.2 Future studies to probe pain modulatory circuitry

Whilst the core conditioning-based circuit contains the necessary components to dynamically elicit and maintain expectations and changes in perceived pain perception, this thesis only establishes its role in placebo analgesia, and only the core brainstem circuits were established for nocebo hyperalgesia. As such, the first investigation necessary to better evaluate whether this proposed circuitry may act independent to the directionality of nociceptive modulation would be to conduct connectivity and biochemical analyses as described in Chapters 3 and 4 during nocebo hyperalgesia. Indeed, if the projection from the hypothalamus-PAG is critical in establishing a modulatory tone in descending brainstem pathways, if this same connection is

involved in the generation of nocebo hyperalgesia it may be of clinical utility to investigate its pharmacological properties, which could be leveraged to promote placebo and attenuate nocebo effects following treatments with a high likelihood of their occurrence. Beyond establishing the core circuity's role in nocebo hyperalgesia, in the context of our findings and what remains to be explored, we propose four future studies which could be performed to advance our understanding of the phenomena:

(1) PET investigation: what are the sites of CB1 and μ-opioid receptor binding in placebo analgesia and nocebo hyperalgesia?

Similar to Scott et al. (2008), conduct dual-tracer PET imaging using a mixed radionuclide specific for endocannabinoid and opioid binding potential. Since we identified lateral PAG activity related to pain modulatory phenomena, conducting this study could resolve the relative influence and localization of these two neurotransmitter systems during top-down pain modulation.

(2) Psychological investigation: can these circuits predict placebo responsivity across time with repeated exposures?

Assess baseline coupling between sites of the affective homeostatic pathway using resting-state fMRI. Collect placebo responses in a group of participants with multiple exposures – similar to Lasagna et al. (1954). Assess if any cortico-brainstem connections constitute biomarkers for consistent placebo responsivity.

(3) Functional imaging investigation: what is the role of the locus coeruleus in placebo analgesia and nocebo hyperalgesia?

Since we found a similar and not parallel engagement of the LC underpinned placebo analgesia and nocebo hyperalgesia, a lingering question from our investigations is the role of this region in mounting pain modulatory phenomena. Whilst noradrenergic supply to cortical executive-control regions or direct-spinal modulation are speculative interpretations, a targeted investigation into LC connectivity using a predefined atlas such as that designed by Ye et al. (2021) could be beneficial to better understanding the role of this neurotransmitter in altering perceived pain.

(4) Functional imaging investigation: do these same circuits underlie the potentiation of other modulatory phenomena such as Offset Analgesia and Conditioned Pain Modulation?

Since the stimulus-independent system we propose is pivotal in setting an excitatory tone of brainstem analgesic pathways necessary to trigger analgesic effects, it is possible that these same regions are at play in alternate phenomena which leverage these pathways. There is evidence that both Offset Analgesia and Conditioned Pain Modulation rely on the PAG-RVM system, and an imaging study where each of these are assessed alongside Placebo Analgesia in a within-subjects design would enable a direct comparison of similarities and differences in the neural circuits underpinning each of these specific pain modulatory phenomena.

In addition to these four studies, it would be advantageous to assess the involvement of this circuitry in other forms of placebo analgesia. As detailed in section 1.3 of this thesis, placebo analgesia can be elicited independent to the effects of conditioning – and can be acquired through social observation and expectation generation alone (Amanzio and Benedetti, 1999, Schenk and Colloca, 2019). In the initial phases of clinical intervention, it is likely that these forms of placebo analgesia predominate over conditioning-based effects, and as such dissecting the relative involvement of each of the four components of our core circuitry under these settings would be useful in facilitating clinical applications of pain modulatory phenomena. However, in the context of chronic pain, where a number of contextual cues regarding an individual's pain have been built over time, conditioning-effects are likely more prevalent. As such, interrogating the core circuitry in conditions where pain has persisted over a period of time could have important therapeutic ramifications and offer an appealing avenue for research.

5.5 Limitations

Whilst this thesis provides a number of novel discoveries as to the cortical and brainstem systems which subserve pain modulatory responses in humans, their mechanisms and potential contributing factors - they are not without limitations which must be considered.

First and foremost, we constrain our insights to healthy populations of individuals, and can only speculate as to the role these regions and circuits may play in chronic pain populations. That being said, there is growing interest in the clinical community surrounding the role of specifically the smaller structures we identify throughout our investigations such as the PAG and

hypothalamus, and their role as potential targets for pain modulation in chronic conditions (Puopolo, 2019). Limited as we are in examining healthy control participants, we suggest that our investigations aid the community in pinpointing the likely routes of action and associated structures that are engaged in placebo analgesia which may in the future also become targets for therapeutic benefit. For nocebo hyperalgesia and avoiding their manifestation in clinical settings, we provide further evidence that they can be brought forward through verbal suggestions and repeated exposures to pain (ie. expectation and conditioning), inviting further investigation on how best to manage and minimize their presence in clinical trials and treatments.

Second, one of the cornerstone results from Chapter 2, indeed, the result that inspired Chapter 3 was the robust negative correlation observed between lateral PAG signal change and placebo analgesia responses. Our interpretation of this finding revolved around a reduction in the activity of GABA-ergic inhibitory interneurons which contact the lPAG, resulting in a net increase in lPAG output to the RVM-DH cascade – promoting an anti-nociceptive state. Another interpretation of this result however is that instead of playing a primary role in the production of placebo analgesia, that lPAG activity rather tracks the intensity of pain an individual is currently experiencing. Despite its small size, the PAG produces an array of physiological and autonomic roles, making assigning a single role to this region in the perception and modulation of pain difficult (Floyd et al., 1996, Lumb, 2002, Keay and Bandler, 2015). Regardless, the support provided in Chapter 3 through DCM and functional connectivity analyses at the very least establishes that during placebo analgesia, the lPAG, via altered descending innervation from the posterior hypothalamus, undergoes a change in excitatory-inhibitory balance – entering a state in which it is more likely to be influenced by top-down (extrinsic) inputs (Friston et al., 2003). This finding closely follows existing preclinical literature of a circuit comprised of the prelimbic cortices (mPFC / rACC homologue), amygdala, and hypothalamus – which is proposed to drive autonomic and homeostatic balance (Ongür et al., 1998).

Additionally, the PAG has been proposed to play a role during pain modulatory phenomena in encoding pain precision and contingency between a conditioned stimulus and response (Grahl et al., 2018). Indeed, our findings in Chapter 4 support this role via communication with the dlPFC, which has also been specifically implicated in prediction-error processing and active inference. Perhaps, a more accurate interpretation of our initial finding in Chapter 2, considering our later findings is that BOLD signal change in the PAG does not directly reflect dis-inhibition, but rather an altered neuronal state of the PAG, set to produce antinociceptive action via a

combination of inputs involving the dlPFC and hypothalamus encoding active inference and autonomic balance.

Finally, the large majority of our results and interpretations were derived from fMRI data – be they activation, connectivity, or dynamic causal modelling. The inherent limitation of fMRI is the relatively poor temporal resolution (in the order of seconds) relative to neural dynamics (in the order of milliseconds). Whilst it is widely accepted that greater connectivity reflects two regions engaging in a similar task, and connectivity reductions reflect regions subserving different or competing functions (Fox et al., 2005), and our interpretation of results align with these principles, it is important to keep in mind that these data are limited by hemodynamic response times (Glover, 2011). Since beginning this thesis, a number of advancements have been made in custom ultra-fast fMRI series – with repetition times approaching that necessary to decode distinct neuronal processes (McDowell and Carmichael, 2019, Nagy et al., 2022, Cabral et al., 2023). One particular method, "direct imaging of neuronal activity" (DIANA) has shown promise in anesthetized mice tracing the flow of somatosensory information along thalamocortical pathways following whisker stimulation (Toi et al., 2022). Techniques such as DIANA exemplify the possibility to probe the ascending and descending pain pathways and their cascade of neural activity surrounding the application of a noxious stimuli. Once these techniques are made available in human MRI scanners, their use in experimental pain modulation will further advance our understanding of how these cortical and subcortical sites act to induce profound changes in the pain percept.

5.6 Conclusions

The core goal of this thesis was the expand the base of knowledge available on the functional neural architecture that underpins pain modulatory responses in healthy humans. When beginning this thesis in 2020, no single study had leverage high field MRI to investigate the role of brainstem circuitry involved in the endogenous inhibition or enhancement of pain. Whilst preclinical evidence had cemented the role of the PAG-RVM system in pain relief some 50 years before (Mayer et al., 1971, Basbaum and Fields, 1984), only a handful of experimental human studies had been able to suggest a direct involvement of these nuclei in the manifestation of pain modulatory responses (Wager et al., 2004, Eippert et al., 2009a, Tinnermann et al., 2017). Building on this work, this thesis catalogues first our efforts to define the likely brainstem and neurochemical pathways that drive both placebo analgesia and nocebo hyperalgesia, as well as

the distinct pathways from cortex to brainstem which leverage these circuits during placebo (chapter 3), and a candidate biomarker which may encode an individual's ability to mount endogenous pain modulatory phenomena (chapter 4).

Our experimental findings first suggest that via a combination of verbal suggestion and response conditioning it is possible to elicit both placebo analgesia and nocebo hyperalgesia in healthy individuals. Further, we suggest that the success of response conditioning to elicit placebo analgesia is inexplicably bound to underlying variability in perceived pain, a quality which could be assessed in a lead-in phase of clinical trials to help manage the distribution of placebo (and nocebo) responses between treatment and no-treatment control groups. Within the brain, our results highlight the importance of two top-down systems, working in parallel to enable effective recruitment of descending analgesic circuitry of the brainstem to ultimately alter the nociceptive input across the DH, one sensitive to sensory input and the other tied with regulating the excitatory-inhibitory tone of the PAG.

Chapter 5 outlines the basic circuitry involved with building strong stimulus-response relationships and mounting these phenomena, offering researchers several specific connections and pathways to investigate in future studies. Further, we suggest some new and powerful techniques which could be employed to interrogate the validity of our proposed circuitry across pain conditions and different modulatory phenomena such as offset analgesia or conditioned pain modulation which are also known to involve the brainstem and associated pathways (Derbyshire and Osborn, 2009, Youssef et al., 2016, Szikszay et al., 2020, Harrison et al., 2022). Such work would assist in our understanding of the neurobiology underpinning endogenous pain modulatory responses in humans, and more importantly, provide potential benefit in developing novel and alternate treatments for people suffering chronic and comorbid pain.

References:

AICHER, S. A., HERMES, S. M., WHITTIER, K. L. & HEGARTY, D. M. 2012. Descending projections from the rostral ventromedial medulla (RVM) to trigeminal and spinal dorsal horns are morphologically and neurochemically distinct. *J Chem Neuroanat,* 43**,** 103-11.

ALMEIDA, A., TJØLSEN, A., LIMA, D., COIMBRA, A. & HOLE, K. 1996. The medullary dorsal reticular nucleus facilitates acute nociception in the rat. *Brain Research Bulletin,* 39**,** 7-15.

AMANZIO, M. & BENEDETTI, F. 1999. Neuropharmacological dissection of placebo analgesia: expectation-activated opioid systems versus conditioning-activated specific subsystems. *J Neurosci,* 19**,** 484-94.

AMANZIO, M., CIPRIANI, G. E. & BARTOLI, M. 2021. How do nocebo effects in placebo groups of randomized controlled trials provide a possible explicative framework for the COVID-19 pandemic? *Expert Rev Clin Pharmacol,* 14**,** 439-444.

AMANZIO, M., CORAZZINI, L. L., VASE, L. & BENEDETTI, F. 2009. A systematic review of adverse events in placebo groups of anti-migraine clinical trials. *Pain,* 146**,** 261-269.

ATLAS, L. Y. & WAGER, T. D. 2014. A meta-analysis of brain mechanisms of placebo analgesia: consistent findings and unanswered questions. *Handb Exp Pharmacol,* 225**,** 37-69.

BAJCAR, E. A., ADAMCZYK, W. M., WIERCIOCH-KUZIANIK, K. & BĄBEL, P. 2020. Nocebo hyperalgesia can be induced by classical conditioning without involvement of expectancy. *PloS one,* 15**,** e0232108-e0232108.

BANDLER, R. & KEAY, K. A. 1996. Chapter 17 Columnar organization in the midbrain periaqueductal gray and the integration of emotional expression. *In:* HOLSTEGE, G., BANDLER, R. & SAPER, C. B. (eds.) *Progress in Brain Research.* Elsevier.

BANDLER, R. & SHIPLEY, M. T. 1994. Columnar organization in the midbrain periaqueductal gray: modules for emotional expression? *Trends Neurosci,* 17**,** 379-89.

BARSKY, A. J., SAINTFORT, R., ROGERS, M. P. & BORUS, J. F. 2002. Nonspecific Medication Side Effects and the Nocebo Phenomenon. *JAMA,* 287**,** 622-627.

BASBAUM, A. I. & FIELDS, H. L. 1984. Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. *Annu Rev Neurosci,* 7**,** 309-38.

BEECHER, H. K. 1955. The powerful placebo. *J Am Med Assoc,* 159**,** 1602-6.

BEHBEHANI, M. M. 1995. Functional characteristics of the midbrain periaqueductal gray. *Progress in Neurobiology,* 46**,** 575-605.

BELL, A. 2018. The neurobiology of acute pain. *Vet J,* 237**,** 55-62.

BENEDETTI, F. 1996. The opposite effects of the opiate antagonist naloxone and the cholecystokinin antagonist proglumide on placebo analgesia. *Pain,* 64, 535-543.

BENEDETTI, F. & AMANZIO, M. 2013. Mechanisms of the placebo response. *Pulmonary Pharmacology & Therapeutics,* 26, 520-523.

BENEDETTI, F., AMANZIO, M., CASADIO, C., OLIARO, A. & MAGGI, G. 1997. Blockade of nocebo hyperalgesia by the cholecystokinin antagonist proglumide. *Pain,* 71, 135-40.

BENEDETTI, F., AMANZIO, M., ROSATO, R. & BLANCHARD, C. 2011. Nonopioid placebo analgesia is mediated by CB1 cannabinoid receptors. *Nature Medicine,* 17, 1228-1230.

BENEDETTI, F., AMANZIO, M., VIGHETTI, S. & ASTEGGIANO, G. 2006. The Biochemical and Neuroendocrine Bases of the Hyperalgesic Nocebo Effect. *The Journal of Neuroscience,* 26, 12014.

BENEDETTI, F. & FRISALDI, E. 2014. Creating placebo responders and nonresponders in the laboratory: boons and banes. *Pain Management,* 4, 165-167.

BENEDETTI, F., THOEN, W., BLANCHARD, C., VIGHETTI, S. & ARDUINO, C. 2013. Pain as a reward: changing the meaning of pain from negative to positive co-activates opioid and cannabinoid systems. *Pain,* 154, 361-367.

BERNSTEIN, M. H. & BROWN, W. A. 2017. The placebo effect in psychiatric practice. *Current psychiatry,* 16, 29.

BERTHELOT, J.-M., NIZARD, J. & MAUGARS, Y. 2019. The negative Hawthorne effect: Explaining pain overexpression. *Joint Bone Spine,* 86, 445-449.

BEST, M. A., NEUHAUSER, D. & SLAVIN, L. 2003. *Benjamin Franklin: Verification and Validation of the Scientific Process in Healthcare As Demonstrated by the Report of the Royal Commission of Animal Magnetism*, Trafford Publishing.

BESTER, H., BOURGEAIS, L., VILLANUEVA, L., BESSON, J. M. & BERNARD, J. F. 1999. Differential projections to the intralaminar and gustatory thalamus from the parabrachial area: a PHA-L study in the rat. *J Comp Neurol,* 405, 421-49.

BINGEL, U., LORENZ, J., SCHOELL, E., WEILLER, C. & BUCHEL, C. 2006. Mechanisms of placebo analgesia: rACC recruitment of a subcortical antinociceptive network. *Pain,* 120, 8-15.

BINGEL, U., WANIGASEKERA, V., WIECH, K., NI MHUIRCHEARTAIGH, R., LEE, M. C., PLONER, M. & TRACEY, I. 2011. The effect of treatment expectation on drug efficacy: imaging the analgesic benefit of the opioid remifentanil. *Sci Transl Med,* 3, 70ra14.

BISWAL, B., ZERRIN YETKIN, F., HAUGHTON, V. M. & HYDE, J. S. 1995. Functional connectivity in the motor cortex of resting human brain using echo-planar mri. *Magnetic Resonance in Medicine,* 34, 537-541.

BLASINI, M., CORSI, N., KLINGER, R. & COLLOCA, L. 2017. Nocebo and pain: An overview of the psychoneurobiological mechanisms. *Pain Rep,* 2.

BLASINI, M., PEIRIS, N., WRIGHT, T. & COLLOCA, L. 2018. The Role of Patient-Practitioner Relationships in Placebo and Nocebo Phenomena. *Int Rev Neurobiol,* 139, 211-231.

BOOTH, C. 2005. The rod of Aesculapios: John Haygarth (1740-1827) and Perkins' metallic tractors. *J Med Biogr,* 13, 155-61.

BRÄSCHER, A. K., KLEINBÖHL, D., HÖLZL, R. & BECKER, S. 2017. Differential Classical Conditioning of the Nocebo Effect: Increasing Heat-Pain Perception without Verbal Suggestions. *Front Psychol,* 8, 2163.

BROOKS, J. & TRACEY, I. 2005. From nociception to pain perception: imaging the spinal and supraspinal pathways. *Journal of anatomy,* 207, 19-33.

BÜCHEL, C., GEUTER, S., SPRENGER, C. & EIPPERT, F. 2014. Placebo Analgesia: A Predictive Coding Perspective. *Neuron,* 81, 1223-1239.

CABRAL, J., FERNANDES, F. F. & SHEMESH, N. 2023. Intrinsic macroscale oscillatory modes driving long range functional connectivity in female rat brains detected by ultrafast fMRI. *Nature Communications,* 14, 375.

CANNON, J. T., PRIETO, G. J., LEE, A. & LIEBESKIND, J. C. 1982. Evidence for opioid and non-opioid forms of stimulation-produced analgesia in the rat. *Brain Res,* 243, 315-21.

CAUZZO, S., SINGH, K., STAUDER, M., GARCÍA-GOMAR, M. G., VANELLO, N., PASSINO, C., STAAB, J., INDOVINA, I. & BIANCIARDI, M. 2022. Functional connectome of brainstem nuclei involved in autonomic, limbic, pain and sensory processing in living humans from 7 Tesla resting state fMRI. *Neuroimage,* 250, 118925.

CAVANAGH, J. F. & SHACKMAN, A. J. 2015. Frontal midline theta reflects anxiety and cognitive control: meta-analytic evidence. *J Physiol Paris,* 109, 3-15.

CHANDLER, D. J. 2016. Evidence for a specialized role of the locus coeruleus noradrenergic system in cortical circuitries and behavioral operations. *Brain research,* 1641, 197-206.

CHAU, B. K. H., JARVIS, H., LAW, C. K. & CHONG, T. T. 2018. Dopamine and reward: a view from the prefrontal cortex. *Behav Pharmacol,* 29, 569-583.

CHEN, Q. & HEINRICHER, M. M. 2019a. Descending Control Mechanisms and Chronic Pain. *Current Rheumatology Reports,* 21, 13.

CHEN, Q. & HEINRICHER, M. M. 2019b. Plasticity in the Link between Pain-Transmitting and Pain-Modulating Systems in Acute and Persistent Inflammation. *J Neurosci,* 39, 2065-2079.

CHEN, Q., ROEDER, Z., LI, M. H., ZHANG, Y., INGRAM, S. L. & HEINRICHER, M. M. 2017. Optogenetic Evidence for a Direct Circuit Linking Nociceptive Transmission through the Parabrachial Complex with Pain-Modulating Neurons of the Rostral Ventromedial Medulla (RVM). *eNeuro,* 4.

CHIANG, M. C., NGUYEN, E. K., CANTO-BUSTOS, M., PAPALE, A. E., OSWALD, A.-M. M. & ROSS, S. E. 2020. Divergent Neural Pathways Emanating from the Lateral Parabrachial Nucleus Mediate Distinct Components of the Pain Response. *Neuron,* 106**,** 927-939.e5.

CHUANG-STEIN, C., KIRBY, S., HIRSCH, I. & ATKINSON, G. 2011. The role of the minimum clinically important difference and its impact on designing a trial. *Pharmaceutical Statistics,* 10**,** 250-256.

COIZET, V., DOMMETT, E. J., KLOP, E. M., REDGRAVE, P. & OVERTON, P. G. 2010. The parabrachial nucleus is a critical link in the transmission of short latency nociceptive information to midbrain dopaminergic neurons. *Neuroscience,* 168**,** 263-72.

COLIZOLI, O., DE GEE, J. W., VAN DER ZWAAG, W. & DONNER, T. H. 2022. Functional magnetic resonance imaging responses during perceptual decision-making at 3 and 7 T in human cortex, striatum, and brainstem. *Hum Brain Mapp,* 43**,** 1265-1279.

COLLOCA, D. L., PETROVIC, D. P., WAGER, D. T., INGVAR, D. M. & BENEDETTI, D. F. 2010. How the number of learning trials affects placebo and nocebo responses. *Pain,* 151**,** 430-439.

COLLOCA, L. & BENEDETTI, F. 2009. Placebo analgesia induced by social observational learning. *Pain,* 144**,** 28-34.

COLLOCA, L. & MILLER, F. G. 2011. The nocebo effect and its relevance for clinical practice. *Psychosom Med,* 73**,** 598-603.

CONNELL, K., BOLTON, N., OLSEN, D., PIOMELLI, D. & HOHMANN, A. G. 2006. Role of the basolateral nucleus of the amygdala in endocannabinoid-mediated stress-induced analgesia. *Neurosci Lett,* 397**,** 180-4.

CRAGG, J. J., WARNER, F. M., FINNERUP, N. B., JENSEN, M. P., MERCIER, C., RICHARDS, J. S., WRIGLEY, P., SOLER, D. & KRAMER, J. L. K. 2016. Meta-analysis of placebo responses in central neuropathic pain: impact of subject, study, and pain characteristics. *Pain,* 157**,** 530-540.

CROSS, S. A. 1994. Pathophysiology of pain. *Mayo Clin Proc,* 69**,** 375-83.

DANIELS, A. M. & SALLIE, R. 1981. Headache, lumbar puncture, and expectation. *Lancet,* 1**,** 1003.

DARRAGH, M., BOOTH, R. J. & CONSEDINE, N. S. 2015. Who responds to placebos? Considering the "placebo personality" via a transactional model. *Psychol Health Med,* 20**,** 287-95.

DE PASCALIS, V., CHIARADIA, C. & CAROTENUTO, E. 2002. The contribution of suggestibility and expectation to placebo analgesia phenomenon in an experimental setting. *Pain,* 96**,** 393-402.

DEPAULIS, A. & BANDLER, R. 2012. *The midbrain periaqueductal gray matter: Functional, anatomical, and neurochemical organization*, Springer Science & Business Media.

DERBYSHIRE, S. W. & OSBORN, J. 2009. Offset analgesia is mediated by activation in the region of the periaqueductal grey and rostral ventromedial medulla. *Neuroimage,* 47**,** 1002-6.

DIEDRICHSEN, J. 2006. A spatially unbiased atlas template of the human cerebellum. *Neuroimage,* 33**,** 127-138.

DIEDRICHSEN, J., BALSTERS, J. H., FLAVELL, J., CUSSANS, E. & RAMNANI, N. 2009. A probabilistic MR atlas of the human cerebellum. *Neuroimage,* 46**,** 39-46.

DIEDRICHSEN, J., MADERWALD, S., KÜPER, M., THÜRLING, M., RABE, K., GIZEWSKI, E. R., LADD, M. E. & TIMMANN, D. 2011. Imaging the deep cerebellar nuclei: a probabilistic atlas and normalization procedure. *Neuroimage,* 54**,** 1786-94.

DRICI, M.-D., RAYBAUD, F., DE LUNARDO, C., IACONO, P. & GUSTOVIC, P. 1995. Influence of the behaviour pattern on the nocebo response of healthy volunteers. *British journal of clinical pharmacology,* 39**,** 204-206.

DUBIN, A. E. & PATAPOUTIAN, A. 2010. Nociceptors: the sensors of the pain pathway. *The Journal of clinical investigation,* 120**,** 3760-3772.

DUKART, J. & BERTOLINO, A. 2014. When structure affects function--the need for partial volume effect correction in functional and resting state magnetic resonance imaging studies. *PLoS One,* 9**,** e114227.

EELEN, P. 2018. Classical Conditioning: Classical Yet Modern. *Psychol Belg,* 58**,** 196-211.

EGOROVA, N., BENEDETTI, F., GOLLUB, R. L. & KONG, J. 2019. Between placebo and nocebo: Response to control treatment is mediated by amygdala activity and connectivity. *Eur J Pain*.

EIPPERT, F., BINGEL, U., SCHOELL, E. D., YACUBIAN, J., KLINGER, R., LORENZ, J. & BÜCHEL, C. 2009a. Activation of the Opioidergic Descending Pain Control System Underlies Placebo Analgesia. *Neuron,* 63**,** 533-543.

EIPPERT, F., FINSTERBUSCH, J., BINGEL, U. & BÜCHEL, C. 2009b. Direct evidence for spinal cord involvement in placebo analgesia. *Science,* 326**,** 404.

ELSENBRUCH, S., KOTSIS, V., BENSON, S., ROSENBERGER, C., REIDICK, D., SCHEDLOWSKI, M., BINGEL, U., THEYSOHN, N., FORSTING, M. & GIZEWSKI, E. R. 2012. Neural mechanisms mediating the effects of expectation in visceral placebo analgesia: An fMRI study in healthy placebo responders and nonresponders. *PAIN,* 153**,** 382-390.

ENCK, P., BENEDETTI, F. & SCHEDLOWSKI, M. 2008. New Insights into the Placebo and Nocebo Responses. *Neuron,* 59**,** 195-206.

ERNST, E. & RESCH, K. L. 1995. Concept of true and perceived placebo effects. *Bmj,* 311**,** 551-3.

FIELDS, H. & HEINRICHER, M. 1985. Anatomy and Physiology of a Nociceptive Modulatory System. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences,* 308**,** 361-74.

FIELDS, H. L., BRY, J., HENTALL, I. & ZORMAN, G. 1983. The activity of neurons in the rostral medulla of the rat during withdrawal from noxious heat. *J Neurosci,* 3**,** 2545-52.

FILLER, A. 2009. The History, Development and Impact of Computed Imaging in Neurological Diagnosis and Neurosurgery: CT, MRI, and DTI. *Nature Precedings*.

FILLINGIM, R. B. 2017. Individual differences in pain: understanding the mosaic that makes pain personal. *Pain,* 158 Suppl 1**,** S11-s18.

FLOYD, N. S., KEAY, K. A., ARIAS, C. M., SAWCHENKO, P. E. & BANDLER, R. 1996. Projections from the ventrolateral periaqueductal gray to endocrine regulatory subdivisions of the paraventricular nucleus of the hypothalamus in the rat. *Neuroscience Letters,* 220**,** 105-108.

FOX, M. D., SNYDER, A. Z., VINCENT, J. L., CORBETTA, M., VAN ESSEN, D. C. & RAICHLE, M. E. 2005. The human brain is intrinsically organized into dynamic, anticorrelated functional networks. *Proceedings of the National Academy of Sciences,* 102**,** 9673-9678.

FOX, M. D., ZHANG, D., SNYDER, A. Z. & RAICHLE, M. E. 2009. The Global Signal and Observed Anticorrelated Resting State Brain Networks. *Journal of Neurophysiology,* 101**,** 3270-3283.

FREEMAN, S., YU, R., EGOROVA, N., CHEN, X., KIRSCH, I., CLAGGETT, B., KAPTCHUK, T. J., GOLLUB, R. L. & KONG, J. 2015. Distinct neural representations of placebo and nocebo effects. *NeuroImage,* 112**,** 197-207.

FRISALDI, E., PIEDIMONTE, A. & BENEDETTI, F. 2015. Placebo and nocebo effects: a complex interplay between psychological factors and neurochemical networks. *Am J Clin Hypn,* 57**,** 267-84.

FRISTON, K., MORAN, R. & SETH, A. K. 2013. Analysing connectivity with Granger causality and dynamic causal modelling. *Current Opinion in Neurobiology,* 23**,** 172-178.

FRISTON, K. J. 2003. Statistical Parametric Mapping. *In:* KÖTTER, R. (ed.) *Neuroscience Databases: A Practical Guide.* Boston, MA: Springer US.

FRISTON, K. J., BUECHEL, C., FINK, G. R., MORRIS, J., ROLLS, E. & DOLAN, R. J. 1997. Psychophysiological and modulatory interactions in neuroimaging. *Neuroimage,* 6**,** 218-29.

FRISTON, K. J., HARRISON, L. & PENNY, W. 2003. Dynamic causal modelling. *NeuroImage,* 19**,** 1273-1302.

FU, J., WU, S., LIU, C., CAMILLERI, J. A., EICKHOFF, S. B. & YU, R. 2021. Distinct neural networks subserve placebo analgesia and nocebo hyperalgesia. *Neuroimage,* 231**,** 117833.

GEBHART, G. F. & TOLEIKIS, J. R. 1978. An evaluation of stimulation-produced analgesia in the cat. *Experimental Neurology,* 62**,** 570-579.

GEERS, A. L., WELLMAN, J. A., FOWLER, S. L., HELFER, S. G. & FRANCE, C. R. 2010. Dispositional optimism predicts placebo analgesia. *The journal of pain : official journal of the American Pain Society,* 11**,** 1165-1171.

GEUTER, S., KOBAN, L. & WAGER, T. D. 2017. The Cognitive Neuroscience of Placebo Effects: Concepts, Predictions, and Physiology. *Annu Rev Neurosci,* 40**,** 167-188.

GLOVER, G. H. 2011. Overview of functional magnetic resonance imaging. *Neurosurg Clin N Am,* 22**,** 133-9, vii.

GOLDENHOLZ, D. M., MOSS, R., SCOTT, J., AUH, S. & THEODORE, W. H. 2015. Confusing placebo effect with natural history in epilepsy: A big data approach. *Ann Neurol,* 78**,** 329-36.

GRACELY, R. H. 1987. Verbal descriptor measures of pain clarify mechanisms of analgesia due to narcotics, brain stimulation, and placebo. *Anesth Prog,* 34**,** 113-6.

GRAHL, A., ONAT, S. & BÜCHEL, C. 2018. The periaqueductal gray and Bayesian integration in placebo analgesia. *Elife,* 7.

GRAVITZ, M. A. 1994. Mesmer and Animal Magnetism: A Chapter in the History of Medicine. Hamilton NY: Edmonston Publishing. *American Journal of Clinical Hypnosis,* 37**,** 60-62.

GREENE, J. A. & PODOLSKY, S. H. 2012. Reform, regulation, and pharmaceuticals--the Kefauver-Harris Amendments at 50. *N Engl J Med,* 367**,** 1481-3.

GREVERT, P., ALBERT, L. H. & GOLDSTEIN, A. 1983. Partial antagonism of placebo analgesia by naloxone. *Pain,* 16**,** 129-43.

GUO, T.-Z., JIANG, J.-Y., BUTTERMANN, A. E. & MAZE, M. 1996. Dexmedetomidine injection into the locus ceruleus produces antinociception. *The Journal of the American Society of Anesthesiologists,* 84**,** 873-881.

HABER, S. N., KIM, K.-S., MAILLY, P. & CALZAVARA, R. 2006. Reward-Related Cortical Inputs Define a Large Striatal Region in Primates That Interface with Associative Cortical Connections, Providing a Substrate for Incentive-Based Learning. *The Journal of Neuroscience,* 26**,** 8368.

HALL, K. T., LEMBO, A. J., KIRSCH, I., ZIOGAS, D. C., DOUAIHER, J., JENSEN, K. B., CONBOY, L. A., KELLEY, J. M., KOKKOTOU, E. & KAPTCHUK, T. J. 2012. Catechol-O-methyltransferase val158met polymorphism predicts placebo effect in irritable bowel syndrome. *PLoS One,* 7**,** e48135.

HALL, K. T., LOSCALZO, J. & KAPTCHUK, T. J. 2015. Genetics and the placebo effect: the placebome. *Trends Mol Med,* 21**,** 285-94.

HARRIS, H. N. & PENG, Y. B. 2020. Evidence and explanation for the involvement of the nucleus accumbens in pain processing. *Neural Regen Res,* 15**,** 597-605.

HARRISON, R., GANDHI, W., VAN REEKUM, C. M. & SALOMONS, T. V. 2022. Conditioned pain modulation is associated with heightened connectivity between the periaqueductal grey and cortical regions. *PAIN Reports,* 7**,** e999.

HASHMI, J. A., KONG, J., SPAETH, R., KHAN, S., KAPTCHUK, T. J. & GOLLUB, R. L. 2014. Functional network architecture predicts psychologically mediated analgesia related to treatment in chronic knee pain patients. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 34**,** 3924-3936.

HÄUSER, W., BARTRAM, C., BARTRAM-WUNN, E. & TÖLLE, T. 2012. Adverse Events Attributable to Nocebo in Randomized Controlled Drug Trials in Fibromyalgia Syndrome and Painful Diabetic Peripheral Neuropathy: Systematic Review. *The Clinical Journal of Pain,* 28**,** 437-451.

HEINRICHER, M. M., BARBARO, N. M. & FIELDS, H. L. 1989. Putative nociceptive modulating neurons in the rostral ventromedial medulla of the rat: firing of on- and off-cells is related to nociceptive responsiveness. *Somatosens Mot Res,* 6**,** 427-39.

HEINRICHER, M. M. & FIELDS, H. L. 2013. Central nervous system mechanisms of pain modulation. *Wall and Melzack's textbook of pain, Ed,* 6**,** 129-142.

HEINRICHER, M. M., MORGAN, M. M., TORTORICI, V. & FIELDS, H. L. 1994. Disinhibition of off-cells and antinociception produced by an opioid action within the rostral ventromedial medulla. *Neuroscience,* 63**,** 279-88.

HEINRICHER, M. M., TAVARES, I., LEITH, J. L. & LUMB, B. M. 2009. Descending control of nociception: Specificity, recruitment and plasticity. *Brain Res Rev,* 60**,** 214-25.

HELMCHEN, C., FU, Q. G. & SANDKÜHLER, J. 1995. Inhibition of spinal nociceptive neurons by microinjections of somatostatin into the nucleus raphe magnus and the midbrain periaqueductal gray of the anesthetized cat. *Neurosci Lett,* 187**,** 137-41.

HENDERSON, L. A., DI PIETRO, F., YOUSSEF, A. M., LEE, S., TAM, S., AKHTER, R., MILLS, E. P., MURRAY, G. M., PECK, C. C. & MACEY, P. M. 2020. Effect of Expectation on Pain Processing: A Psychophysics and Functional MRI Analysis. *Frontiers in neuroscience,* 14**,** 6-6.

HILLMAN, E. M. 2014. Coupling mechanism and significance of the BOLD signal: a status report. *Annu Rev Neurosci,* 37**,** 161-81.

HINES, T. 2017. Delusions of the imagination: how the "tractor"--an early 19th century medical quack device--was debunked by one of the earliest single blind placebo studies. *Skeptic (Altadena, CA),* 22**,** 18+.

HIRSCHBERG, S., LI, Y., RANDALL, A., KREMER, E. J. & PICKERING, A. E. 2017. Functional dichotomy in spinal- vs prefrontal-projecting locus coeruleus modules splits descending noradrenergic analgesia from ascending aversion and anxiety in rats. *eLife,* 6.

HOFFMAN, G. A., HARRINGTON, A. & FIELDS, H. L. 2005. Pain and the placebo: what we have learned. *Perspectives in biology and medicine,* 48**,** 248-265.

HOLLAND, P. & GOADSBY, P. J. 2007. The Hypothalamic Orexinergic System: Pain and Primary Headaches. *Headache: The Journal of Head and Face Pain,* 47**,** 951-962.

HOOPER, R. 1817. *Quincy's Lexicon-medicum: A New Medical Dictionary; Containing an Explanation of the Terms in Anatomy, Physiology, Practice of Physic, Materia Medica, Chymistry, Pharmacy, Surgery, Midwifery, and the Various Branches of Natural Philosophy Connected with Medicine*, E. & R. Parker [etc.] Griggs & Company, printers.

HOWICK, J., FRIEDEMANN, C., TSAKOK, M., WATSON, R., TSAKOK, T., THOMAS, J., PERERA, R., FLEMING, S. & HENEGHAN, C. 2013. Are treatments more effective than placebos? A systematic review and meta-analysis. *PLoS One,* 8**,** e62599.

HRÓBJARTSSON, A. & GØTZSCHE, P. C. 2010. Placebo interventions for all clinical conditions. *Cochrane Database Syst Rev,* 2010**,** Cd003974.

HRÓBJARTSSON, A., KAPTCHUK, T. J. & MILLER, F. G. 2011. Placebo effect studies are susceptible to response bias and to other types of biases. *Journal of clinical epidemiology,* 64, 1223-1229.

HUNT, S. P. & MANTYH, P. W. 2001. The molecular dynamics of pain control. *Nat Rev Neurosci,* 2, 83-91.

IANNETTI, G. D. & MOURAUX, A. 2010. From the neuromatrix to the pain matrix (and back). *Experimental Brain Research,* 205, 1-12.

IGELSTRÖM, K. M., WEBB, T. W., KELLY, Y. T. & GRAZIANO, M. S. 2016. Topographical Organization of Attentional, Social, and Memory Processes in the Human Temporoparietal Cortex. *eNeuro,* 3.

ISLAM, K. U. S., MELI, N. & BLAESS, S. 2021. The Development of the Mesoprefrontal Dopaminergic System in Health and Disease. *Frontiers in Neural Circuits,* 15.

IVERSEN, L. 2003. Cannabis and the brain. *Brain,* 126, 1252-1270.

JENSEN, K. B., KAPTCHUK, T. J., CHEN, X., KIRSCH, I., INGVAR, M., GOLLUB, R. L. & KONG, J. 2015. A Neural Mechanism for Nonconscious Activation of Conditioned Placebo and Nocebo Responses. *Cerebral cortex (New York, N.Y. : 1991),* 25, 3903-3910.

JENSEN, K. B., KAPTCHUK, T. J., KIRSCH, I., RAICEK, J., LINDSTROM, K. M., BERNA, C., GOLLUB, R. L., INGVAR, M. & KONG, J. 2012. Nonconscious activation of placebo and nocebo pain responses. *Proceedings of the National Academy of Sciences of the United States of America,* 109, 15959-15964.

JILCH, S., SEL, R. & SHARIAT, S. F. 2020. Medical practice and placebo response: an inseparable bond? *Wiener klinische Wochenschrift,* 132, 228-231.

KANDEL, E. R., SCHWARTZ, J. H., JESSELL, T. M., SIEGELBAUM, S., HUDSPETH, A. J. & MACK, S. 2000. *Principles of neural science*, McGraw-hill New York.

KANUI, T. I., TJØLSEN, A., LUND, A., MJELLEM-JOLY, N. & HOLE, K. 1993. Antinociceptive effects of intrathecal administration of α -adrenoceptor antagonists and clonidine in the formalin test in the mouse. *Neuropharmacology,* 32, 367-371.

KATZ, R. 2004. FDA: evidentiary standards for drug development and approval. *NeuroRx,* 1, 307-16.

KEAY, K. A. & BANDLER, R. 2001. Parallel circuits mediating distinct emotional coping reactions to different types of stress. *Neurosci Biobehav Rev,* 25, 669-78.

KEAY, K. A. & BANDLER, R. 2015. Chapter 10 - Periaqueductal Gray. *In:* PAXINOS, G. (ed.) *The Rat Nervous System (Fourth Edition)*. San Diego: Academic Press.

KENNEDY, W. P. 1961. The nocebo reaction. *Medical world,* 95, 203-205.

KERR, C., MILNE, I. & KAPTCHUK, T. 2008. William Cullen and a missing mind-body link in the early history of placebos. *Journal of the Royal Society of Medicine,* 101**,** 89-92.

KHALID, S. & TUBBS, R. S. 2017. Neuroanatomy and Neuropsychology of Pain. *Cureus,* 9**,** e1754-e1754.

KHASABOV, S. G., MALECHA, P., NOACK, J., TABAKOV, J., OKAMOTO, K., BEREITER, D. A. & SIMONE, D. A. 2015. Activation of rostral ventromedial medulla neurons by noxious stimulation of cutaneous and deep craniofacial tissues. *Journal of neurophysiology,* 113**,** 14-22.

KISSIWAA, S. A. & BAGLEY, E. E. 2018. Central sensitization of the spino-parabrachial-amygdala pathway that outlasts a brief nociceptive stimulus. *J Physiol,* 596**,** 4457-73.

KNOFF, W. F. 1970. A history of the concept of neurosis, with a memoir of William Cullen. *Am J Psychiatry,* 127**,** 80-4.

KONG, J., GOLLUB, R. L., POLICH, G., KIRSCH, I., LAVIOLETTE, P., VANGEL, M., ROSEN, B. & KAPTCHUK, T. J. 2008. A Functional Magnetic Resonance Imaging Study on the Neural Mechanisms of Hyperalgesic Nocebo Effect. *The Journal of Neuroscience,* 28**,** 13354.

KRUMMENACHER, P., CANDIA, V., FOLKERS, G., SCHEDLOWSKI, M. & SCHÖNBÄCHLER, G. 2010. Prefrontal cortex modulates placebo analgesia. *PAIN,* 148**,** 368-374.

KULKARNI, B., BENTLEY, D. E., ELLIOTT, R., YOUELL, P., WATSON, A., DERBYSHIRE, S. W., FRACKOWIAK, R. S., FRISTON, K. J. & JONES, A. K. 2005. Attention to pain localization and unpleasantness discriminates the functions of the medial and lateral pain systems. *Eur J Neurosci,* 21**,** 3133-42.

LANSKA, D. J. 2019. Perkins's patent metallic "Tractors": Development, adoption, and early dissemination of an eighteenth-century therapeutic fad. *Journal of the History of the Neurosciences,* 28**,** 122-146.

LASAGNA, L., MOSTELLER, F., VON FELSINGER, J. M. & BEECHER, H. K. 1954. A study of the placebo response. *The American Journal of Medicine,* 16**,** 770-779.

LATAGLIATA, E. C., SACCOCCIO, P., MILIA, C. & PUGLISI-ALLEGRA, S. 2016. Norepinephrine in prelimbic cortex delays extinction of amphetamine-induced conditioned place preference. *Psychopharmacology,* 233**,** 973-982.

LAU, B. K. & VAUGHAN, C. W. 2014. Descending modulation of pain: the GABA disinhibition hypothesis of analgesia. *Current Opinion in Neurobiology,* 29**,** 159-164.

LE BARS, D., VILLANUEVA, L., BOUHASSIRA, D. & WILLER, J. C. 1992. Diffuse noxious inhibitory controls (DNIC) in animals and in man. *Patol Fiziol Eksp Ter***,** 55-65.

LEVINE, J. D. & GORDON, N. C. 1984. Influence of the method of drug administration on analgesic response. *Nature,* 312, 755-756.

LEVINE, J. D., GORDON, N. C., BORNSTEIN, J. C. & FIELDS, H. L. 1979. Role of pain in placebo analgesia. *Proc Natl Acad Sci U S A,* 76, 3528-31.

LEVINE, J. D., GORDON, N. C. & FIELDS, H. L. 1978. The mechanism of placebo analgesia. *Lancet,* 2, 654-7.

LEWIS, V. A. & GEBHART, G. 1977. Evaluation of the periaqueductal central gray (PAG) as a morphine-specific locus of action and examination of morphine-induced and stimulation-produced analgesia at coincident PAG loci. *Brain research,* 124, 283-303.

LI, Z., CHEN, Z., FAN, G., LI, A., YUAN, J. & XU, T. 2018. Cell-Type-Specific Afferent Innervation of the Nucleus Accumbens Core and Shell. *Front Neuroanat,* 12, 84.

LLORCA-TORRALBA, M., BORGES, G., NETO, F., MICO, J. A. & BERROCOSO, E. 2016. Noradrenergic Locus Coeruleus pathways in pain modulation. *Neuroscience,* 338, 93-113.

LONG, D. M., UEMATSU, S. & KOUBA, R. B. 1989. Placebo responses to medical device therapy for pain. *Stereotactic and functional neurosurgery,* 53, 149-156.

LOPEZ, C. A. 1993. Franklin and Mesmer: an encounter. *Yale J Biol Med,* 66, 325-31.

LOVICK, T. A. 1985. Ventrolateral medullary lesions block the antinociceptive and cardiovascular responses elicited by stimulating the dorsal periaqueductal grey matter in rats. *Pain,* 21, 241-252.

LOVICK, T. A. 1993. The periaqueductal gray-rostral medulla connection in the defence reaction: efferent pathways and descending control mechanisms. *Behav Brain Res,* 58, 19-25.

LUI, J. H., NGUYEN, N. D., GRUTZNER, S. M., DARMANIS, S., PEIXOTO, D., WAGNER, M. J., ALLEN, W. E., KEBSCHULL, J. M., RICHMAN, E. B., REN, J., NEWSOME, W. T., QUAKE, S. R. & LUO, L. 2021. Differential encoding in prefrontal cortex projection neuron classes across cognitive tasks. *Cell,* 184, 489-506.e26.

LUMB, B. M. 2002. Inescapable and Escapable Pain is Represented in Distinct Hypothalamic-Midbrain Circuits: Specific Roles for a δ - and C-Nociceptors. *Experimental Physiology,* 87, 281-286.

MA, W. & PESCHANSKI, M. 1988. Spinal and trigeminal projections to the parabrachial nucleus in the rat: electron-microscopic evidence of a spino-ponto-amygdalian somatosensory pathway. *Somatosens Res,* 5, 247-57.

MACKINNON, D. P., FAIRCHILD, A. J. & FRITZ, M. S. 2007. Mediation analysis. *Annu Rev Psychol,* 58, 593-614.

MAKOVAC, E., VENEZIA, A., HOHENSCHURZ-SCHMIDT, D., DIPASQUALE, O., JACKSON, J. B., MEDINA, S., O'DALY, O., WILLIAMS, S. C. R., MCMAHON, S. B. & HOWARD, M. A. 2021. The association between pain-induced autonomic reactivity and descending pain control is mediated by the periaqueductal grey. *The Journal of Physiology,* n/a.

MALLORY, G. W., ABULSEOUD, O., HWANG, S. C., GORMAN, D. A., STEAD, S. M., KLASSEN, B. T., SANDRONI, P., WATSON, J. C. & LEE, K. H. 2012. The nucleus accumbens as a potential target for central poststroke pain. *Mayo Clin Proc,* 87**,** 1025-31.

MANTYH, P. W. 1983. Connections of midbrain periaqueductal gray in the monkey. II. Descending efferent projections. *J Neurophysiol,* 49**,** 582-94.

MARREIROS, A. C., KIEBEL, S. J. & FRISTON, K. J. 2008. Dynamic causal modelling for fMRI: A two-state model. *NeuroImage,* 39**,** 269-278.

MARREIROS, A. C., STEPHAN, K. E. & FRISTON, K. J. 2010. Dynamic causal modeling. *Scholarpedia,* 5**,** 9568.

MARS, R. B., SALLET, J., SCHÜFFELGEN, U., JBABDI, S., TONI, I. & RUSHWORTH, M. F. 2012. Connectivity-based subdivisions of the human right "temporoparietal junction area": evidence for different areas participating in different cortical networks. *Cereb Cortex,* 22**,** 1894-903.

MARTIN, W. J., COFFIN, P. O., ATTIAS, E., BALINSKY, M., TSOU, K. & WALKER, J. M. 1999. Anatomical basis for cannabinoid-induced antinociception as revealed by intracerebral microinjections. *Brain Research,* 822**,** 237-242.

MARTINS, I., PINTO, M., WILSON, S. P., LIMA, D. & TAVARES, I. 2008. Dynamic of migration of HSV-1 from a medullary pronociceptive centre: antinociception by overexpression of the preproenkephalin transgene. *Eur J Neurosci,* 28**,** 2075-83.

MARTINS, I. & TAVARES, I. 2017. Reticular Formation and Pain: The Past and the Future. *Front Neuroanat,* 11**,** 51.

MATSUMOTO, M. & HIKOSAKA, O. 2009. Two types of dopamine neuron distinctly convey positive and negative motivational signals. *Nature,* 459**,** 837-41.

MAYER, D. J., WOLFLE, T. L., AKIL, H., CARDER, B. & LIEBESKIND, J. C. 1971. Analgesia from electrical stimulation in the brainstem of the rat. *Science,* 174**,** 1351-4.

MCDONALD, C. J., MAZZUCA, S. A. & MCCABE, G. P., JR. 1983. How much of the placebo 'effect' is really statistical regression? *Stat Med,* 2**,** 417-27.

MCDOWELL, A. R. & CARMICHAEL, D. W. 2019. Optimal repetition time reduction for single subject event-related functional magnetic resonance imaging. *Magn Reson Med,* 81**,** 1890-1897.

MEISSNER, K. & LINDE, K. 2018. Are Blue Pills Better Than Green? How Treatment Features Modulate Placebo Effects. *Int Rev Neurobiol,* 139, 357-378.

MELZACK, R. & CASEY, K. L. 1968. Sensory, motivational, and central control determinants of pain: a new conceptual model. *The skin senses,* 1, 423-43.

MELZACK, R. & WALL, P. D. 1965. Pain mechanisms: a new theory. *Science,* 150, 971-9.

MENSE, S. 1993. Nociception from skeletal muscle in relation to clinical muscle pain. *Pain,* 54, 241-289.

MILLAN, M. J. 2002. Descending control of pain. *Progress in Neurobiology,* 66, 355-474.

MILTON, G. W. 1973. Self-willed death or the bone-pointing syndrome. *Lancet,* 1, 1435-6.

MISCHKOWSKI, D., PALACIOS-BARRIOS, E. E., BANKER, L., DILDINE, T. C. & ATLAS, L. Y. 2018. Pain or nociception? Subjective experience mediates the effects of acute noxious heat on autonomic responses. *PAIN,* 159, 699-711.

MITSIKOSTAS, D. D., MANTONAKIS, L. I. & CHALARAKIS, N. G. 2011. Nocebo is the enemy, not placebo. A meta-analysis of reported side effects after placebo treatment in headaches. *Cephalalgia,* 31, 550-61.

MONTGOMERY, G. H. & KIRSCH, I. 1997. Classical conditioning and the placebo effect. *Pain,* 72, 107-13.

MUELLER, D., PORTER, J. T. & QUIRK, G. J. 2008. Noradrenergic signaling in infralimbic cortex increases cell excitability and strengthens memory for fear extinction. *J Neurosci,* 28, 369-75.

MUNN, B. R., MÜLLER, E. J., WAINSTEIN, G. & SHINE, J. M. 2021. The ascending arousal system shapes neural dynamics to mediate awareness of cognitive states. *Nature Communications,* 12, 6016.

NAGY, Z., HUTTON, C., DAVID, G., HINTERHOLZER, N., DEICHMANN, R., WEISKOPF, N. & VANNESJO, S. J. 2022. HiHi fMRI: a data-reordering method for measuring the hemodynamic response of the brain with high temporal resolution and high SNR. *Cereb Cortex.*

NAPADOW, V., SCLOCCO, R. & HENDERSON, L. A. 2019. Brainstem neuroimaging of nociception and pain circuitries. *Pain reports,* 4, e745-e745.

NASSER, H. M., CALU, D. J., SCHOENBAUM, G. & SHARPE, M. J. 2017. The Dopamine Prediction Error: Contributions to Associative Models of Reward Learning. *Frontiers in psychology,* 8, 244-244.

NOURBAKHSH, M. R. & OTTENBACHER, K. J. 1994. The Statistical Analysis of Single-Subject Data: A Comparative Examination. *Physical Therapy,* 74, 768-776.

O'REILLY, J. X., WOOLRICH, M. W., BEHRENS, T. E., SMITH, S. M. & JOHANSEN-BERG, H. 2012. Tools of the trade: psychophysiological interactions and functional connectivity. *Soc Cogn Affect Neurosci,* 7**,** 604-9.

OGAWA, S., LEE, T. M., KAY, A. R. & TANK, D. W. 1990. Brain magnetic resonance imaging with contrast dependent on blood oxygenation. *Proceedings of the National Academy of Sciences,* 87**,** 9868.

OLSEN, M. F., BJERRE, E., HANSEN, M. D., TENDAL, B., HILDEN, J. & HRÓBJARTSSON, A. 2018. Minimum clinically important differences in chronic pain vary considerably by baseline pain and methodological factors: systematic review of empirical studies. *J Clin Epidemiol,* 101**,** 87-106.e2.

ONGÜR, D., AN, X. & PRICE, J. L. 1998. Prefrontal cortical projections to the hypothalamus in macaque monkeys. *J Comp Neurol,* 401**,** 480-505.

PAGNINI, F., BARBIANI, D., CAVALERA, C., VOLPATO, E., GROSSO, F., MINAZZI, G. A., VAILATI RIBONI, F., GRAZIANO, F., DI TELLA, S., MANZONI, G. M., SILVERI, M. C., RIVA, G. & PHILLIPS, D. 2023. Placebo and Nocebo Effects as Bayesian-Brain Phenomena: The Overlooked Role of Likelihood and Attention. *Perspect Psychol Sci***,** 17456916221141383.

PALAZZO, E., LUONGO, L., NOVELLIS, V. D., ROSSI, F. & MAIONE, S. 2010. The Role of Cannabinoid Receptors in the Descending Modulation of Pain. *Pharmaceuticals (Basel, Switzerland),* 3**,** 2661-2673.

PAN, Y.-Z., LI, D.-P., CHEN, S.-R. & PAN, H.-L. 2004. Activation of μ -opioid receptors excites a population of locus coeruleus-spinal neurons through presynaptic disinhibition. *Brain Research,* 997**,** 67-78.

PAULI, W. M., LARSEN, T., COLLETTE, S., TYSZKA, J. M., SEYMOUR, B. & O'DOHERTY, J. P. 2015. Distinct Contributions of Ventromedial and Dorsolateral Subregions of the Human Substantia Nigra to Appetitive and Aversive Learning. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 35**,** 14220-14233.

PECIÑA, M., AZHAR, H., LOVE, T. M., LU, T., FREDRICKSON, B. L., STOHLER, C. S. & ZUBIETA, J. K. 2013. Personality trait predictors of placebo analgesia and neurobiological correlates. *Neuropsychopharmacology,* 38**,** 639-46.

PECIÑA, M., LOVE, T., STOHLER, C. S., GOLDMAN, D. & ZUBIETA, J. K. 2015. Effects of the Mu opioid receptor polymorphism (OPRM1 A118G) on pain regulation, placebo effects and associated personality trait measures. *Neuropsychopharmacology,* 40**,** 957-65.

PECIÑA, M., MARTÍNEZ-JAUAND, M., HODGKINSON, C., STOHLER, C. S., GOLDMAN, D. & ZUBIETA, J. K. 2014. FAAH selectively influences placebo effects. *Molecular psychiatry,* 19, 385-391.

PERTOVAARA, A. 2006. Noradrenergic pain modulation. *Progress in Neurobiology,* 80, 53-83.

PETROVIC, P., KALSO, E., PETERSSON, K. M. & INGVAR, M. 2002. Placebo and Opioid Analgesia-- Imaging a Shared Neuronal Network. *Science,* 295, 1737.

PFINGSTEN, M., LEIBING, E., HARTER, W., KRÖNER-HERWIG, B., HEMPEL, D., KRONSHAGE, U. & HILDEBRANDT, J. 2001. Fear-avoidance behavior and anticipation of pain in patients with chronic low back pain: a randomized controlled study. *Pain Med,* 2, 259-66.

PLANÈS, S., VILLIER, C. & MALLARET, M. 2016. The nocebo effect of drugs. *Pharmacology Research & Perspectives,* 4, e00208.

PRICE, D. D., FILLINGIM, R. B. & ROBINSON, M. E. 2006. Placebo analgesia: friend or foe? *Curr Rheumatol Rep,* 8, 418-24.

PRICE, D. D., FINNISS, D. G. & BENEDETTI, F. 2008. A Comprehensive Review of the Placebo Effect: Recent Advances and Current Thought. *Annual Review of Psychology,* 59, 565-590.

PUOPOLO, M. 2019. The hypothalamic-spinal dopaminergic system: a target for pain modulation. *Neural Regen Res,* 14, 925-930.

PURVES, D., AUGUSTINE, G., FITZPATRICK, D., KATZ, L., LAMANTIA, A., MCNAMARA, J. & WILLIAMS, S. 2001. Central pain pathways: the spinothalamic tract. *Neuroscience*, 8-10.

QUIRK, G. J., LIKHTIK, E., PELLETIER, J. G. & PARÉ, D. 2003. Stimulation of medial prefrontal cortex decreases the responsiveness of central amygdala output neurons. *J Neurosci,* 23, 8800-7.

REYNOLDS, D. V. 1969. Surgery in the rat during electrical analgesia induced by focal brain stimulation. *Science,* 164, 444-5.

SCHAFER, S. M., COLLOCA, L. & WAGER, T. D. 2015. Conditioned Placebo Analgesia Persists When Subjects Know They Are Receiving a Placebo. *Journal of Pain,* 16, 412-420.

SCHAFER, S. M., GEUTER, S. & WAGER, T. D. 2018. Mechanisms of placebo analgesia: A dual-process model informed by insights from cross-species comparisons. *Prog Neurobiol,* 160, 101-122.

SCHENK, L. A. & COLLOCA, L. 2019. The neural processes of acquiring placebo effects through observation. *Neuroimage*, 116510.

SCHENK, L. A., SPRENGER, C., ONAT, S., COLLOCA, L. & BÜCHEL, C. 2017. Suppression of Striatal Prediction Errors by the Prefrontal Cortex in Placebo Hypoalgesia. *J Neurosci,* 37, 9715-9723.

SCHIENLE, A., HÖFLER, C., ÜBEL, S. & WABNEGGER, A. 2018. Emotion-specific nocebo effects: an fMRI study. *Brain imaging and behavior,* 12, 180-187.

SCHMID, J., BINGEL, U., RITTER, C., BENSON, S., SCHEDLOWSKI, M., GRAMSCH, C., FORSTING, M. & ELSENBRUCH, S. 2015. Neural underpinnings of nocebo hyperalgesia in visceral pain: A fMRI study in healthy volunteers. *Neuroimage,* 120, 114-22.

SCHULTZ, W. 2002. Getting Formal with Dopamine and Reward. *Neuron,* 36, 241-263.

SCHWEINHARDT, P., SEMINOWICZ, D. A., JAEGER, E., DUNCAN, G. H. & BUSHNELL, M. C. 2009. The Anatomy of the Mesolimbic Reward System: A Link between Personality and the Placebo Analgesic Response. *The Journal of Neuroscience,* 29, 4882-4887.

SCOTT, D. J., STOHLER, C. S., EGNATUK, C. M., WANG, H., KOEPPE, R. A. & ZUBIETA, J. K. 2008. Placebo and nocebo effects are defined by opposite opioid and dopaminergic responses. *Arch Gen Psychiatry,* 65, 220-31.

SEELEY, W. W., MENON, V., SCHATZBERG, A. F., KELLER, J., GLOVER, G. H., KENNA, H., REISS, A. L. & GREICIUS, M. D. 2007. Dissociable intrinsic connectivity networks for salience processing and executive control. *Journal of Neuroscience,* 27, 2349-2356.

SEGAL, M. & SANDBERG, D. 1977. Analgesia produced by electrical stimulation of catecholamine nuclei in the rat brain. *Brain Res,* 123, 369-72.

SEVEL, L. S., CRAGGS, J. G., PRICE, D. D., STAUD, R. & ROBINSON, M. E. 2015. Placebo Analgesia Enhances Descending Pain-Related Effective Connectivity: A Dynamic Causal Modeling Study of Endogenous Pain Modulation. *The Journal of Pain,* 16, 760-768.

SHI, Y., CUI, S., ZENG, Y., HUANG, S., CAI, G., YANG, J. & WU, W. 2021. Brain Network to Placebo and Nocebo Responses in Acute Experimental Lower Back Pain: A Multivariate Granger Causality Analysis of fMRI Data. *Front Behav Neurosci,* 15, 696577.

SHI, Y., HUANG, S., ZHAN, H., WANG, Y., ZENG, Y., CAI, G., YANG, J. & WU, W. 2020. Personality Differences of Brain Networks in Placebo Analgesia and Nocebo Hyperalgesia: A Psychophysiological Interaction (PPI) Approach in fMRI. *Neural Plast,* 2020, 8820443.

SIKORA, M., HEFFERNAN, J., AVERY, E. T., MICKEY, B. J., ZUBIETA, J. K. & PECIÑA, M. 2016. Salience Network Functional Connectivity Predicts Placebo Effects in Major Depression. *Biol Psychiatry Cogn Neurosci Neuroimaging,* 1, 68-76.

SMITH, S. M. 2004. Overview of fMRI analysis. *The British Journal of Radiology,* 77, S167-S175.

SPISAK, T., KINCSES, B., SCHLITT, F., ZUNHAMMER, M., SCHMIDT-WILCKE, T., KINCSES, Z. T. & BINGEL, U. 2020. Pain-free resting-state functional brain connectivity predicts individual pain sensitivity. *Nature Communications,* 11**,** 187.

STROMAN, P. W., IOACHIM, G., POWERS, J. M., STAUD, R. & PUKALL, C. 2018. Pain processing in the human brainstem and spinal cord before, during, and after the application of noxious heat stimuli. *Pain,* 159**,** 2012-2020.

STROMAN, P. W., POWERS, J. M., IOACHIM, G., WARREN, H. J. M. & MCNEIL, K. 2021. Investigation of the neural basis of expectation-based analgesia in the human brainstem and spinal cord by means of functional magnetic resonance imaging. *Neurobiology of Pain,* 10**,** 100068.

SZIKSZAY, T. M., ADAMCZYK, W. M., CARVALHO, G. F., MAY, A. & LUEDTKE, K. 2020. Offset analgesia: somatotopic endogenous pain modulation in migraine. *PAIN,* 161**,** 557-564.

TANG, W., JBABDI, S., ZHU, Z., COTTAAR, M., GRISOT, G., LEHMAN, J. F., YENDIKI, A. & HABER, S. N. 2019. A connectional hub in the rostral anterior cingulate cortex links areas of emotion and cognitive control. *Elife,* 8.

TANGREA, J. A., ADRIANZA, M. E. & HELSEL, W. E. 1994. Risk factors for the development of placebo adverse reactions in a multicenter clinical trial. *Annals of Epidemiology,* 4**,** 327-331.

TAYLOR, B. K. & WESTLUND, K. N. 2017. The noradrenergic locus coeruleus as a chronic pain generator. *J Neurosci Res,* 95**,** 1336-1346.

TAYLOR, N. E., PEI, J., ZHANG, J., VLASOV, K. Y., DAVIS, T., TAYLOR, E., WENG, F.-J., VAN DORT, C. J., SOLT, K. & BROWN, E. N. 2019. The Role of Glutamatergic and Dopaminergic Neurons in the Periaqueductal Gray/Dorsal Raphe: Separating Analgesia and Anxiety. *eNeuro,* 6**,** ENEURO.0018-18.2019.

TÉTREAULT, P., MANSOUR, A., VACHON-PRESSEAU, E., SCHNITZER, T. J., APKARIAN, A. V. & BALIKI, M. N. 2016. Brain Connectivity Predicts Placebo Response across Chronic Pain Clinical Trials. *PLOS Biology,* 14**,** e1002570.

THOMAIDOU, M. A., PEERDEMAN, K. J., KOPPESCHAAR, M. I., EVERS, A. W. M. & VELDHUIJZEN, D. S. 2021. How Negative Experience Influences the Brain: A Comprehensive Review of the Neurobiological Underpinnings of Nocebo Hyperalgesia. *Front Neurosci,* 15**,** 652552.

THOMAS, K. B. 1987. General practice consultations: is there any point in being positive? *Br Med J (Clin Res Ed),* 294**,** 1200-2.

TINNERMANN, A., GEUTER, S., SPRENGER, C., FINSTERBUSCH, J. & BÜCHEL, C. 2017. Interactions between brain and spinal cord mediate value effects in nocebo hyperalgesia. *Science,* 358, 105-108.

TIWARI, A. K., ZAI, C. C., SAJEEV, G., ARENOVICH, T., MÜLLER, D. J. & KENNEDY, J. L. 2013. Analysis of 34 candidate genes in bupropion and placebo remission. *Int J Neuropsychopharmacol,* 16, 771-81.

TODD, K. H., FUNK, K. G., FUNK, J. P. & BONACCI, R. 1996. Clinical significance of reported changes in pain severity. *Ann Emerg Med,* 27, 485-9.

TOI, P. T., JANG, H. J., MIN, K., KIM, S. P., LEE, S. K., LEE, J., KWAG, J. & PARK, J. Y. 2022. In vivo direct imaging of neuronal activity at high temporospatial resolution. *Science,* 378, 160-168.

TU, Y., WILSON, G., CAMPRODON, J., DOUGHERTY, D. D., VANGEL, M., BENEDETTI, F., KAPTCHUK, T. J., GOLLUB, R. L. & KONG, J. 2021. Manipulating placebo analgesia and nocebo hyperalgesia by changing brain excitability. *Proc Natl Acad Sci U S A,* 118.

TU, Y., ZHANG, L. & KONG, J. 2022. Placebo and nocebo effects: from observation to harnessing and clinical application. *Translational Psychiatry,* 12, 524.

UEMATSU, A., TAN, B. Z., YCU, E. A., CUEVAS, J. S., KOIVUMAA, J., JUNYENT, F., KREMER, E. J., WITTEN, I. B., DEISSEROTH, K. & JOHANSEN, J. P. 2017. Modular organization of the brainstem noradrenaline system coordinates opposing learning states. *Nature Neuroscience,* 20, 1602-1611.

VACHON-PRESSEAU, E., BERGER, S. E., ABDULLAH, T. B., HUANG, L., CECCHI, G. A., GRIFFITH, J. W., SCHNITZER, T. J. & APKARIAN, A. V. 2018. Brain and psychological determinants of placebo pill response in chronic pain patients. *Nat Commun,* 9, 3397.

VAGHELA, V., KESAVADAS, C. & THOMAS, B. 2010. Functional magnetic resonance imaging of the brain: a quick review. *Neurol India,* 58, 879-85.

VAN DEN HEUVEL, M. P. & HULSHOFF POL, H. E. 2010. Exploring the brain network: a review on resting-state fMRI functional connectivity. *Eur Neuropsychopharmacol,* 20, 519-34.

VAN WIJK, G. & VELDHUIJZEN, D. S. 2010. Perspective on Diffuse Noxious Inhibitory Controls as a Model of Endogenous Pain Modulation in Clinical Pain Syndromes. *The Journal of Pain,* 11, 408-419.

VELO, P., LEIRAS, R. & CANEDO, A. 2013. Electrophysiological study of supraspinal input and spinal output of cat's subnucleus reticularis dorsalis (SRD) neurons. *PLoS One,* 8, e60686.

VOGT, B. A. 2005. Pain and emotion interactions in subregions of the cingulate gyrus. *Nature reviews. Neuroscience,* 6, 533-544.

VOUDOURIS, N. J., PECK, C. L. & COLEMAN, G. 1985. Conditioned placebo responses. *J Pers Soc Psychol,* 48, 47-53.

VOUDOURIS, N. J., PECK, C. L. & COLEMAN, G. 1989. Conditioned response models of placebo phenomena: further support. *Pain,* 38, 109-116.

VOUDOURIS, N. J., PECK, C. L. & COLEMAN, G. 1990. The role of conditioning and verbal expectancy in the placebo response. *Pain,* 43, 121-128.

WAGER, T. D. & ATLAS, L. Y. 2015. The neuroscience of placebo effects: connecting context, learning and health. *Nature Reviews Neuroscience,* 16, 403-418.

WAGER, T. D., ATLAS, L. Y., LINDQUIST, M. A., ROY, M., WOO, C. W. & KROSS, E. 2013. An fMRI-based neurologic signature of physical pain. *N Engl J Med,* 368, 1388-97.

WAGER, T. D., DAVIDSON, M. L., HUGHES, B. L., LINDQUIST, M. A. & OCHSNER, K. N. 2008. Prefrontal-subcortical pathways mediating successful emotion regulation. *Neuron,* 59, 1037-50.

WAGER, T. D., RILLING, J. K., SMITH, E. E., SOKOLIK, A., CASEY, K. L., DAVIDSON, R. J., KOSSLYN, S. M., ROSE, R. M. & COHEN, J. D. 2004. Placebo-induced changes in FMRI in the anticipation and experience of pain. *Science,* 303, 1162-1167.

WAGER, T. D., SCOTT, D. J. & ZUBIETA, J. K. 2007. Placebo effects on human mu-opioid activity during pain. *Proc Natl Acad Sci U S A,* 104, 11056-61.

WAGNER, I. C., RÜTGEN, M., HUMMER, A., WINDISCHBERGER, C. & LAMM, C. 2020. Placebo-induced pain reduction is associated with negative coupling between brain networks at rest. *NeuroImage,* 219, 117024.

WALL, P. D. 1979. On the relation of injury to pain. The John J. Bonica lecture. *Pain,* 6, 253-264.

WAMPOLD, B. E., IMEL, Z. E. & MINAMI, T. 2007. The story of placebo effects in medicine: evidence in context. *J Clin Psychol,* 63, 379-90; discussion 405-8.

WANG, L. H., DING, W. Q. & SUN, Y. G. 2022a. Spinal ascending pathways for somatosensory information processing. *Trends Neurosci,* 45, 594-607.

WANG, Y., CHAN, E., DORSEY, S. G., CAMPBELL, C. M. & COLLOCA, L. 2022b. Who are the placebo responders? A cross-sectional cohort study for psychological determinants. *Pain,* 163, 1078-1090.

WANG, Z. X., ZHANG, N. N., ZHAO, H. X. & SONG, J. 2022c. Nocebo effect in multiple system atrophy: systematic review and meta-analysis of placebo-controlled clinical trials. *Neurol Sci,* 43, 899-905.

WHITNEY, C. W. & VON KORFF, M. 1992. Regression to the mean in treated versus untreated chronic pain. *Pain,* 50, 281-285.

WIECH, K. 2016. Deconstructing the sensation of pain: The influence of cognitive processes on pain perception. *Science,* 354, 584-587.

WILCOX, C. E., MAYER, A. R., TESHIBA, T. M., LING, J., SMITH, B. W., WILCOX, G. L. & MULLINS, P. G. 2015. The Subjective Experience of Pain: An FMRI Study of Percept-Related Models and Functional Connectivity. *Pain medicine (Malden, Mass.),* 16, 2121-2133.

WOO, C.-W., ROY, M., BUHLE, J. T. & WAGER, T. D. 2015. Distinct Brain Systems Mediate the Effects of Nociceptive Input and Self-Regulation on Pain. *PLOS Biology,* 13, e1002036.

YE, R., RUA, C., O'CALLAGHAN, C., JONES, P. S., HEZEMANS, F. H., KAALUND, S. S., TSVETANOV, K. A., RODGERS, C. T., WILLIAMS, G., PASSAMONTI, L. & ROWE, J. B. 2021. An in vivo probabilistic atlas of the human locus coeruleus at ultra-high field. *NeuroImage,* 225, 117487.

YOSHIDA, W., SEYMOUR, B., KOLTZENBURG, M. & DOLAN, R. J. 2013. Uncertainty increases pain: evidence for a novel mechanism of pain modulation involving the periaqueductal gray. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 33, 5638-5646.

YOUSSEF, A. M., MACEFIELD, V. G. & HENDERSON, L. A. 2016. Pain inhibits pain; human brainstem mechanisms. *Neuroimage,* 124, 54-62.

YU, R., GOLLUB, R. L., VANGEL, M., KAPTCHUK, T., SMOLLER, J. W. & KONG, J. 2014. Placebo analgesia and reward processing: integrating genetics, personality, and intrinsic brain activity. *Hum Brain Mapp,* 35, 4583-93.

ZAKI, J., OCHSNER, K. N., HANELIN, J., WAGER, T. D. & MACKEY, S. C. 2007. Different circuits for different pain: patterns of functional connectivity reveal distinct networks for processing pain in self and others. *Soc Neurosci,* 2, 276-91.

ZAMAN, J., CHALKIA, A., ZENSES, A.-K., BILGIN, A. S., BECKERS, T., VERVLIET, B. & BODDEZ, Y. 2021. Perceptual variability: Implications for learning and generalization. *Psychonomic Bulletin & Review,* 28, 1-19.

ZHANG, C., YANG, S. W., GUO, Y. G., QIAO, J. T. & DAFNY, N. 1997. Locus coeruleus stimulation modulates the nociceptive response in parafascicular neurons: an analysis of descending and ascending pathways. *Brain Res Bull,* 42, 273-8.

ZHUO, M. & GEBHART, G. F. 1997. Biphasic modulation of spinal nociceptive transmission from the medullary raphe nuclei in the rat. *J Neurophysiol,* 78**,** 746-58.

ZUBIETA, J. K., BUELLER, J. A., JACKSON, L. R., SCOTT, D. J., XU, Y., KOEPPE, R. A., NICHOLS, T. E. & STOHLER, C. S. 2005. Placebo effects mediated by endogenous opioid activity on mu-opioid receptors. *J Neurosci,* 25**,** 7754-62.

ZUNHAMMER, M., SPISÁK, T., WAGER, T. D., BINGEL, U., ATLAS, L., BENEDETTI, F., BÜCHEL, C., CHOI, J. C., COLLOCA, L., DUZZI, D., EIPPERT, F., ELLINGSEN, D.-M., ELSENBRUCH, S., GEUTER, S., KAPTCHUK, T. J., KESSNER, S. S., KIRSCH, I., KONG, J., LAMM, C., LEKNES, S., LUI, F., MÜLLNER-HUBER, A., PORRO, C. A., RÜTGEN, M., SCHENK, L. A., SCHMID, J., THEYSOHN, N., TRACEY, I., WROBEL, N., ZEIDAN, F. & THE PLACEBO IMAGING, C. 2021. Meta-analysis of neural systems underlying placebo analgesia from individual participant fMRI data. *Nature Communications,* 12**,** 1391.

Appendix A:

Brain activity changes associated with pain perception variability. Cerebral Cortex [online ahead of print].

OXFORD

Cerebral Cortex, 2022, 1–11
https://doi.org/10.1093/cercor/bhac332
Original Article

Brain activity changes associated with pain perception variability

L. Crawford [1], E. Mills[1], N. Meylakh[1], P.M. Macey[2], V.G. Macefield [3,4], L.A. Henderson[1,*]

[1]Department of Anatomy and Histology, School of Medical Sciences, Brain and Mind Centre, University of Sydney, New South Wales 2006, Australia,
[2]UCLA School of Nursing, University of California, Los Angeles, California 90095, United States,
[3]Baker Heart and Diabetes Institute, Melbourne, Victoria 3004, Australia,
[4]Department of Anatomy & Physiology, University of Melbourne, Melbourne, Victoria, 3052, Australia
*Corresponding author: Department of Anatomy and Histology, 94 Mallett Street Camperdown, School of Medical Sciences, Brain and Mind Centre, University of Sydney, Sydney, New South Wales 2006, Australia. Email: lukeh@anatomy.usyd.edu.au

Pain perception can be modulated by several factors. Phenomena like temporal summation leads to increased perceived pain, whereas behavioral conditioning can result in analgesic responses. Furthermore, during repeated, identical noxious stimuli, pain intensity can vary greatly in some individuals. Understanding these variations is important, given the increase in investigations that assume stable baseline pain for accurate response profiles, such as studies of analgesic mechanisms. We utilized functional magnetic resonance imaging to examine the differences in neural circuitry between individuals displaying consistent pain ratings and those who experienced variable pain during a series of identical noxious stimuli. We investigated 63 healthy participants: 31 were assigned to a "consistent" group, and 32 were assigned to a "variable" group dependent on pain rating variability. Variable pain ratings were associated with reduced signal intensity in the dorsolateral prefrontal cortex (dlPFC). Furthermore, the dlPFC connectivity with the primary somatosensory cortex and temperoparietal junction was significantly reduced in variable participants. Our results suggest that investigators should consider variability of baseline pain when investigating pain modulatory paradigms. Additionally, individuals with consistent and variable pain ratings differ in their dlPFC activity and connectivity with pain-sensitive regions during noxious stimulation, possibly reflecting the differences in attentional processing and catastrophizing during pain.

Key words: acute pain; connectivity; dorsolateral prefrontal cortex; noxious stimuli; pain catastrophizing.

Introduction

It is well understood that pain perception can be modulated by a number of intrinsic and extrinsic factors. For example, the perceived intensity of incoming noxious information can be modified by stress, attention, emotional, and cognitive processes (Tracey and Mantyh 2007; Ploner et al. 2011). During somatosensation, the salience of incoming information determines whether the stimulus is perceived or not, and often incoming potentially noxious information does not to reach painful levels (for example during warm or pricking sensations). Conversely, within the pain-processing system, repeated application of identical noxious stimuli at relatively high frequencies can lead to a gradual increase in the intensity of perceived pain, i.e. temporal summation (Price et al. 2002), and in individuals with chronic pain, sensitization within the central nervous system results in increased sensitivity to noxious stimuli (Latremoliere and Woolf 2009).

Interindividual differences in the perceived pain intensity to noxious stimuli of identical intensities have been well described and correspond with differential activation in the primary somatosensory and prefrontal cortices (Coghill et al. 2003; Fillingim 2005). However, it is also the case that during repeated identical noxious stimuli, subsequent pain intensity ratings can vary substantially in some individuals but remain relatively consistent in others. Examining variations in perceived pain intensities is important, given the rapid increase in investigations exploring phenomena, such as endogenous analgesic mechanisms, which rely on stable baseline pain intensity ratings for accurate response profiles. Furthermore, determining the neural circuitry responsible for changes in the perceived pain intensity in the context of persistent pain may provide a potential target for pain modulation.

The neural mechanism responsible for large, relatively rapid variations in the pain intensity perception within some individuals has not been explored. Given that, in typical experiments, the perceived pain intensity can vary up and down and noxious stimuli are often presented at relatively long interstimulus intervals (ISIs), such instability is not likely related to gradual changes in receptor sensitivities, or phenomena such as wind-up and temporal summation (Price et al. 2002; Latremoliere and Woolf 2009). Mechanisms associated with higher-order brain function, such as salience and attention, can alter the intensity of perceived pain (Tracey et al. 2002), and these higher-order processes can fluctuate even during relatively rapid and repeated noxious stimuli (Miron et al. 1989; Borsook et al. 2013). For example, in a recent investigation, it was reported that "mind wandering" during noxious stimulation was associated with altered activity in higher-order processing regions such as the dorsolateral prefrontal and insula cortices (Kucyi et al. 2013). Whether such mechanisms are responsible for variations in pain intensity perception remains unknown, although changes in higher-order processing associated with such pain perception variability would be consistent with this interpretation.

The aim of this retrospective investigation was to use functional magnetic resonance imaging (fMRI) to determine the

Received: May 16, 2022. **Revised:** July 29, 2022. **Accepted:** July 30, 2022

differences in the underlying neural circuitry function in individuals who display consistent pain intensity ratings compared with those who display variable pain intensities during a series of noxious thermal stimuli of identical intensities. We hypothesized that participants who perceive repeated identical noxious stimuli as having variable pain intensities will display different activation in higher-order processing regions, such as the dorsolateral prefrontal, insula, and primary somatosensory cortices, compared to participants who experience consistent pain intensity ratings.

Materials and methods

Participants

Sixty-three pain-free participants (26 males, mean [±SD] age: 24.5 ± 6.3 years) were recruited for the study. Informed written consent was obtained for all procedures, which were conducted under the approval by the University of Sydney Human Research Ethics Committees and satisfied the Declaration of Helsinki. Using G*Power 3 (Faul et al. 2007), an a priori power analysis was performed using results from a previous imaging study investigating pain rating variability and temporal summation (Rogachov et al. 2016). This revealed that a total sample size of 55 would be necessary to detect similar effect sizes with 90% power ($\rho = -0.373$, $\alpha = 0.05$, power = 0.90).

Magnetic resonance imaging scans

Prior to entering the magnetic resonance imaging (MRI) scanner, a 3 × 3-cm MRI-compatible Peltier-element thermode (Medoc) was secured to the skin of the right side of the lower lip. The original investigation from which this retrospective dataset arose was exploring a pain modulatory paradigm, and as such the lower lip was elected as the thermal stimulation site as to enable investigation of the entire ascending and descending orofacial pain pathway from the spinal trigeminal nucleus rostrally (Youssef et al. 2016a, 2016b). To determine a temperature that evoked a moderate pain rating in each individual, the thermode temperature was raised with a Thermal Sensory Analyzer (TSA-II, Medoc) from a resting temperature of 32 °C to various temperatures at 0.5 °C intervals between 44 and 49 °C. Temperatures were randomly applied in 15-s intervals for a duration of 10 s during which each participant continuously rated their pain intensity (0 = no pain, 10 = worse imaginable pain) in real time using a computerized visual analog scale (CoVAS, Medoc). The temperature which generated a pain intensity rating of approximately 6 out of 10 was then used for the remainder of the experiment.

Each participant was then positioned supine onto the MRI scanner bed and was placed into a 3 Tesla MRI scanner (Intera, Philips Medical Systems, The Netherlands), with the head immobilized in a 32-channel transmit-receive head coil to which padding was added to prevent head movement. With the participant relaxed, a fMRI series consisting of 140 gradient echo echo-planar image sets with blood oxygen level–dependent contrast covering the entire brain was collected (38 axial slices, repetition time = 2,500 ms, echo time = 40 ms, flip angle = 90°, turbo factor = 45, raw voxel size = 1.5 × 1.5 × 4.0 mm thick). During this fMRI series, following a 30-volume baseline period, 8 noxious thermal stimuli of "identical temperatures" were delivered. Each noxious stimulus was delivered for 15 s (including ramp up and down periods of 2.5 s each), followed by a 6-volume, 15-s baseline (32 °C) period. During the entire scan period, participants used an MR-compatible continuous slider to continuously report their perceived pain perception. The CoVAS scale was shown on a reflected digital screen at the end of the magnet bore, and participants controlled the position of a slider to report their pain continuously by holding the left (moved slider toward zero) or right (moved slider toward ten) with their left middle and index fingers. Pain rating data were recorded in 2.5-s periods overlapping with each of the collected volumes of the scanning sequence. Finally, a T1-weighted anatomical image was also collected (288 axial slices, repetition time = 5,600 ms, raw voxel size = 0.87 × 0.87 × 0.87 mm thick).

For each participant, the mean pain intensity ratings during each of the 8 noxious stimulus periods were calculated. In each participant, the standard deviation (SD) of the 8 pain intensity ratings was then calculated as an indicator of their pain rating variability. These SDs were then plotted for all 63 participants and a median spilt used to separate the groups into consistent and variable groups. Additionally, consistent and variable participants VAS responses during each volume of the scan were binarized (1 = any volume with a nonzero VAS response; 0 = any volume with a zero VAS response) and were converted to a percentage of the group showing a VAS response in each functional volume overlapping with a noxious stimulus or ramp period to determine if any temporal effects of latency in pain perception related to the allocation of a participant as either consistent or variable (Supplementary Fig. 1). Finally, prior to the scanning session, each participant completed the Pain Catastrophizing Questionnaire (Sullivan et al. 1995).

MRI scan analysis

Using Statistical Parametric Mapping (SPM12) (Friston et al. 1994) and custom software, the fMRI image sets were realigned and movement parameters examined to ensure no participant displayed > 1 mm volume-to-volume movement in the x, y, and z planes and 0.05 radians in the pitch, roll, and yaw directions. Cardiac (frequency band of 60–120 beats per minute +1 harmonic) and respiratory (frequency band of 8–25 breaths per minute +1 harmonic) noise was modeled and removed using the Dynamic Retrospective Filtering toolbox (Särkkä et al. 2012) and images sets were then linear detrended to remove global signal intensity changes. Movement parameters were modeled and removed from the fMRI signal by removing any signal correlated with the movement parameters, similar to the linear model of global signal detrending method developed by Macey et al. (2004). The fMRI images were then coregistered to each participant's T1-weighted anatomical image. The T1-weighted image was spatially normalized to the Montreal Neurological Institute (MNI) template and the normalization parameters were applied to the fMRI images. This process resulted in the fMRI images being resliced into 2 × 2 × 2 mm voxels. The fMRI images were then spatially smoothed using a 6-mm full-width at half maximum Gaussian filter.

For each participant, significant changes in the signal intensity during the repeated noxious stimuli during the fMRI scan were determined using a repeated box-car model convolved with a canonical hemodynamic response function. The resulting contrast maps for the consistent and variable groups were placed into 2 separate 1 group, random-effects, second-level analyses, and significant increases in either group were determined with age, sex, and elected moderate temperature as nuisance variables ($P < 0.05$, false discovery rate–corrected, cluster extent threshold = 10 contiguous voxels). Binary maps of these significant increases were made and masked together using the SPM12 "imcalc" function to identify the overlap in regional increases across both groups. In addition, significant differences in signal intensity changes during the 8 noxious stimuli between the consistent and variable groups were determined with age, sex,

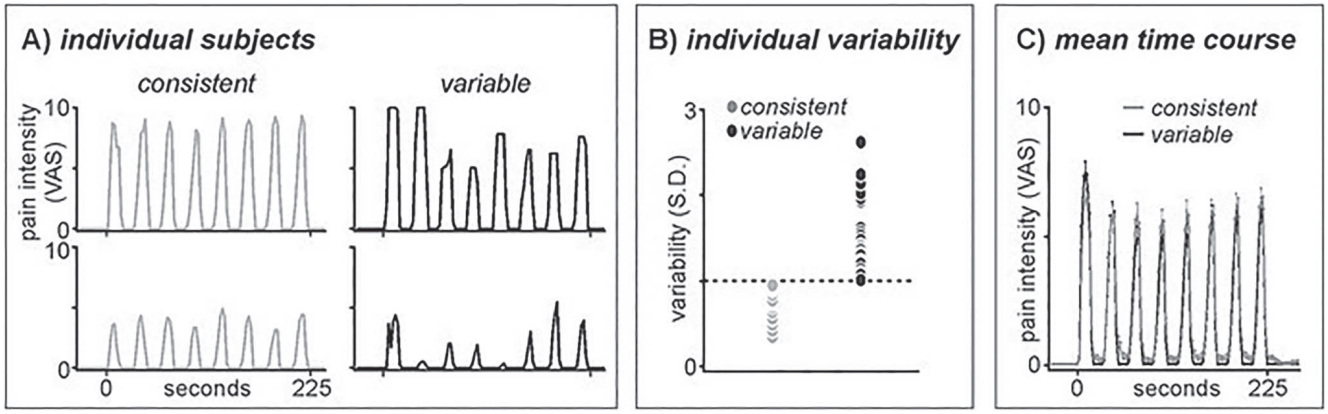

Fig. 1. Pain intensity rating variability in individuals receiving 8 identical noxious heat stimuli to the right side of the mouth. Notably, in the "consistent" group, there was very little variation in their pain intensity ratings, whereas in the "variable" group, the ratings could vary dramatically during successive noxious stimuli of identical intensities. A) On-line pain intensity ratings measured on a 10-cm visual analog scale (VAS) from 0 = no pain to 10 = most intense pain imaginable during 8 noxious thermal stimuli of equal intensities in 4 participants. The 2 participants to the left display consistent pain intensity ratings during each of the 8 noxious stimuli (gray), whereas the 2 participants to the right display variable responses despite identical thermal stimuli intensities during each of the 8 stimuli (black). B) Participants pain intensity variability responses were plotted and a median split resulted in a group of participants in which their ratings were considered consistent ($n = 31$) or variable ($n = 32$) if the SD of their 8 pain intensity rating was either $<$ or >1, respectively. C) Mean ± SEM pain intensity ratings in consistent ($n = 31$, gray) and variable ($n = 32$, black) pain intensity rating participants. Despite differences in the variability of pain intensity ratings at an individual participant level, both groups display almost identical overall pain intensity ratings.

and elected moderate temperature as nuisance variables (2-sample t-test, $P < 0.05$, false discovery rate–corrected, cluster extent threshold = 10 contiguous voxels). This analysis resulted in a single significant cluster in the dorsolateral prefrontal cortex (dlPFC) from which the percentage changes, relative to the 75-s baseline period, in the signal intensity for each volume were extracted and the mean of the consistent and variable groups was plotted. In addition, the mean ± SEM signal intensity changes during the noxious periods were plotted for each group and the signal intensity change during each of the 8 noxious stimulus periods was determined. From these first 2 analyses, a forward lag in peak dlPFC activity was identified in variable compared to consistent participants, and so we further conducted a temporal analysis comparing the latency in stimulus onset to peak dlPFC activity in both consistent and variable participants during each of the 8 stimulus periods (inclusive of ramp up and down volumes). To add rigor that this region was responsible for the consistent perception of identical noxious stimuli, we conducted an extreme value analysis with the 10 most variable and 10 least variable participants and calculated the mean ± SEM signal intensity changes in the dlPFC (2-sample t-tests, $P < 0.05$).

Finally, we performed 2 separate psychophysiological interaction (PPI) analyses within SPM12 software (Ashburner et al. 2014) to determine task-specific and ISI changes in the functional connectivity between the dlPFC cluster derived from the 2-group analysis described above and all other voxels in the brain. In each individual, the percentage signal intensity change was extracted from the dlPFC cluster and multiplied by the either the repeated box-car model (task-specific changes), or time points of ISI within the scanning sequence to create a PPI regressor. Essentially, this results in a regressor in which we are searching for voxels that are correlated with the timecourse of the dlPFC during the noxious stimulation periods and are anticorrelated during the periods between noxious stimuli, and it is vice versa for the ISI PPI regressor. The resulting PPI brain maps were then entered into second-level random-effects analyses and differences between consistent and variable participants were determined with age, sex, and elected moderate temperature entered as nuisance variables. There were no significant differences following correction for multiple comparisons, so we subsequently lowered the significance threshold to $P < 0.001$, uncorrected. We set the minimum contiguous cluster size to 10 voxels to reduce the chances of a type 1 error.

Results

Psychophysics

Each participant rated all of the 8 thermal stimuli as painful, with the average pain intensity rating being 6.1 ± 2.0 out of 10 (mean VAS ± SD). Plots of the mean peak intensity rating during each of the 8 noxious stimuli in each participant revealed that the variance of these ratings varied considerably from a SD of 0.2 to 3.3 (Fig. 1A). The median SD was 1.0 and participants were subsequently divided into consistent (SD < 1) and variable (SD > 1) groups: 31 participants were placed in the consistent group and 32 were placed in the variable group (Supplementary Table 1). Figure 1A shows plots of on-line pain intensity ratings during these 8 noxious stimuli in 2 consistent and 2 variable participants. Volume-to-volume movement did not exceed our criteria in any of our 63 participants, nor significantly differ, between our "Consistent" (32) and "Variable" (31) groups: x-axis: Consistent = 0.01 ± 0.12; Variable = 0.12 ± 0.41, $P = 0.18$, y-axis: Consistent = 0.09 ± 0.32; Variable = 0.17 ± 0.26, $P = 0.35$; z-axis: Consistent = 0.16 ± 0.74, Variable = 0.32 ± 0.51, $P = 0.34$; Roll: Consistent = 0.001 ± 0.009, Variable = 0.005 ± 0.008, $P = 0.12$; Pitch: Consistent = 0.001 ± 0.005, Variable = 0.001 ± 0.005, $P = 0.40$; Yaw: Consistent = 0.001 ± 0.005, Variable = 0.003 ± 0.009, $P = 0.18$ (mean ± SD, paired t-test). By definition, in the consistent group, there was very little variation in their pain intensity ratings, whereas, in the variable group, the ratings could vary dramatically during the successive noxious stimuli of identical intensities. Figure 1B is a scatter plot of the SD of pain intensity ratings during 8 identical noxious stimuli in all 63 participants, and Fig. 1C shows the mean ± SEM pain intensity ratings for the consistent and variable groups. Across all participants, the first stimuli in the series were rated significantly more painful than the remaining 7 stimuli $F(7,496) = 5.67$, $P = 0.000003$. However, the remaining 7 stimuli were not rated as significantly different to

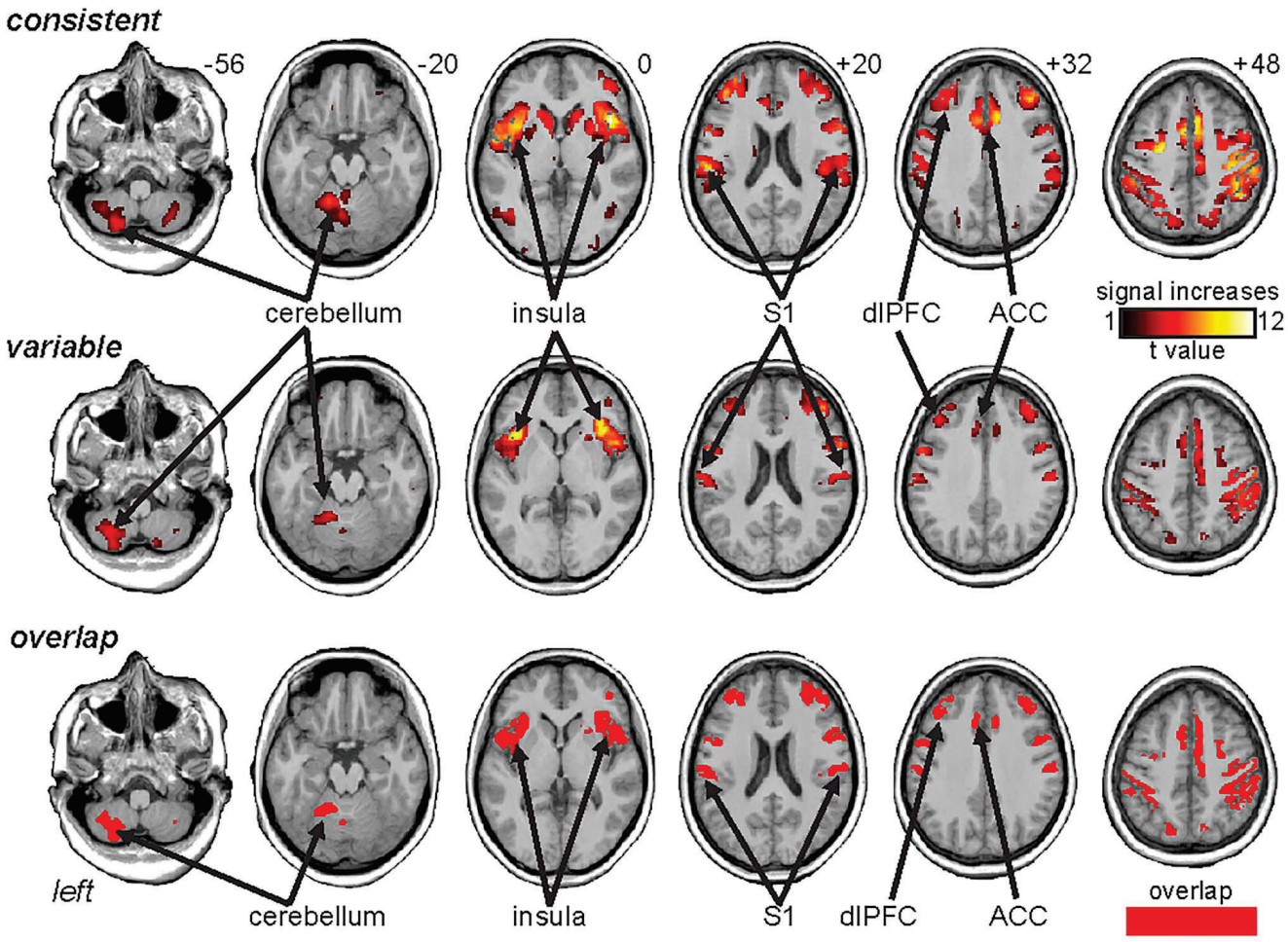

Fig. 2. Signal intensity increases (hot color scale) overlaid onto axial slices of a mean T1-weighted anatomical image during 8 noxious thermal stimuli applied to the right side of the lips. Slice locations in MNI space are indicated at the top right of each slice. The top 2 rows show significant signal intensity increases in the consistent and variable groups, respectively. The bottom row shows an imcalc overlay of the maps shown in the upper and middle rows converted into a binarized map, allowing easy interpretation of the overlapping regions (red shading) where both groups displayed significant signal intensity increases. ACC: anterior cingulate cortex.

each other $F(6,434) = 0.91$, $P = 0.49$. As the "variable" group were delineated based on the variability in their pain ratings, this effect was more pronounced in the variable group ($P = 0.0004$) than in the consistent ($P = 0.46$) group. Additionally, there was no significant relationships between the test temperature applied and either the SD of pain ratings ($R = 0.14$, $P = 0.26$), or the mean pain ratings ($R = 0.18$, $P = 0.16$) across all participants. Although, overall, the 2 participant groups displayed almost identical mean pain intensity ratings during all 8 noxious stimuli, at an individual level, participants displayed either consistent or variable pain intensity ratings. There was no significant difference between the consistent and variable groups with respect to age (mean ± SEM; consistent: 24.9 ± 1.0, variable: 24.2 ± 1.2, $P = 0.63$), sex (consistent: 13 males, variable: 13 males, $P = 0.75$), thermode temperature (mean ± SEM °C: consistent: 47.7 ± 0.15, variable: 48.0 ± 0.13, $P = 0.08$), or time between the stimulus and pain onset for each stimulus period (stimulus 1: $P = 0.92$; stimulus 2: $P = 0.84$; stimulus 3: $P = 0.77$; stimulus 4: $P = 0.79$; stimulus 5: $P = 0.91$; stimulus 6: $P = 0.98$; stimulus 7: $P = 0.97$; stimulus 8: $P = 0.87$; Supplementary Fig. 1; consistent vs. variable participants; 2-sample *t*-test). However, the consistent group reported significantly higher pain catastrophizing scores compared to the variable group (mean ± SEM catastrophizing score: consistent: 15.4 ± 0.3, variable 10.3 ± 1.4, $P = 0.03$), and there was a significant negative relationship between pain catastrophizing and pain intensity variability ($r = -0.26$, $P = 0.04$).

Signal intensity changes

Analysis of signal intensity changes in all participants during noxious stimulation revealed signal intensity increases in a number of brain regions (Fig. 2). In both the variable and consistent groups, increases in signal intensity during noxious thermal stimuli occurred in the left (contralateral) cerebellar cortex, and bilaterally in the insula, primary somatosensory cortex (S1), anterior cingulate cortex, and the dlPFC.

Direct comparison of signal intensity changes in consistent compared with variable participant groups revealed a single region in which signal intensity increased significantly more in the consistent group compared with the variable group. This cluster was located in the region of the left dlPFC ($P = 0.043$ FDR-corrected) (Fig. 3, Table 1). The reverse contrast was also investigated but revealed no significant cluster in which signal increase was significantly more in variable compared with consistent participants. Extraction of beta values revealed a significant inverse relationship between the dlPFC activity and pain rating variability across all 63 participants ($R = 0.34$, $P = 0.007$; Fig. 3A). Moreover, extraction of percentage signal intensity changes in

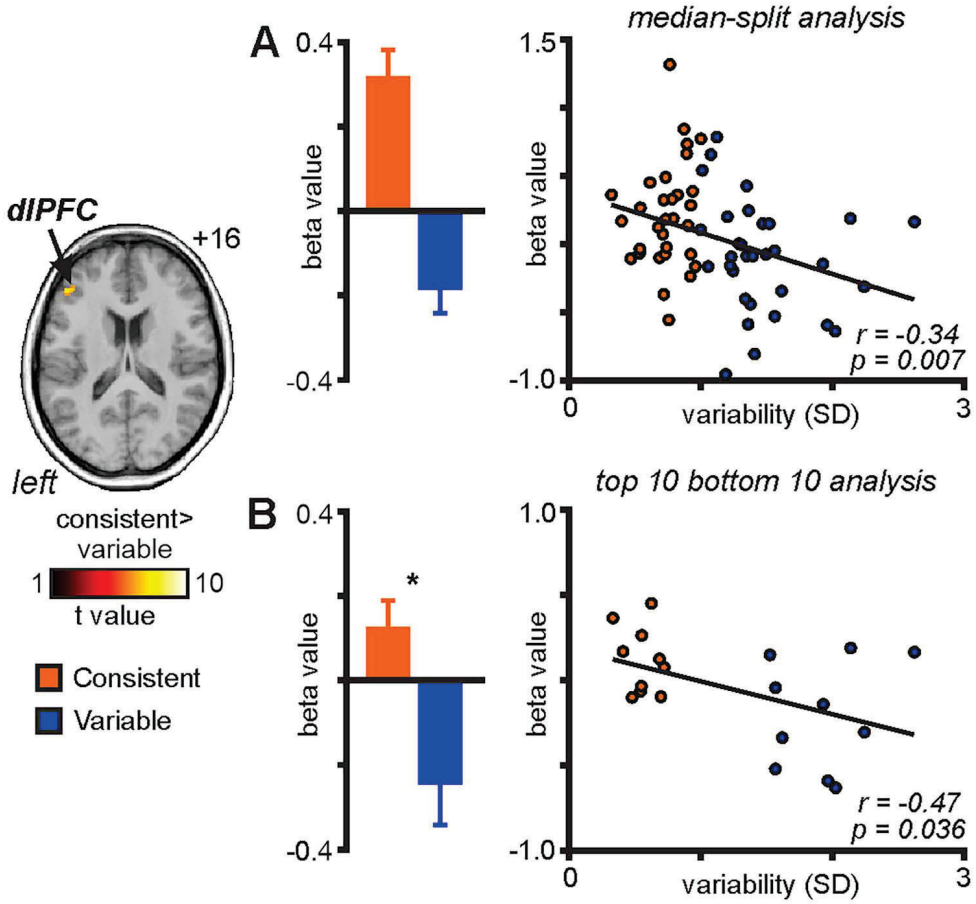

Fig. 3. Region in which there was a significant difference in signal intensity during 8 noxious thermal stimuli in participants who display consistent versus those who display variable pain intensity ratings. The region of significant difference in the left dlPFC is color-coded (hot color scale) and overlaid onto a mean T1-weighted anatomical axial slice. A) Extraction of beta values from the left dlPFC revealed a significant increase in signal intensity in consistent participants compared to variable participants. Additionally, across all 63 participants, a significant inverse relationship was observed between pain rating variability and left dlPFC signal intensity. B) An "extreme-value" analysis isolating the 10 lowest (consistent) and 10 highest (variable) pain rating variability participants revealed similar results as when all 63 participants were investigated. That is, dlPFC activity was significantly greater in consistent compared to variable participants (*$P < 0.05$, 2-sample t-test). Slice location in MNI space is indicated at the top right. Bars and scatter plot points indicate consistent (orange) and variable (blue) pain participants.

this region revealed that during each noxious stimulus, signal intensity increased in the consistent group (mean ± SEM % signal intensity: stimulus 1: 0.57 ± 0.10, 2: 0.22 ± 0.10; 3: 0.17 ± 0.08; 4: 0.26 ± 0.09; 5: 0.19 ± 0.09; 6: 0.25 ± 0.08; 7: 0.19 ± 0.10; 8: 0.17 ± 0.08). By contrast, as shown in Fig. 4A, in the variable group, signal intensity either decreased or did not change from baseline during the second–eighth stimuli, and the magnitude of these changes during each of the 8 stimuli periods were of reduced magnitude when compared to the consistent group (mean ± SEM % signal intensity: stimulus 1: 0.25 ± 0.10, 2: 0.01 ± 0.08; 3: −0.01 ± 0.11; 4: −0.06 ± 0.09; 5: 0.01 ± 0.07; 6: −0.05 ± 0.09; 7: −0.07 ± 0.06; 8: 0.03 ± 0.08). Further, our extreme value analysis that isolated the 10 participants who demonstrated the lowest pain rating variability (Consistent'), and the 10 participants who demonstrated the highest pain rating variability (Variable') revealed similar results to when including all 63 participants (Fig. 3B). Consistent' participants demonstrated significantly greater left dlPFC activation compared to Variable' participants: Consistent' = 0.12 ± 0.06; Variable' = −0.21 ± 0.09; $P = 0.01$ (mean ± SEM beta value), and we observed a significant negative relationship between the pain rating variability and dlPFC activation in these 20 extreme participants ($R = 0.47$, $P = 0.03$). Although signal intensity increased during the first stimulus in this region in the variable group, this signal intensity change was also notably lower than that of the consistent group. Extraction of percentage signal intensity changes in this region during the entire scanning period revealed that, in contrast to the consistent group in which signal intensity increased during each stimulus period, in the variable group, signal intensity increased during the first noxious stimulus but then decreased during the subsequent 7 noxious stimulus periods (Fig. 4B). Indeed, our temporal analysis (Fig. 4C) confirmed that the mean signal intensity in the variable pain group was delayed, however, not significantly in any of the 8 stimulus periods (mean ± SEM seconds—stimulus 1: consistent = 5.32 ± 0.73, variable = 6.95 ± 0.81, $P = 0.15$; stimulus 2: consistent = 6.45 ± 0.81, variable = 6.17 ± 0.74, $P = 0.97$; stimulus 3: consistent = 6.13 ± 0.87; variable = 6.17 ± 0.74, $P = 0.97$; stimulus 4: consistent = 7.26 ± 0.82, variable = 8.91 ± 0.63, $P = 0.12$; stimulus 5: consistent = 5.81 ± 0.78, variable = 6.56 ± 0.85, $P = 0.52$; stimulus 6: consistent = 6.61 ± 0.75, variable = 8.20 ± 0.73, $P = 0.14$; stimulus 7: consistent = 5.89 ± 0.77, variable = 6.09 ± 0.73, $P = 0.85$; stimulus 8: consistent = 6.21 ± 0.75, variable = 7.34 ± 0.76, $P = 0.31$) such that the peak activity in this region occurs in the periods between stimuli—necessitating our inclusion of 2 separate PPI analyses to fully understand the cortical mechanisms involving the dlPFC between consistent and variable groups.

Table 1. Location of significant differences in signal intensity changes and PPI between individuals who display consistent pain intensity ratings and individuals who display variable pain ratings during a series of identical noxious heat stimuli ($P < 0.001$ uncorrected for multiple comparisons). Locations are in MNI space.

	MNI coordinates				
	x	y	z	Cluster size	Peak voxel *t*-score
Signal intensity increases					
Consistent > variable					
Left dlPFC	−46	32	16	25	5.29[a]
PPI differences					
During noxious stimulation					
Consistent > variable					
Right dlPFC	46	48	0	13	4.30
Left dlPFC	−46	26	6	22	3.58
Right PI	40	−18	12	13	3.63
Left TPJ	−54	−42	18	20	3.41
Right premotor cortex	44	6	22	58	4.19
Left premotor cortex	−48	10	22	22	3.42
Right S1	60	−12	32	11	4.22
Between noxious stimulation					
Consistent > variable					
Right primary motor/right S1	34	−28	55	437	4.01
Right S1	46	−18	56	148	4.01

[a]Significant at FDR $P < 0.05$.

PPI differences

PPI during noxious stimulation

Our first PPI analysis revealed significant differences in the dlPFC connectivity during the noxious stimuli between the consistent and variable participant groups (Fig. 5A, Table 1). The consistent participants displayed greater stimulus-dependent connectivity between the left dlPFC and the right S1 (mean ± SEM eigenvariate—consistent: 0.21 ± 0.05, variable: −0.11 ± 0.03), left temperoparietal junction (TPJ; consistent: 0.19 ± 0.04, variable: −0.04 ± 0.04), right dlPFC (consistent: 0.17 ± 0.07, variable: −0.20 ± 0.06), left premotor cortex (consistent: 0.21 ± 0.06, variable: −0.09 ± 0.05), right premotor cortex (consistent: 0.17 ± 0.04, variable: −0.12 ± 0.05), right posterior insula (PI: consistent: 0.22 ± 0.07, variable: −0.21 ± 0.08), and a more caudal area of the left dlPFC relative to the seed (consistent: 0.13 ± 0.04, variable: −0.11 ± 0.04; Fig. 5B; $P < 0.001$ uncorrected for multiple comparisons). Plots of the dlPFC stimulus-dependent connectivity against variability in the pain intensity ratings during the 8 noxious stimuli revealed significant negative linear relationships in the right S1 ($r = -0.48$, $P = 0.00007$), left TPJ ($r = -0.43$, $P = 0.0003$), left premotor cortex ($r = -0.45$, $P = 0.0002$), right premotor cortex ($r = -0.47$, $P = 0.0007$), right PI ($r = -0.37$, $P = 0.003$), and both the left dlPFC ($r = -0.45$, $P = 0.0001$) and right dlPFC ($r = -0.36$, $P = 0.004$; $P < 0.001$ uncorrected for multiple comparisons).

PPI outside of noxious stimulation

Our second PPI analysis revealed relatively fewer regions which significantly differed in the dlPFC connectivity between periods of noxious stimulus application in the consistent and variable participant groups (Fig. 5C, Table 1). Consistent participants displayed greater connectivity between the dlPFC and the right S1 (mean ± SEM eigenvariate—consistent: 0.18 ± 0.09, variable: −0.28 ± 0.07) as well as a more rostral cluster spanning both the primary somatosensory and motor cortices (consistent: 0.17 ± 0.10, variable: −0.27 ± 0.06; Fig. 5C). The inverse contrast image revealed no significant clusters. That is, in no brain region did variable participants display greater dlPFC connectivity than consistent participants in this ISI PPI analysis. Further, by saving significant clusters from the initial PPI analysis as masks and extracting connectivity values from these masks using the secondary PPI contrast images, we confirmed that these regions did not differ in dlPFC connectivity between the variable and consistent participants between periods of noxious stimulation (mean ± SEM eigenvariate—right S1: consistent: −0.12 ± 0.05, variable: −0.08 ± 0.07; left TPJ: consistent: 0.01 ± 0.05, variable: −0.04 ± 0.05; right PI: consistent: −0.02 ± 0.11, variable: 0.06 ± 0.08; left dlPFC: consistent: −0.01 ± 0.05, variable: 0.04 ± 0.05; $P > 0.05$, 2-sample *t*-test), nor did their dlPFC connectivity values correlate with pain rating variability across the entire experimental cohort (right S1: $r = 0.01$; left TPJ: $r = 0.06$; right PI: $r = 0.02$; left dlPFC: $r = 0.07$) ($P > 0.05$, linear regression; Fig. 5D).

Discussion

This study reports several notable findings. First, we confirm that the repeated noxious thermal stimuli applied to the same region at the same temperature often do not evoke consistent pain intensity ratings. Second, we found that a series of noxious stimuli evoke a nonsignificant yet delayed dlPFC activation time series in individuals who experience variable pain intensities compared to those who experience consistent pain intensities, and these results were similar when investigating in isolation participants who demonstrated extreme consistency and variability. Notably, as revealed from our 2 separate PPI analyses modeling periods of noxious stimulation and ISI, respectively, this lagged engagement of the dlPFC in variable participants did not relate to the recruitment of similar brain regions at later time points, or even a different cortical network, potentially suggesting that the lagged nature of dlPFC activation in variable participants represented a failure to recruit pain-attentional regions effectively when a stimulus is being applied. Finally, during the application of noxious stimuli, individuals with variable pain ratings demonstrate reduced dlPFC functional connectivity with areas involved in processing the saliency of pain intensity, that is, the insula, the TPJ, and the S1,

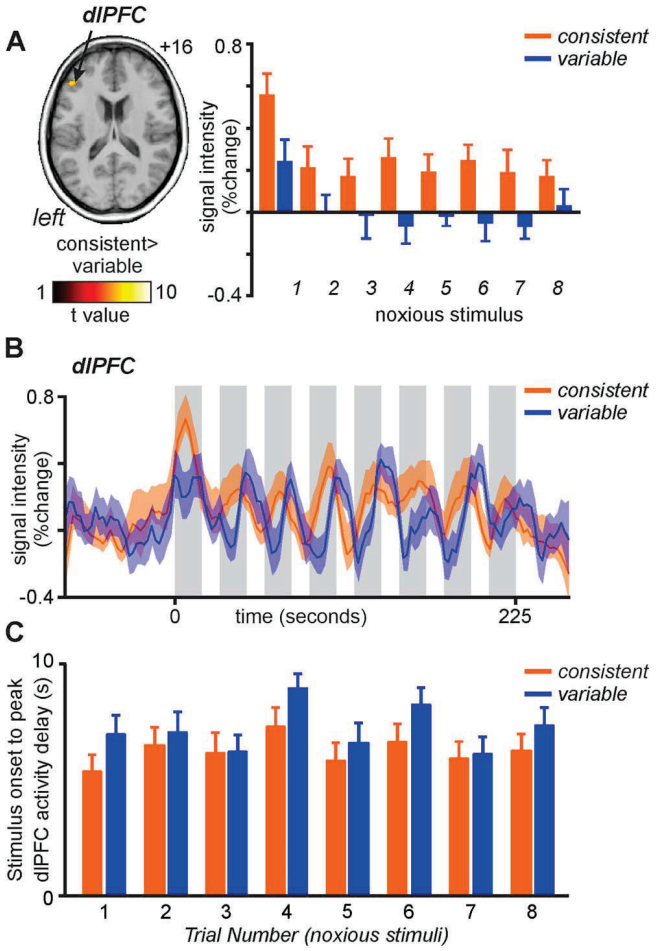

Fig. 4. A) Percentage signal intensity changes in the left dlPFC during each of the 8 identical noxious stimuli periods in consistent (orange) and variable (blue) pain groups. While consistent participants show signal increases in the dlPFC during each noxious stimulus, the variable participants overall show decreases or no signal changes. B) Percentage signal intensity changes in the left dlPFC over the entire scanning period in consistent (orange) and variable (blue) pain groups. Note that overall, variable participants exhibit a shift in overall dlPFC activity such that peaks in signal intensity change occur outside periods of noxious stimuli applied. The vertical gray bars indicate the periods of noxious stimulation, and shaded areas of orange and blue indicate the ±SEM in the consistent and variable groups, respectively. C) Mean time delay in stimulus onset to peak dlPFC activity change for each stimulus period (ramp volumes included) in consistent (orange bars) and variable (blue bars) participants.

compared with those who do show consistent pain ratings. We additionally observed a negative relationship between the dlPFC functional connectivity and pain intensity variability in these same areas such that individuals with greater pain variability experience weaker dlPFC connectivity strength in a continuum. The 2 areas of the left dlPFC we identified in our activation and PPI analyses encompassed the borders as outlined in Mai and colleagues' "Atlas of the human brain," bounded superiorly and inferiorly by the middle and inferior frontal sulci, respectively (Mai et al. 2015). Overall, these results indicate that many individuals experience large variations in their experience of repeated noxious stimuli and that this may be mediated by the dlPFC and its connections with the TPJ, insula, and S1.

Over the past decade, there have been many investigations exploring how various phenomena alter perceived pain intensity. For the most part, these studies have explored methods to reduce pain intensity with the goal of alleviating pain in chronic conditions. Recently, we and others have explored the neural underpinnings of the analgesic mechanism known as conditioned pain modulation (CPM), whereby a conditioning noxious stimulus reduces the perceived intensity of a series of test noxious stimuli (Yarnitsky 2010; Granovsky 2013; Youssef et al. 2016b). When performing CPM paradigms, as well as experiments testing placebo analgesia and nocebo hyperalgesia, it is important to begin with a stable baseline where individuals experience similar pain intensity ratings during multiple noxious test stimuli—i.e. an unconditioned stimulus. However, our present findings show that this is not always the case, and in our CPM experiments, had we not taken baseline pain variability into account, over half of our participants would have been categorized as displaying a CPM response despite not even receiving the conditioning stimulus. By including only those participants with a series of consistent pain ratings, the validity of our results is improved, as we can attribute the reduction in pain to the application of the conditioning stimulus and not to the individual's pain perception variability. Ultimately, pooling data from multiple participants without first determining whether they show consistent pain ratings will dilute observed modulatory effects.

In addition to assessing pain variability when exploring the neural mechanisms underlying phenomena, such as CPM, placebo, and nocebo, understanding the variability itself is important. While it is possible that noxious stimulus intensity variability can result from mechanisms, such as central sensitization at the primary afferent synapse (Latremoliere and Woolf 2009; Arendt-Nielsen 2015), the present finding that average pain intensity ratings did not differ between consistent and variable participants argues against the involvement of this mechanism. Additionally, the 15-s interval between stimuli far exceeds the 1.5- or 2.5-s interval that is frequently used to test thermal temporal summation in pain-free individuals (Fillingim et al. 1998; Nahman-Averbuch et al. 2013), arguing against the role of the primary afferent synapse in producing these effects.

Alongside mechanisms at the primary afferent synapse, many higher-order processes contribute to our experience of pain, including cognitive, motivational, and emotional factors (Ploner et al. 2011). The dlPFC is a key part of an executive-attention network that directs attention during task performance and during the experience of sensory stimuli (Kane and Engle 2002; Curtis and D'Esposito 2003; Lorenz et al. 2003). This region is considered as an interface between cognitive processing and pain modulation and is associated with pain catastrophizing, that is, difficulty disengaging from pain (Seminowicz and Davis 2006; Seminowicz and Moayedi 2017). The dlPFC modulates pain intensity perception since its transient disruption can increase tolerance to noxious cold stimuli (Graff-Guerrero et al. 2005) and can completely abolish placebo analgesia (Krummenacher et al. 2010). There is also evidence that stimulation of the left dlPFC specifically can increase pain thresholds in healthy individuals (Borckardt et al. 2007; Tu et al. 2021) and can ameliorate migraine symptoms (Brighina et al. 2004). In keeping with this role in pain modulation, the present findings suggest that the left dlPFC is involved in modulating an individual's pain intensity even within a series of brief, identical stimuli.

It is conceivable that the altered engagement of the dlPFC we observed between consistent and variable participants reflected attentional processes, directing attention toward or away from the applied noxious stimuli and engaging cortical regions to modulate perceived pain intensity (Tracey et al. 2002; Villemure and Bushnell 2002; Tracey and Mantyh 2007). During our experimental procedure, we collected pain rating data consistently throughout

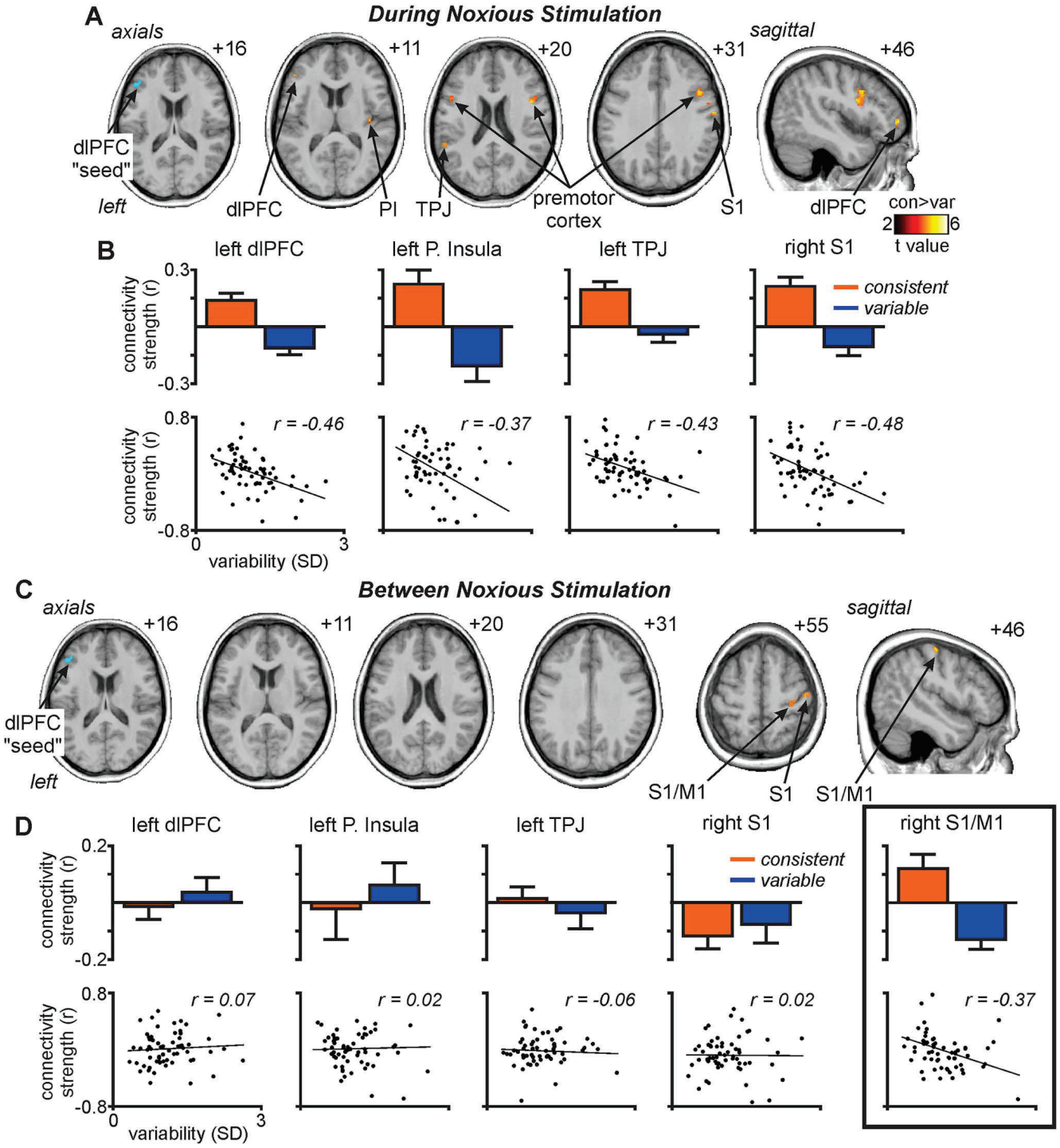

Fig. 5. A) PPI results showing brain regions which show altered connectivity strengths with the left dlPFC (light blue shading "seed") during the noxious stimuli compared with baseline periods ($P < 0.001$ uncorrected for multiple comparisons). Areas of greater dlPFC connectivity in consistent (con) compared with variable (var) participants are overlaid onto a series of mean T1-weighted anatomical images (hot color scale). Slice locations in MNI space are indicated at the top right of each slice. B) Plots of mean ± SEM connectivity strengths for 4 clusters in the consistent (orange) and variable (blue) participant groups. The lower row are plots of connectivity strength compared with pain intensity perception variability. C) PPI results showing brain regions which altered in connectivity strength with the left dlPFC during ISI periods compared with periods of noxious stimulation ($P < 0.001$ uncorrected for multiple comparisons). M1: primary motor cortex. D) Plots of mean ± SEM dlPFC connectivity strengths during ISI periods for 4 clusters revealed in the initial PPI analysis as well as for 1 cluster which was revealed in the ISI PPI analysis. Note that no cluster demonstrating altered dlPFC stimulus-dependent connectivity demonstrates similar or inverse patterns of dlPFC connectivity change between variable and consistent participants during periods of ISI.

the scanning sequence, requiring subjects to both perceive and accurately report their current pain at all times. This methodology was chosen to reduce the likelihood of series position effects or response biases associated with collecting pain rating data after a series of stimuli (Murdock Jr 1962; Hróbjartsson et al. 2011). However, this decision may have in part contributed further to phenomena of pain rating variability, aiding us in revealing the role of the dlPFC and its cortical projections in both attending to and perceiving pain intensity. Our results suggest that such a circuitry that may be altered in those less proficient in monitoring their pain in response to consistent moderate intensity stimuli. Indeed, in a previous investigation, Kucyi and colleagues reported that individuals can experience short-term spontaneous shifts in attention toward and away from pain even during a series of brief identical electrical stimuli (Kucyi et al. 2013). When individuals reported that their mind was on "something else," they experienced significantly reduced signal intensity changes in the dlPFC and TPJ compared to trials when they reported high attention to the painful stimulus. Consistent with this previous work, we found the reduced dlPFC signal intensity changes during noxious stimuli in participants with variable pain ratings were associated with reduced connectivity strengths between the dlPFC and S1 and TPJ during the noxious stimuli. These data are consistent with the idea that the dlPFC can influence the S1 and TPJ function on a trial-to-trial basis and that this may underpin the attentional modification between successive stimuli.

Additionally, Kucyi and colleagues also found that an individual's intrinsic attention to pain during 1 session correlated with their attention to pain in a follow-up session, suggesting that this is a trait-like quality (Kucyi et al. 2013). Interestingly, our variable pain rating participants reported lower pain catastrophizing scores than the consistent participants, which is consistent with the idea that the variable pain group is more capable of disengaging from pain; this may also be a trait characteristic (Van Damme et al. 2004). As such, the rightward delay in mean dlPFC signal in the variable participants may also underlie their altered engagement with the stimulus. It is likewise conceivable that the strong dlPFC activations in the consistent group are reflective of relatively stable attention toward and engagement with each stimulus, which thereby contributes to a steady perception of pain. This could also be explained by the greater pain catastrophizing scores in consistent compared to variable participants, as this attentional component of pain catastrophizing has been tied with altered dlPFC activity (MacDonald 3rd et al. 2000; Bishop 2009; Galambos et al. 2019). Furthermore, the PPI analysis revealed a negative relationship between dlPFC connectivity strength and pain intensity variability in the PI and S1 specifically during pain periods. This may also reflect the stable attentional control over pain in individuals with lower variability, as both the PI and S1 are involved in encoding sensory-discriminative components of a painful stimulus (Peyron et al. 2000) and are modulated by attentional processes (Fardo et al. 2017).

It is important to note some limitations. First, throughout our analyses, we observed bilateral activity and connectivity changes in somatosensory networks despite stimulation occurring on the right lower lip only. While it is well described that somatosensory innervation in the brain is predominantly contralateral to stimulus input, it has been previously demonstrated that the orofacial noxious stimulation particularly produces significant bilateral activation at both the level of the thalamus and somatosensory cortices (Nash et al. 2010; Henssen et al. 2016). While our results do reflect our current understanding of orofacial noxious organization in the brain, future investigations interested in pain rating variability on particularly somatosensory networks may benefit from electing an alternate stimulation site with solely contralateral cortical input. Next, it is possible that the pain intensity fluctuations and associated dlPFC activity and connectivity changes do indeed reflect alterations in an individual's attentional processes rather that an inherent ability to perceive identical noxious stimuli as either consistent or variable. Each participant rated the pain intensity on-line during the entire fMRI scan and thus their focus was directed toward this task. We observed a negative relationship between the dlPFC connectivity strength and pain intensity variability within the left TPJ, a region that is pivotal in the salience network and is involved in processing the behavioral relevance and emotional dimension of stimuli (Seeley et al. 2007; Kucyi et al. 2012). Indeed, the behavioral importance of a stimulus often informs the individual of how much attention they need to pay to that stimulus (Legrain et al. 2009). It is possible that the positive signal intensity changes within the dlPFC during the initial stimulus in both consistent and variable groups reflect a high degree of saliency and attention capture during the first noxious input, similar to positive signal intensity changes that are observed in the TPJ and anterior cingulate cortex in response to novel stimuli (Downar et al. 2002). We also observed across all participants that the initial stimulus was rated as significantly more intense than subsequent stimuli, and so it is possible that only during the subsequent noxious stimuli that the neural mechanisms of the 2 groups diverge and differences in the underlying neural circuitry become observable. Third, in order to collect a full coverage of the cortex and brainstem within a suitable timeframe, we acquired relatively large voxels. We then upsampled our images, which may have led to the introduction of partial volume effects from the neighboring tissue. However, the area of the dlPFC we identified in our paired analysis which was then used for a seed in the PPI was not directly bordered by either white matter, brain ventricles, or cisterns. We therefore suggest that partial volume effects did not play a substantial role in influencing the overall results of this study. Finally, our connectivity analysis was conducted at a relatively less stringent correction level of $P < 0.001$, uncorrected for multiple comparisons. This correction level prevents us from controlling for false positive voxels of significance, however, all regions which emerged from this analysis are known for their role in pain perception and modulation (Kulkarni et al. 2005; Kong et al. 2010), leaving us confident they represent true neural effects encoding an individual's consistent perception of moderately intense noxious stimuli.

Conclusions

The results of this study show that a series of brief, noxious thermal stimuli often do not evoke consistent pain intensity ratings within an individual. This strongly suggests that investigators should take baseline pain intensity variability into account when determining how an intervention affects baseline pain ratings, for example, during CPM paradigms. Furthermore, individuals with consistent and variable pain ratings differ in the dlPFC activity and connectivity with the TPJ and S1 during noxious stimulation, possibly reflecting the differences in attentional processes, pain catastrophizing, and attention-salience network interactions in the consistent and variable groups.

Supplementary material

Supplementary material is available at *Cerebral Cortex* online.

Funding

This work was supported by funding from the Australian National Health and Medical Research Council (Grant ID 1130280).

Conflict of interest: None declared.

References

Arendt-Nielsen L. Central sensitization in humans: assessment and pharmacology. In: Schaible H-G, editors. editor*Pain control*. Berlin, Heidelberg: Springer; 2015. pp. 79–102.

Ashburner J, Barnes G, Chen C-C, Daunizeau J, Flandin G, Friston K, Kiebel S, Kilner J, Litvak V, Moran R. *SPM12 manual*. London, UK: Wellcome Trust Centre for Neuroimaging; 2014. p. 2464.

Bishop SJ. Trait anxiety and impoverished prefrontal control of attention. *Nat Neurosci*. 2009:12(1):92–98.

Borckardt JJ, Smith AR, Reeves ST, Weinstein M, Kozel FA, Nahas Z, Shelley N, Branham RK, Thomas KJ, George MS. Fifteen minutes of left prefrontal repetitive transcranial magnetic stimulation acutely increases thermal pain thresholds in healthy adults. *Pain Res Manag*. 2007:12(4):287–290.

Borsook D, Edwards R, Elman I, Becerra L, Levine J. Pain and analgesia: the value of salience circuits. *Prog Neurobiol*. 2013:104:93–105.

Brighina F, Piazza A, Vitello G, Aloisio A, Palermo A, Daniele O, Fierro B. rTMS of the prefrontal cortex in the treatment of chronic migraine: a pilot study. *J Neurol Sci*. 2004:227(1):67–71.

Coghill RC, McHaffie JG, Yen YF. Neural correlates of interindividual differences in the subjective experience of pain. *Proc Natl Acad Sci U S A*. 2003:100(14):8538–8542.

Curtis CE, D'Esposito M. Persistent activity in the prefrontal cortex during working memory. *Trends Cogn Sci*. 2003:7(9):415–423.

Downar J, Crawley AP, Mikulis DJ, Davis KD. A cortical network sensitive to stimulus salience in a neutral behavioral context across multiple sensory modalities. *J Neurophysiol*. 2002:87(1):615–620.

Fardo F, Auksztulewicz R, Allen M, Dietz MJ, Roepstorff A, Friston KJ. Expectation violation and attention to pain jointly modulate neural gain in somatosensory cortex. *NeuroImage*. 2017:153:109–121.

Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods*. 2007:39(2):175–191.

Fillingim RB. Individual differences in pain responses. *Curr Rheumatol Rep*. 2005:7(5):342–347.

Fillingim RB, Maixner W, Kincaid S, Silva S. Sex differences in temporal summation but not sensory-discriminative processing of thermal pain. *Pain*. 1998:75(1):121–127.

Friston KJ, Holmes AP, Worsley KJ, Poline JP, Frith CD, Frackowiak RSJ. Statistical parametric maps in functional imaging: a general linear approach. *Hum Brain Mapp*. 1994:2(4):189–210.

Galambos A, Szabó E, Nagy Z, Édes AE, Kocsel N, Juhász G, Kökönyei G. A systematic review of structural and functional MRI studies on pain catastrophizing. *J Pain Res*. 2019:12:1155–1178.

Graff-Guerrero A, González-Olvera J, Fresán A, Gómez-Martín D, Carlos Méndez-Núñez J, Pellicer F. Repetitive transcranial magnetic stimulation of dorsolateral prefrontal cortex increases tolerance to human experimental pain. *Cogn Brain Res*. 2005:25(1):153–160.

Granovsky Y. Conditioned pain modulation: a predictor for development and treatment of neuropathic pain. *Curr Pain Headache Rep*. 2013:17(9):361.

Henssen DJ, Kurt E, Kozicz T, van Dongen R, Bartels RH, Cappellen van Walsum AM. New insights in trigeminal anatomy: a double orofacial tract for nociceptive input. *Front Neuroanat*. 2016:10:53.

Hróbjartsson A, Kaptchuk TJ, Miller FG. Placebo effect studies are susceptible to response bias and to other types of biases. *J Clin Epidemiol*. 2011:64(11):1223–1229.

Kane MJ, Engle RW. The role of prefrontal cortex in working-memory capacity, executive attention, and general fluid intelligence: an individual-differences perspective. *Psychon Bull Rev*. 2002:9(4):637–671.

Kong J, Loggia ML, Zyloney C, Tu P, Laviolette P, Gollub RL. Exploring the brain in pain: activations, deactivations and their relation. *Pain*. 2010:148(2):257–267.

Krummenacher P, Candia V, Folkers G, Schedlowski M, Schonbachler G. Prefrontal cortex modulates placebo analgesia. *Pain*. 2010:148(3):368–374.

Kucyi A, Hodaie M, Davis KD. Lateralization in intrinsic functional connectivity of the temporoparietal junction with salience- and attention-related brain networks. *J Neurophysiol*. 2012:108(12):3382–3392.

Kucyi A, Salomons TV, Davis KD. Mind wandering away from pain dynamically engages antinociceptive and default mode brain networks. *Proc Natl Acad Sci U S A*. 2013:110(46):18692–18697.

Kulkarni B, Bentley DE, Elliott R, Youell P, Watson A, Derbyshire SW, Frackowiak RS, Friston KJ, Jones AK. Attention to pain localization and unpleasantness discriminates the functions of the medial and lateral pain systems. *Eur J Neurosci*. 2005:21(11):3133–3142.

Latremoliere A, Woolf CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain*. 2009:10(9):895–926.

Legrain V, Damme SV, Eccleston C, Davis KD, Seminowicz DA, Crombez G. A neurocognitive model of attention to pain: behavioral and neuroimaging evidence. *Pain*. 2009:144(3):230–232.

Lorenz J, Minoshima S, Casey KL. Keeping pain out of mind: the role of the dorsolateral prefrontal cortex in pain modulation. *Brain*. 2003:126(Pt 5):1079–1091.

MacDonald AW 3rd, Cohen JD, Stenger VA, Carter CS. Dissociating the role of the dorsolateral prefrontal and anterior cingulate cortex in cognitive control. *Science*. 2000:288(5472):1835–1838.

Macey PM, Macey KE, Kumar R, Harper RM. A method for removal of global effects from fMRI time series. *NeuroImage*. 2004:22(1):360–366.

Mai JK, Majtanik M, Paxinos G. *Atlas of the human brain*. Cambridge, MA: Academic Press; 2015

Miron D, Duncan GH, Bushnell MC. Effects of attention on the intensity and unpleasantness of thermal pain. *Pain*. 1989:39(3):345–352.

Murdock BB Jr. The serial position effect of free recall. *J Exp Psychol*. 1962:64(5):482.

Nahman-Averbuch H, Yarnitsky D, Granovsky Y, Gerber E, Dagul P, Granot M. The role of stimulation parameters on the conditioned pain modulation response. *Scand J Pain*. 2013:4(1):10–14.

Nash PG, Macefield VG, Klineberg IJ, Gustin SM, Murray GM, Henderson LA. Bilateral activation of the trigeminothalamic tract by acute orofacial cutaneous and muscle pain in humans. *Pain*. 2010:151(2):384–393.

Peyron R, Laurent B, García-Larrea L. Functional imaging of brain responses to pain. A review and meta-analysis (2000). *Neurophysiol Clin/Clin Neurophysiol*. 2000:30(5):263–288.

Ploner M, Lee MC, Wiech K, Bingel U, Tracey I. Flexible cerebral connectivity patterns subserve contextual modulations of pain. *Cereb Cortex*. 2011:21(3):719–726.

Price DD, Staud R, Robinson ME, Mauderli AP, Cannon R, Vierck CJ. Enhanced temporal summation of second pain and its central modulation in fibromyalgia patients. *Pain*. 2002:99(1–2):49–59.

Rogachov A, Cheng JC, Erpelding N, Hemington KS, Crawley AP, Davis KD. Regional brain signal variability: a novel indicator of pain sensitivity and coping. *Pain*. 2016:157(11):2483–2492.

Särkkä S, Solin A, Nummenmaa A, Vehtari A, Auranen T, Vanni S, Lin F-H. Dynamic retrospective filtering of physiological noise in BOLD fMRI: DRIFTER. *NeuroImage*. 2012:60(2):1517–1527.

Seeley WW, Menon V, Schatzberg AF, Keller J, Glover GH, Kenna H, Reiss AL, Greicius MD. Dissociable intrinsic connectivity

networks for salience processing and executive control. *J Neurosci*. 2007:27(9):2349–2356.

Seminowicz DA, Davis KD. Cortical responses to pain in healthy individuals depends on pain catastrophizing. *Pain*. 2006:120(3): 297–306.

Seminowicz DA, Moayedi M. The dorsolateral prefrontal cortex in acute and chronic pain. *J Pain*. 2017:18(9):1027–1035.

Sullivan MJL, Bishop SR, Pivik J. The pain catastrophizing scale: development and validation. *Psychol Assess*. 1995:7(4):524–532.

Tracey I, Mantyh PW. The cerebral signature for pain perception and its modulation. *Neuron*. 2007:55(3):377–391.

Tracey I, Ploghaus A, Gati JS, Clare S, Smith S, Menon RS, Matthews PM. Imaging attentional modulation of pain in the periaqueductal gray in humans. *J Neurosci*. 2002:22(7):2748.

Tu Y, Wilson G, Camprodon J, Dougherty DD, Vangel M, Benedetti F, Kaptchuk TJ, Gollub RL, Kong J. Manipulating placebo analgesia and nocebo hyperalgesia by changing brain excitability. *Proc Natl Acad Sci U S A*. 2021:118(19):e2101273118.

Van Damme S, Crombez G, Eccleston C. Disengagement from pain: the role of catastrophic thinking about pain. *Pain*. 2004:107(1): 70–76.

Villemure C, Bushnell CM. Cognitive modulation of pain: How do attention and emotion influence pain processing? *Pain*. 2002: 95(3):195–199.

Yarnitsky D. Conditioned pain modulation (the diffuse noxious inhibitory control-like effect): its relevance for acute and chronic pain states. *Curr Opin Anaesthesiol*. 2010:23(5):611–615.

Youssef AM, Macefield VG, Henderson LA. Cortical influences on brainstem circuitry responsible for conditioned pain modulation in humans. *Hum Brain Mapp*. 2016a:37(7):2630–2644.

Youssef AM, Macefield VG, Henderson LA. Pain inhibits pain; human brainstem mechanisms. *NeuroImage*. 2016b:124(Pt A):54–62.

Appendix B:

The pain conductor: brainstem modulation in acute and chronic pain. Current Opinions in Supportive and Palliative Care 16(2): 71-77.

REVIEW

The pain conductor: brainstem modulation in acute and chronic pain

AQ1

Lewis S. Crawford, Damien C. Boorman, Kevin A. Keay, and Luke A. Henderson

Purpose of review
It is well established in experimental settings that brainstem circuits powerfully modulate the multidimensional experience of pain. This review summarizes current understanding of the roles of brainstem nuclei in modulating the intensity of pain, and how these circuits might be recruited therapeutically for pain relief in chronic and palliative settings.

Recent findings
The development of ultra-high field magnetic resonance imaging and more robust statistical analyses has led to a more integrated understanding of brainstem function during pain. It is clear that a number of brainstem nuclei and their overlapping pathways are recruited to either enhance or inhibit incoming nociceptive signals. This review reflects on early preclinical research, which identified in detail brainstem analgesic function, putting into context contemporary investigations in humans that have identified the role of specific brainstem circuits in modulating pain, their contribution to pain chronicity, and even the alleviation of palliative comorbidities.

Summary
The brainstem is an integral component of the circuitry underpinning pain perception. Enhanced understanding of its circuitry in experimental studies in humans has, in recent years, increased the possibility for better optimized pain-relief strategies and the identification of vulnerabilities to postsurgical pain problems. When integrated into the clinical landscape, these experimental findings of brainstem modulation of pain signalling have the potential to contribute to the optimization of pain management and patient care from acute, to chronic, to palliative states.

Keywords
analgesia, medulla, palliative treatment, patient care, placebo

INTRODUCTION

The perception of pain is a complex phenomenon, and includes sensory-discriminative, affective-motivational, and cognitive-evaluative components [1]. The critical role of pain in learning and survival has led to a broadening appreciation of Patrick Wall's challenge to pain neurobiologists that its central representations should resemble more the homeostatic circuitry that regulates thirst and hunger than the purely sensory circuits that process vision or audition. The idea that pain sensitivity can be increased (hyperalgesia), when for example certain behaviours might exacerbate existing injuries, or decreased (hypoalgesia/analgesia), when for example pain may be unavoidable or during more immediate threats to survival, is largely accepted. In each case, a series of brainstem nuclei are essential for driving the modulation of pain sensitivity. They do this primarily by modifying incoming pain signals from the periphery (i.e., nociception) at the site of their first nervous system relay, either in the dorsal horn (DH) of the spinal cord (for the body) or the spinal trigeminal nucleus (SpV) in the caudal brainstem (for the head). These pain signals then ascend to higher brain regions for further processing and ultimately the perception of pain. The pain modulatory regions of the brainstem are positioned between these spinal sites and the higher forebrain

School of Medical Sciences (Neuroscience), and Brain and Mind Centre, University of Sydney, NSW, Australia AQ2

Correspondence to Luke A. Henderson, School of Medical Sciences (Neuroscience), Brain and Mind Centre, University of Sydney, NSW 2050, Australia. Tel: +61 2 9351 7063; e-mail: luke.henderson@sydney.edu.au AQ3

Curr Opin Support Palliat Care 2022, 16:000–000
DOI:10.1097/SPC.0000000000000598

KEY POINTS

- The brainstem is uniquely positioned to alter pain signalling.
- Brainstem nuclei contain the necessary neurochemicals for altering acute, chronic, and palliative pain states.
- An integrated view of brainstem function is pivotal in understanding how experimental interventions and clinical treatments inhibit or enhance pain.

regions and serve as both a relay and gateway for ascending sensory and descending motor signals.

Brainstem circuitry involved in pain transmission and modulation

Early preclinical laboratory studies using electrophysiological, pharmacological, and lesioning approaches in cats, rats, and mice identified a number of pain processing and pain modulatory regions in the brainstem [2,3]. These regions include the periaqueductal gray (PAG) in the midbrain; the parabrachial complex (PB) and locus coeruleus (LC) in the pons; and the rostral ventromedial medulla (RVM) and subnucleus reticularis dorsalis (SRD) in the medulla. Neuroanatomical tract tracing revealed that each of these brainstem regions projects directly to, or receives projections from, both the DH and SpV [4–8]. Modern brain imaging has revealed that these pathways are likely conserved in humans, and with the advent of ultra-high field magnetic resonance imaging (MRI), we have begun to better define their contributions to pain modulation in both experimental and clinical settings, as well start to robustly characterize the manner in which these brainstem sites interact with one another [9▪▪].

The earliest to be defined and the best characterized of the brainstem's pain modulatory circuits are the PAG→RVM→DH/SpV pathways. Direct electrical stimulation or morphine application to the ventrolateral region of the PAG (vlPAG) produces analgesia, and work from Jean-Marie Besson's Lab in Paris, and John Liebeskind's group at UCLA, first characterized this functional circuitry. The discovery of the critical role of vlPAG projections to the RVM in mediating these pain modulatory responses followed in work led by Howard Fields, Alan Basbaum and Mary Heinricher [10,11]. In brief, vlPAG neurons project onto spinally-projecting RVM neurons, which release serotonin into the DH/SpV that acts to inhibit ascending nociceptive transmission [12]. The PAG also interacts with LC and PB, each of which projects either directly, or indirectly via either the RVM or SRD, to the DH/SpV. Although these brainstem circuits can evoke analgesia, their relative contributions appear to differ in different conditions. More importantly, the ability of these regions to produce analgesic responses differs markedly between individuals, and descending inputs from higher brain structures can strongly modify the overall efficacy of these brainstem analgesic circuits. Moreover, these analgesic brainstem functions are known to be disrupted in several clinical populations, including in a number of chronic pain conditions [13,14].

When and how does the brainstem modulate pain?

The brainstem circuitry described above provides a number of routes via which noxious information can be modulated at the level of the DH/SpV. Over the past 50 years, experimental animal studies and more recent human investigations have begun to determine the relative contributions of each of these brainstem regions to analgesic responses evoked under different conditions. The following is a description of three of the most frequently investigated analgesia-evoking phenomena and the likely brainstem circuitry involved.

Conditioned pain modulation

When pain from one body location reduces the intensity of pain at another, remote location, that is, *pain inhibits pain*. Experimental animal investigations revealed that conditioned pain modulation (CPM) analgesic responses are eliminated following lesions to the SRD nucleus in the caudal brainstem, whilst lesions in the PAG and RVM have no significant effects [15,16]. Consistent with these findings, a recent human brain imaging study reported that signal intensity changes in the SRD and PB were associated with CPM analgesic responses [17] (Figure 1a and 2).

Placebo analgesia

When pain intensity is reduced by an individual's belief in the analgesic properties of an intervention, even when that intervention does not contain biologically active components (pharmacological or otherwise). Although placebo analgesia is considered difficult to explore in experimental animals, it was recently reported that pharmacologically conditioned placebo analgesia can be elicited from both female and male rats including in those with chronic pain opening up a way to explore this phenomenon from cellular to systems levels

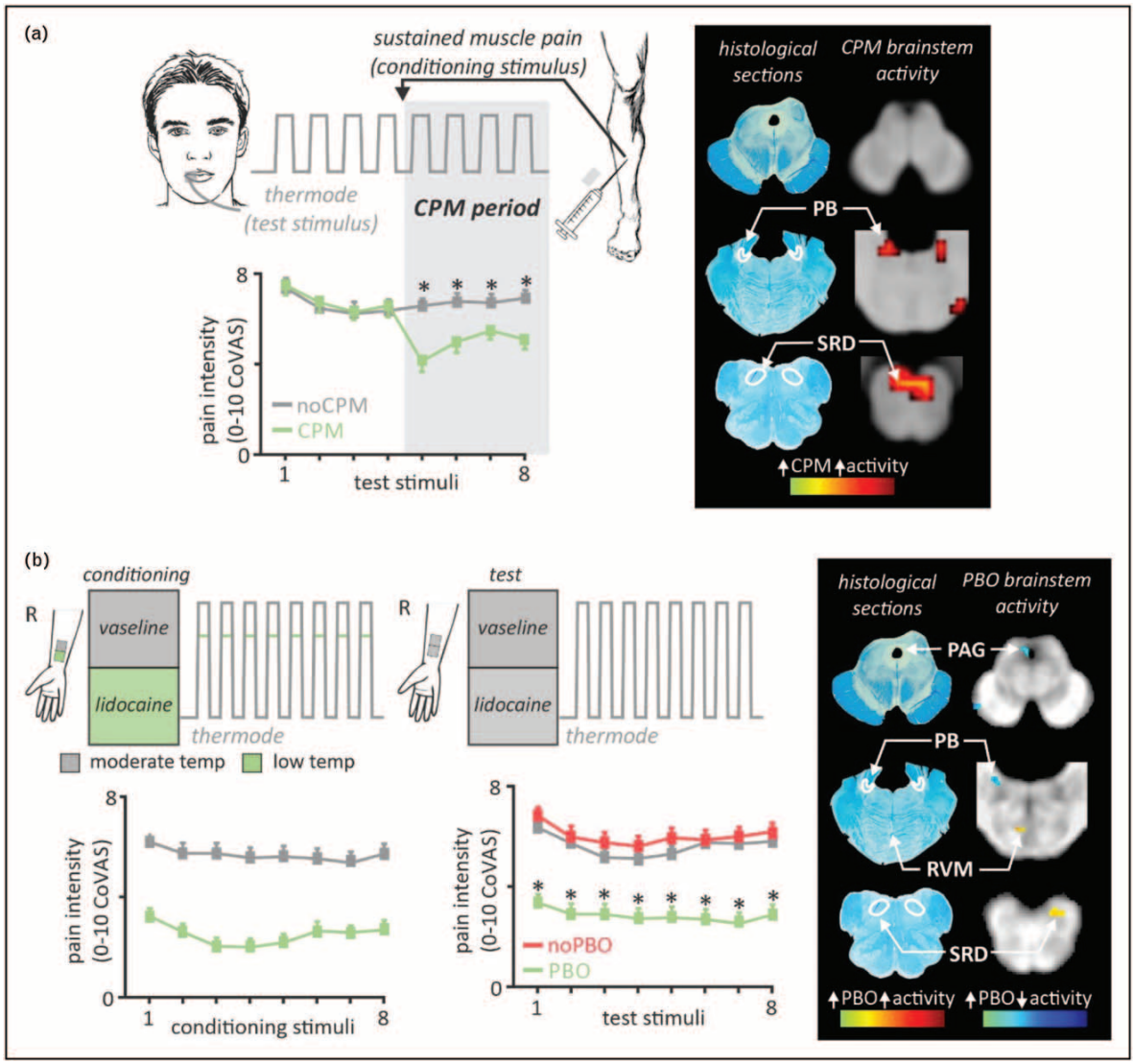

FIGURE 1. Human functional MRI of the brainstem during conditioned pain modulation (CPM) and placebo analgesia (PBO). (a) CPM analgesia is induced by applying brief noxious stimuli on the lips at the same time as a sustained pain in the leg. Approximately 50% of the participants displayed a CPM analgesic response. Functional MRI of the brainstem during CPM analgesia identified signal changes in the region encompassing the parabrachial complex (PB) and subnucleus reticularis dorsalis (SRD) only in those participants that displayed a CPM analgesia. (b) Placebo analgesia is induced by conditioning participants over time that an inert substance holds pain relieving qualities, typically by deceptively applying lower noxious intensity stimuli to the placebo-treated body site. Again, approximately 50% of participants displayed significant placebo analgesia. Functional MRI revealed that several nuclei are recruited during placebo analgesia including the midbrain periaqueductal gray matter (PAG), rostral ventromedial medulla (RVM), PB, and SRD. During both these phenomena, activity in these nuclei is either positively related to greater pain modulation (yellow colour bars), or inversely related to greater pain modulation (blue colour bars). Adapted from Youssef *et al.* (2016) and Crawford *et al.* (2021).

[18▪▪]. Human brain imaging studies have reported changes in brainstem and spinal cord activity during conditioned placebo analgesia [19,20]. A recent ultra-high field functional MRI study identified the lateral/dorsolateral PAG and the RVM as the critical brainstem circuitry responsible for placebo analgesia (Figure 1b and 2) [21▪▪]. Interestingly, regardless of whether the placebo is induced by classical conditioning or expectation, the same brainstem circuitry appears to be involved [22,23].

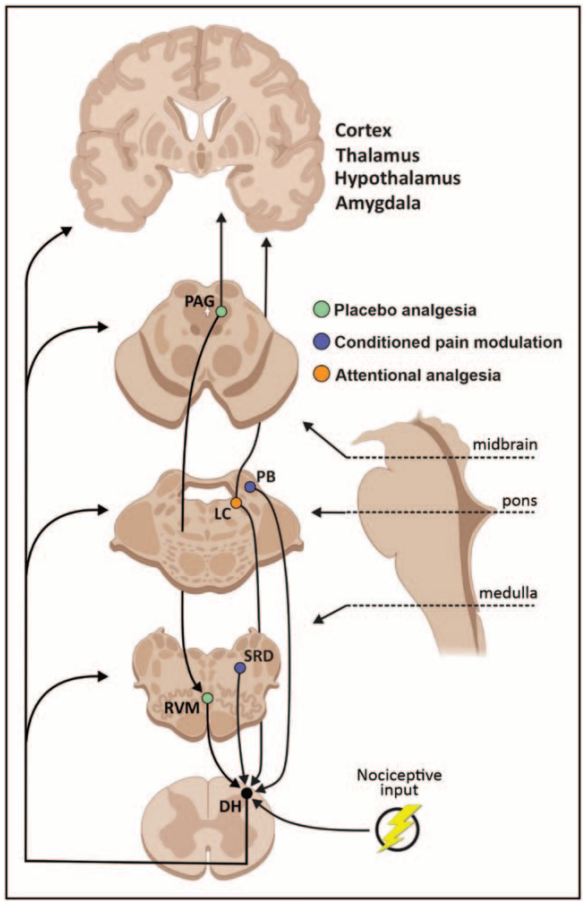

FIGURE 2. Brainstem circuitry involved in the modulation of pain. Nociceptive information is first relayed to the dorsal horn (DH)/spinal trigeminal nucleus (SpV), and subsequently to multiple brainstem and forebrain structures. Descending inputs from the rostroventromedial medulla (RVM), subnucleus reticularis dorsalis (SRD), parabrachial complex (PB), and locus coeruleus (LC) to the DH/SpV can enhance or inhibit incoming noxious information and alter the intensity of perceived pain. Key nodes in this same circuitry are recruited during pain modulatory experimental interventions, including placebo analgesia (green), conditioned pain modulation (blue), and attentional analgesia (orange).

Attentional and stress-induced analgesia

When high cognitive loads (attentional analgesia) or acutely stressful events (stress-induced analgesia) result in a reduction in pain. Similar to placebo analgesia, both of these analgesic responses appear to involve the PAG→RVM→DH/SpV pathway [24]. In addition, recent human imaging studies have reported that the level of stress-induced analgesia is related to the degree of activity synchrony (functional connectivity) between the LC, PAG, and RVM [25,26▪] and further, that attentional analgesia involves a reciprocal connection between the LC and the anterior cingulate cortex (Fig. 2) [26▪]. Indeed, noradrenergic drive from the LC as well as dopaminergic drive from the substantia nigra and ventral tegmental area have also been reported to be critical for the cortical recruitment of the descending pain modulatory pathways [27,28]. Similarly, it is important to note that that placebo analgesia is also associated with changes in the substantia nigra and LC [21▪▪].

Each of these brainstem analgesic responses are driven by and/or modulated by descending inputs from various forebrain regions including the hypothalamus, amygdala, thalamus, prefrontal and cingulate cortices [29,30]. Analgesic responses such as placebo, attentional, and stress-induced analgesia are strongly driven from cognitive and evaluative mechanisms within the cerebral cortex, whereas CPM analgesia relies more strongly on sensory inputs [31], although is still likely modified by cortical inputs [32].

Brainstem pain modulation in chronic pain

Chronic pain occurs at surprisingly high rates following physical trauma such as amputation or planned surgical intervention, treatments such as chemotherapy, or disease processes that occur in conditions such as diabetes, multiple sclerosis and Parkinson's disease [33–35]. Chronic pain is also considered a disease entity itself and results from neuronal and glial adaptations in both pain-recipient and pain modulatory brain regions [36]. It is often associated with altered responses to acute pain [37], as well as impaired CPM analgesia [13,38,39]. In fact, an individual's ability to effectively recruit CPM-related brainstem circuits is proposed as a prognostic risk factor in postsurgical development of chronic pain [40,41].

Consistent with this idea, recent human brain imaging studies have demonstrated alterations in brainstem activity and activity synchrony (functional connectivity) that likely reflect a shift towards pro-nociceptive states in chronic pain [42▪]. For instance, greater presurgical activity in the RVM correlates with greater likelihood of developing chronic pain following osteoarthritis surgery [43], changes in cortical-PB functional connectivity have been tied with chronic migraine [44], and increased LC, PAG, RVM, and SRD functional connectivity occurs in individual's with chronic orofacial pain [45]. Similarly, animal models of chronic pain have revealed that increased RVM activity contributes to increased pain sensitivity [46], and PAG or RVM inhibition reduces pain-related behaviours [47].

LC neurons also play an important role in the maintenance of chronic pain, as increased LC input to the spinal cord promotes sensory hypersensitivity [48].

Can we modify brainstem analgesic circuits for pain relief?

While brainstem pain modulatory systems may contribute to chronic pain, clinical targeting of these systems provides an opportunity for novel development of analgesic treatments. Currently available pharmacological interventions such as opioids and cannabinoids each target brainstem pain modulatory circuits, however these compounds can have significant deleterious adverse effects and addictive potential [49–51]. In contrast, there are a number of emerging techniques that may 'naturally' recruit cortical sites that modulate brainstem pain modulatory circuits to ultimately produce pain relief.

The recruitment of placebo analgesia for pain relief has been of interest to clinicians for decades. Of course, gold-standard clinical trial design uses a placebo control group paired to the pharmacological treatment of interest. It is well established that chronic pain patients in placebo groups experience on average, approximately a 20–35% reduction in pain [52,53]. Such placebo analgesia varies considerably between individuals, and this variability likely reflects differences in the efficacy of brainstem circuit function and/or modulation. Although many consider it unethical to use placebo analgesic techniques in clinical practice due to their inherent deception, placebo effects can be promoted without deception. It is ethical to, for example, promote a positive expectation combined with an honest disclosure of expected treatment effects via patient–clinician interactions in an attempt to enhance placebo effects [54]. Furthermore, it might even be possible to elicit a direct placebo effect without deception. Recent studies report evidence of *open-label* placebo analgesia, where one is aware they are being administered an inert treatment, and a proportion of individuals still express a placebo analgesia response [55]. This open-label technique may provide a pathway for the use of placebo analgesia in the treatment of pain in an open and ethical way.

In addition to placebo analgesia, other *less conventional* ways to recruit the brainstem pain modulatory systems are being explored. The oldest way known to relieve pain is music and it has been appreciated for some time that the analgesic qualities of music lie in its ability to modulate emotional states [56]. It appears that music chosen by a patient has a greater analgesic effect than music chosen by the researcher [57], with personal preference and familiarity being important factors in music analgesia [56,58]. Music can elicit cognitive and emotional changes including distraction [59], pleasure [60], and a sense of control [61] – all effects that likely modulate brainstem analgesic circuits. It has been proposed that the effects of music-induced analgesia occur via modulation of brainstem analgesic circuits, and recent evidence supports this idea. A human functional MRI study reported that music-induced analgesic responses were associated with activity changes in the PAG, RVM, and DH, in addition to higher brain centres such as the dorsolateral prefrontal cortex [62]. In a more recent brain imaging study, music-induced analgesia was associated with altered connectivity between the dorsolateral prefrontal cortex and amygdala in individuals with fibromyalgia [63]. These same brainstem and forebrain regions have been consistently shown to be involved in both CPM and placebo analgesic effects [32,64].

Finally, olfactory stimuli have also been shown to produce odour-induced analgesia [65]. For example, aromatherapy using lavender essence has been reported to reduce pain after episiotomy [66], reduce the severity of migraine attacks [67], and can reduce demand for postoperative opioids [68]. Neurons in the olfactory cortices project directly and indirectly via the amygdala to the hypothalamus [69], an area that is known to modulate pain via connections with the brainstem, in particular the PAG [70,71]. Indeed, a recent study reported altered amygdala activation and connectivity patterns during odour-induced changes in experimental heat pain [72▪▪], and another reported activation in major olfactory brain structures, as well as the prefrontal cortex and hypothalamus during lavender administration [73]. The amygdala and hypothalamus have been shown to be involved in the relief of pain [74], improvement of sleep quality [75], and reduction in arousal markers [76], consistent with changes observed in those responsive to aromatherapy treatments. This supports a similar role for the amygdala and its brainstem afferents driving aromatherapeutic benefit in humans [77].

CONCLUSIONS

The brainstem contains a number of sites that are critical for the transmission and modulation of nociceptive signals. These nuclei form circuits that can either inhibit or enhance nociceptive signals as they ascend to the forebrain, and thus powerfully modulate one's perception of pain. A range of analgesic phenomena, including placebo analgesia, attentional analgesia, CPM, and stress-induced analgesia recruit these circuits to produce their effects. In chronic pain conditions, these brainstem circuits

display altered and aberrant activity, which can contribute to the development and maintenance of these pathological states. Promisingly, recent evidence has demonstrated that while these brainstem pain modulatory systems might by dysregulated in chronic pain conditions, descending analgesic circuitry remains largely intact. Harnessing this system via noninvasive, pharmacologically inert treatment strategies (e.g., placebo, music therapy, and positive odours) could allow for the development of personalized and more effective patient care. This is especially pertinent given the growing need for and improved accessibility to remote patient care, such as video conferencing [78] and virtual reality hardware [79].

Acknowledgements

None.

Financial support and sponsorship

The present work was funded by the National Health and Medical Council of Australia: Grants 1130280 and 2002877.

Conflicts of interest

There are no conflicts of interests.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:
- ■ of special interest
- ■■ of outstanding interest

1. Coninx S. A multidimensional phenomenal space for pain: structure, primitiveness, and utility. Phenomenol Cogn Sci 2022; 21:223–243.
2. Heinricher MM, Fields HL. Central nervous system mechanisms of pain modulation. Wall and Melzack's textbook of pain 2013; Vol. 6:129–142.
3. Basbaum AI, Fields HL. Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. Annu Rev Neurosci 1984; 7:309–338.
4. Velo P, Leiras R, Canedo A. Electrophysiological study of supraspinal input and spinal output of cat's subnucleus reticularis dorsalis (SRD) neurons. PLoS One 2013; 8:e60686.
5. Llorca-Torralba M, Borges G, Neto F, *et al.* Noradrenergic locus coeruleus pathways in pain modulation. Neuroscience 2016; 338:93–113.
6. Ma W, Peschanski M. Spinal and trigeminal projections to the parabrachial nucleus in the rat: electron-microscopic evidence of a spino-ponto-amygdalian somatosensory pathway. Somatosens Res 1988; 5:247–257.
7. Aicher SA, Hermes SM, Whittier KL, Hegarty DM. Descending projections from the rostral ventromedial medulla (RVM) to trigeminal and spinal dorsal horns are morphologically and neurochemically distinct. J Chem Neuroanat 2012; 43:103–111.
8. Keay KA, Bandler R. Chapter 10 – Periaqueductal gray. In: Paxinos G, editor. The rat nervous system, 4th ed. San Diego: Academic Press; 2015pp. 207–221.
9. ■■ Cauzzo S, Singh K, Stauder M, *et al.* Functional connectome of brainstem nuclei involved in autonomic, limbic, pain and sensory processing in living humans from 7 Tesla resting state fMRI. Neuroimage 2022; 250:118925.
Resting-state functional connectivity combined with ultra-high field 7-Tesla MRI was used to discern 'the functional connectome of the human brainstem'. Largely, known connections from preclinical investigations were preserved in humans, with the brainstem serving as both a relay and supplier of neuromodulatory signals.
10. Fields H, Heinricher M. Anatomy and physiology of a nociceptive modulatory system. Philos Trans R Soc Lond B Biol Sci 1985; 308:361–374.
11. Heinricher MM, Barbaro NM, Fields HL. Putative nociceptive modulating neurons in the rostral ventromedial medulla of the rat: firing of on- and off-cells is related to nociceptive responsiveness. Somatosens Mot Res 1989; 6:427–439.
12. Heinricher MM, Morgan MM, Tortorici V, Fields HL. Disinhibition of off-cells and antinociception produced by an opioid action within the rostral ventromedial medulla. Neuroscience 1994; 63:279–288.
13. Lewis GN, Rice DA, McNair PJ. Conditioned pain modulation in populations with chronic pain: a systematic review and meta-analysis. J Pain 2012; 13:936–944.
14. Löffler M, Schneider P, Schuh-Hofer S, *et al.* Stress-induced analgesia in patients with chronic musculoskeletal pain and healthy controls. arXiv preprint arXiv:190207795 2019. AQ4
15. Le Bars D, Villanueva L, Bouhassira D, Willer JC. Diffuse noxious inhibitory controls (DNIC) in animals and in man. Patol Fiziol Eksp Ter 1992; (4):55–65.
16. Bouhassira D, Bing Z, Le Bars D. Studies of brain structures involved in diffuse noxious inhibitory controls in the rat: the rostral ventromedial medulla. J Physiol 1993; 463:667–687.
17. Youssef AM, Macefield VG, Henderson LA. Pain inhibits pain; human brainstem mechanisms. Neuroimage 2016; 124(Pt A):54–62.
18. ■■ Boorman DC, Keay KA. Morphine-conditioned placebo analgesia in female and male rats with chronic neuropathic pain: c-Fos expression in the rostral ventromedial medulla. Neuroscience 2021; 457:51–73.
Pharmacological conditioning elicited placebo analgesia from 25% of female and 36% of male rats with chronic neuropathic pain.
19. Eippert F, Bingel U, Schoell ED, *et al.* Activation of the opioidergic descending pain control system underlies placebo analgesia. Neuron 2009; 63:533–543.
20. Grahl A, Onat S, Büchel C. The periaqueductal gray and Bayesian integration in placebo analgesia. Elife 2018; 7:. AQ5
21. ■■ Crawford LS, Mills EP, Hanson T, *et al.* Brainstem mechanisms of pain modulation: a within-subjects 7T fMRI study of placebo analgesic and nocebo hyperalgesic responses. J Neurosci 2021; 41:9794–9806.
A 7-Tesla MRI experiment where both placebo and nocebo responses were elicited in the same group of subjects. Brainstem activity was compared between the two phenomena and was found to rely on overlapping brainstem pathways, principally the PAG-RVM circuit.
22. Stroman PW, Powers JM, Ioachim G, *et al.* Investigation of the neural basis of expectation-based analgesia in the human brainstem and spinal cord by means of functional magnetic resonance imaging. Neurobiol Pain 2021; 10:100068.
23. Wiech K, Eippert F, Vandekerckhove J, *et al.* Cortico-brainstem mechanisms of biased perceptual decision-making in the context of pain. J Pain 2021; 23:680–692.
24. Butler RK, Finn DP. Stress-induced analgesia. Prog Neurobiol 2009; 88:184–202.
25. Oliva V, Hartley-Davies R, Moran R, *et al.* Simultaneous brain, brainstem, and spinal cord pharmacological-fMRI reveals involvement of an endogenous opioid network in attentional analgesia. Elife 2022; 11:.
26. ■ Oliva V, Gregory R, Davies W-E, *et al.* Parallel cortical-brainstem pathways to attentional analgesia. NeuroImage 2021; 226:117548.
Experimental investigation of attentional analgesia utilizing a brainstem-optimized approach to discern the involvement of the PAG-RVM system, and ascending LC input, for driving attentional analgesia.
27. Koban L, Gianaros PJ, Kober H, Wager TD. The self in context: brain systems linking mental and physical health. Nat Rev Neurosci 2021; 22:309–322.
28. Ashar YK, Chang LJ, Wager TD. Brain mechanisms of the placebo effect: an affective appraisal account. Annu Rev Clin Psychol 2017; 13:73–98.
29. Tracey I, Mantyh PW. The cerebral signature for pain perception and its modulation. Neuron 2007; 55:377–391.
30. Heinricher MM. Pain modulation and the transition from acute to chronic pain. Adv Exp Med Biol 2016; 904:105–115.
31. Bernaba M, Johnson KA, Kong JT, Mackey S. Conditioned pain modulation is minimally influenced by cognitive evaluation or imagery of the conditioning stimulus. J Pain Res 2014; 7:689–697.
32. Youssef AM, Macefield VG, Henderson LA. Cortical influences on brainstem circuitry responsible for conditioned pain modulation in humans. Hum Brain Mapp 2016; 37:2630–2644.
33. Blanchet PJ, Brefel-Courbon C. Chronic pain and pain processing in Parkinson's disease. Prog Neuro-Psychopharmacol Biol Psychiatry 2018; 87:200–206.
34. Urits I, Adamian L, Fiocchi J, *et al.* Advances in the understanding and management of chronic pain in multiple sclerosis: a comprehensive review. Curr Pain Headache Rep 2019; 23:1–11.
35. Fischer TZ, Waxman SG. Neuropathic pain in diabetes—evidence for a central mechanism. Nat Rev Neurol 2010; 6:462–466.
36. Tang J, Bair M, Descalzi G. Reactive astrocytes: critical players in the development of chronic pain. Front Psychiatry 2021; 12:682056.
37. Crofford LJ. Chronic pain: where the body meets the brain. Trans Am Clin Climatol Assoc 2015; 126:167–183.
38. Marciszewski KK, Meylakh N, Di Pietro F, *et al.* Changes in brainstem pain modulation circuitry function over the migraine cycle. J Neurosci 2018; 38:10479–10488.
39. Yarnitsky D. Conditioned pain modulation (the diffuse noxious inhibitory control-like effect): its relevance for acute and chronic pain states. Curr Opin Anaesthesiol 2010; 23:611–615.
40. Wilder-Smith OH, Schreyer T, Scheffer GJ, Arendt-Nielsen L. Patients with chronic pain after abdominal surgery show less preoperative endogenous pain inhibition and more postoperative hyperalgesia: a pilot study. J Pain Palliat Care Pharmacother 2010; 24:119–128.

41. Kennedy DL, Kemp HI, Ridout D, *et al.* Reliability of conditioned pain modulation: a systematic review. Pain 2016; 157:2410–2419.

42. Mills EP, Keay KA, Henderson LA. Brainstem pain-modulation circuitry and its ■ plasticity in neuropathic pain: insights from human brain imaging investigations. Front Pain Res 2021; 2:705345.

A review article highlighting the recent advancements in understanding of brainstem function for the manifestation and persistence of chronic pain.

43. Soni A, Wanigasekera V, Mezue M, *et al.* Central sensitization in knee osteoarthritis: relating presurgical brainstem neuroimaging and PainDETECT-based patient stratification to arthroplasty outcome. Arthritis Rheumatol 2019; 71:550–560.

44. Schulte LH, May A. The migraine generator revisited: continuous scanning of the migraine cycle over 30 days and three spontaneous attacks. Brain 2016; 139:1987–1993.

AQ6 **45.** Mills EP DPF, Alshelh Z, Peck CC, *et al.* Brainstem pain control circuitry connectivity in chronic neuropathic pain. J Neurosci 2017.

46. Silva M, Amorim D, Almeida A, *et al.* Pronociceptive changes in the activity of rostroventromedial medulla (RVM) pain modulatory cells in the streptozotocin-diabetic rat. Brain Res Bull 2013; 96:39–44.

47. Wang R, King T, De Felice M, *et al.* Descending facilitation maintains long-term spontaneous neuropathic pain. J Pain 2013; 14:845–853.

48. Kaushal R, Taylor BK, Jamal AB, *et al.* GABA-A receptor activity in the noradrenergic locus coeruleus drives trigeminal neuropathic pain in the rat; contribution of NAα1 receptors in the medial prefrontal cortex. Neuroscience 2016; 334:148–159.

49. Henderson LA, Keay KA. Imaging acute and chronic pain in the human brainstem and spinal cord. Neuroscientist 2018; 24:84–96.

50. MacCallum CA, Eadie L, Barr AM, *et al.* Practical strategies using medical cannabis to reduce harms associated with long term opioid use in chronic pain. Front Pharmacol 2021; 12:633168.

51. Kosten TR, George TP. The neurobiology of opioid dependence: implications for treatment. Sci Pract Perspect 2002; 1:13–20.

52. Enck P, Klosterhalfen S, Weimer K, *et al.* The placebo response in clinical trials: more questions than answers. Philos Trans R Soc Lond B Biol Sci 2011; 366:1889–1895.

53. Tuttle AH, Tohyama S, Ramsay T, *et al.* Increasing placebo responses over time in U.S. clinical trials of neuropathic pain. Pain 2015; 156:2616–2626.

54. Finniss DG, Kaptchuk TJ, Miller F, Benedetti F. Biological, clinical, and ethical advances of placebo effects. Lancet 2010; 375:686–695.

55. Klinger R, Stuhlreyer J, Schwartz M, *et al.* Clinical use of placebo effects in patients with pain disorders. Int Rev Neurobiol 2018; 139:107–128.

56. Garza-Villarreal EA, Wilson AD, Vase L, *et al.* Music reduces pain and increases functional mobility in fibromyalgia. Front Psychol 2014; 5:90.

57. Garza-Villarreal EA, Pando V, Vuust P, Parsons C. Music-induced analgesia in chronic pain conditions: a systematic review and meta-analysis. Pain Physician 2017; 20:597–610.

58. Mitchell LA, MacDonald RA. An experimental investigation of the effects of preferred and relaxing music listening on pain perception. J Music Ther 2006; 43:295–316.

59. Fauerbach JA, Lawrence JW, Haythornthwaite JA, Richter L. Coping with the stress of a painful medical procedure. Behav Res Ther 2002; 40:1003–1015.

60. Roy M, Peretz I, Rainville P. Emotional valence contributes to music-induced analgesia. Pain 2008; 134:140–147.

61. Brown CJ, Chen ACN, Dworkin SF. Music in the control of human pain. Music Ther 1989; 8:47–60.

62. Dobek CE, Beynon ME, Bosma RL, Stroman PW. Music modulation of pain perception and pain-related activity in the brain, brain stem, and spinal cord: a functional magnetic resonance imaging study. J Pain 2014; 15:1057–1068.

63. Pando-Naude V, Barrios FA, Alcauter S, *et al.* Functional connectivity of music-induced analgesia in fibromyalgia. Sci Rep 2019; 9:15486.

64. Wager TD, Scott DJ, Zubieta J-K. Placebo effects on human μ-opioid activity during pain. Proc Natl Acad Sci USA 2007; 104:11056–11061.

65. Pehlivan S, Karadakovan A. Effects of aromatherapy massage on pain, functional state, and quality of life in an elderly individual with knee osteoarthritis. Jpn J Nurs Sci 2019; 16:450–458.

66. Vakilian K, Atarha M, Bekhradi R, Chaman R. Healing advantages of lavender essential oil during episiotomy recovery: a clinical trial. Complement Ther Clin Pract 2011; 17:50–53.

67. Sasannejad P, Saeedi M, Shoeibi A, *et al.* Lavender essential oil in the treatment of migraine headache: a placebo-controlled clinical trial. Eur Neurol 2012; 67:288–291.

68. Kim JT, Wajda M, Cuff G, *et al.* Evaluation of aromatherapy in treating postoperative pain: pilot study. Pain Pract 2006; 6:273–277.

69. Price JL, Slotnick BM, Revial MF. Olfactory projections to the hypothalamus. J Comp Neurol 1991; 306:447–461.

70. Coelho LS, Correa-Netto NF, Masukawa MY, *et al.* Inhaled *Lavandula angustifolia* essential oil inhibits consolidation of contextual- but not tone-fear conditioning in rats. J Ethnopharmacol 2018; 215:34–41.

71. Ongür D, An X, Price JL. Prefrontal cortical projections to the hypothalamus in macaque monkeys. J Comp Neurol 1998; 401:480–505.

72. Jo H-G, Wudarczyk O, Leclerc M, *et al.* Effect of odor pleasantness on heat-■■ induced pain: an fMRI study. Brain Imaging Behav 2021; 15:1300–1312.

Functional imaging study utilizing positive and negative odours to modulate the experience of pain. Positive odour inhibited and negative odour enhanced perceived pain compared to a control 'no odour' condition. Irrespective of valence, odour induced amygdala activation. Furthermore, functional connectivity between the amygdala and prefrontal cortices related to the pain experience – demonstrating common systems between olfaction and pain.

73. Wang J, Eslinger PJ, Smith MB, Yang QX. Functional magnetic resonance imaging study of human olfaction and normal aging. J Gerontol A Biol Sci Med Sci 2005; 60:510–514.

74. Masaoka Y, Takayama M, Yajima H, *et al.* Analgesia is enhanced by providing information regarding good outcomes associated with an odor: placebo effects in aromatherapy? Evid Based Complement Alternat Med 2013; 2013:921802.

75. Ju MS, Lee S, Bae I, *et al.* Effects of aroma massage on home blood pressure, ambulatory blood pressure, and sleep quality in middle-aged women with hypertension. Evid Based Complement Alternat Med 2013; 2013:403251.

76. Cho MY, Min ES, Hur MH, Lee MS. Effects of aromatherapy on the anxiety, vital signs, and sleep quality of percutaneous coronary intervention patients in intensive care units. Evid Based Complement Alternat Med 2013; 2013:381381.

77. LoBisco SA. The Power of Olfaction in the Relief of Pain Naturopathic Doctor News & Review: ndnr; 2014. Available at: https://ndnr.com/pain-medicine/the-smell-of-pain/.

78. Read Paul L, Salmon C, Sinnarajah A, Spice R. Web-based videoconferencing for rural palliative care consultation with elderly patients at home. Support Care Cancer 2019; 27:3321–3330.

79. Groninger H, Stewart D, Wesley D, *et al.* Virtual reality for management of cancer pain: study rationale and design. Contemp Clin Trials Commun 2022; 26:100895.