Stress-sensitive neurosignalling in depression: an integrated network biology approach to candidate gene selection for genetic association analysis

J. Anke M. van Eekelen,[1] Justine A. Ellis,[2] Craig E. Pennell,[3] Jeff Craig,[4] Richard Saffery,[4] Eugen Mattes,[1] Craig A. Olsson[5]

[1]Developmental Neuroscience, Telethon Institute for Child Health Research and Centre for Child Health Research, University of Western Australia, Perth; [2]Environmental and Genetic Epidemiology, Murdoch Childrens Research Institute, The Royal Children's Hospital and Department of Physiology, University of Melbourne; [3]The School of Women's and Infants' Health, University of Western Australia at King Edward Memorial Hospital; [4]Developmental Epigenetics, Early Development and Disease, Department of Paediatrics, Murdoch Childrens Research Institute, Royal Children's Hospital; [5]School of Psychology, Deakin University Australia; Murdoch Childrens Research Institute; University of Melbourne, Australia

Abstract

Genetic risk for depressive disorders is poorly understood despite consistent suggestions of a high heritable component. Most genetic studies have focused on risk associated with single variants, a strategy which has so far only yielded small (often non-replicable) risks for depressive disorders. In this paper we argue that more substantial risks are likely to emerge from genetic variants acting in synergy within and across larger neurobiological systems (polygenic risk factors). We show how knowledge of major integrated neurobiological systems provides a robust basis for defining and testing theoretically defensible polygenic risk factors. We do this by describing the architecture of the overall stress response. Maladaptation via impaired stress responsiveness is central to the aetiology of depression and anxiety and provides a framework for a systems biology approach to candidate gene selection. We propose principles for identifying genes and gene networks within the neurosystems involved in the stress response and for defining polygenic risk factors based on the neurobiology of stress-related behaviour. We conclude that knowledge of the neurobiology of the stress response system is likely to play a central role in future efforts to improve genetic prediction of depression and related disorders.

Introduction

Twin studies have consistently suggested high heritability of common mental disorders (40-50% for depression and anxiety).[1] However, despite significant investment in genome wide association studies (GWAS) and specific studies of biologically relevant candidate genes, to date, progress in understanding the molecular contribution to these disorders has been slow.[1] Even in comparison to other complex diseases, mental disorders lag behind in terms of understanding the underlying genetic mechanisms. Among the published findings,[2] there are few examples where significant results have been replicated indicating a propensity within the field to yield false positives. One of the significant challenges of psychiatric genetic research, and indeed research on complex diseases more generally, is that risk associated with individual variants [typically single nucleotide polymorphisms (SNPs) >1% prevalence] has been consistently shown to be small.[1] This raises important methodological challenges around the detection of variants of small effects and questions about the applied utility of such variants when detected. Reliable detection of small effects is critically dependent high quality and well powered studies, heterogeneity of which continues to be a major cause of the lack of replication in the field.[3] Even for genetic variants with demonstrated functional effects (*e.g.* 5HTTLPR), cross-study replication has been difficult and individual functional loci have likewise not translated into large effects on risk for mental disorders.[4,5] In this general context of small effects and associated difficulties with outcome replication, there has been a growing interest in other forms of genetic risk that might have higher aetiological significance. For example, with enhanced deep sequencing capabilities provided by new generation technologies, there has been a shift in interest from common to rare variants on the understanding that rare variants might play a more substantial aetiological role in complex disease.[6,7] However, even if higher predictive values are observed for rare variants, these would only apply to a small proportion of the population and arguably are characterised by more extreme *clinical* phenotypes. It would provide little insight into the genetic determinants of the more common mental disorders, which drive the global burden of disease. Another idea, which has applicability to a broader range of phenotypes, has attracted considerable attention: synergistic actions between multiple (common) *loci* of small effect may define more substantial genetic risk for common mental disorders. This has lead to interest in assembling SNPs into SNP composites, or more precisely, polygenic risk pathways.[8] The essential idea is that joint effects have the potential to confer aetiological impacts beyond the sum of their individual parts.[9-13] The importance of integrated approaches to investigate composite genetic risk is gaining increasing leverage in other areas (such as cancer genetics) and methodol-

Correspondence: Craig A. Olsson, Murdoch Childrens Research Institute, Flemington Road Parkville, VIC 3052, Australia.
Tel: +61.393456250 - Fax: +61.393456502.
E-mail: craig.olsson@rch.org.au

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ogy development is an active field of research.[14] However, in the presence of millions of variants available within for example a GWAS dataset, and billions of unique combinations between variants, the challenge is develop methods that enable identification of polygenic risk factors. One approach is to use conventional interaction models to *atheoretically* scan for interaction between variants within, for example, a GWAS dataset. The problem with this approach is that sample size requirements and multiple testing burdens increase exponentially with interaction complexity and place severe constraints on investigations. This limit is fundamental and cannot be avoided by purely statistical means.[15] An alternative is to examine cumulative effects by summing risk alleles to create a continuous *profile* score. This polygenic profile can then be associated with phenotypes of interest.[16] Profile scoring was pioneered in the context of GWAS by Wray and co-workers and represents a do-able way of commencing investigations into polygenic effects while other methodologies continue to be developed.[17] Polygenic profiling is particularly suited to investigations within well defined biological fields, such as the study of specific apoptosis pathways in cancer and growth pathways predicting birth weight.[18,19] This strategy is informed by a *systems biology* approach. The neurobiology of the stress response is a well-defined biological system capable of providing robust guidance to profile scoring methods for investigating polygenic risk factors relevant to common mental disorders. A systems biology approach brings additional advantages to research on complex disease: i) it reduces multi-testing burden by restricting the focus of analysis to meaningful biological pathways and ii) it provides a basis for identifying genes and gene networks of higher aetiological impact because of their position and role within known biological pathways.[3] The purpose of this review is to describe how knowledge of major integrated neurobiological systems underlying stress-related behaviour could be used to guide a systems biology approach to identifying and testing theoretically defensible polygenic risk factors for common mental disorders - in particular, depressive and anxiety disorders. To do this, we first describe the physiological architecture of major neurobiological systems underlying the regulation of stress responsiveness and stress-sensitive behaviour: the *Hypothalamic-Pituitary-Adrenocortical (HPA) axis*, the *Meso-Corticolimbic System (MCLS),* the *Hindbrain Autonomic Regulatory System (HARS)* and the *Renin-Angiotensin System (RAS)*. We then describe four principles for candidate gene selection, which is based on the cumulative weight of evidence for the role of the gene products in each of these neurosignalling systems and as part of their interactions.

Neurobiological systems important to stress-sensitive mood regulation

Mental health relies on the ability to regulate cognition, to control emotion and behaviour and to cope with stress. The *HPA axis, the MCLS, the HARS and the RAS* have all been implicated in the management of executive cognitive functioning, behavioural inhibition and emotional and stress reactivity.[20] Pivotal neurotransmitters involved in these systems are dopamine (DA), serotonin (5HT), norepinephrine (NE), epinephrine (E) and γ-aminobutyric acid (GABA). Other indispensable neuroactive peptides and hormones are angiotensin (ANG) II, corticotrophin releasing hormone (CRH), arginine vasopressin (AVP), cortisol (CORT) and adrenalin. There is clear overlap in forebrain target sites of innervation by these signalling systems (Figure 1A). Furthermore, the mode of action of each of these systems is particularly sensitive to stress. Together, these signalling systems form the basis of an intricate network that links the corticolimbic system to the hypothalamus in the forebrain, the autonomic regions in the hindbrain and stress hormone secreting glands outside the brain (Figure 1B) to control mood and its associated behaviour.

The hypothalamic-pituitary-adrenocortical axis

Psychological stress plays an important role in the aetiology of mood disorders and tends to result in hyperactivity of the HPA axis, which elevates the level of the stress hormone CORT in the circulation (Figure 1B). This neuroendocrine phenomenon is frequently observed in various forms of depression and anxiety and explains why these specific mental health illnesses are often referred to as stress-related disorders associated with impaired regulation of stress hormones.[21]

The cortex of the adrenal glands is the primary site of biosynthesis of CORT. Regulation of CORT synthesis and release by the adrenal glands commences with synthesis of CRH in the parvocellular neurons of the paraventricular nucleus in the hypothalamus of the forebrain (Figure 1B).[22,23] CRH is released into the portal blood system around the pituitary gland, where it binds to CRHR1 receptors on a specific type of cells within the anterior subdivision of the pituitary, the corticotrophic cells. Here, binding of CRH to its receptors triggers the expression of pro-opiomelanocortin and the release of its derivative adrenocorticotrophic hormone (ACTH) into the blood circulation. In turn, ACTH can reach the adrenal glands situated on top of the kidneys, to stimulate the release of CORT from the adrenal cortex. Corticosteroid binding globulin (CBG) in blood is able to temporarily inactivate CORT, which identifies a distinct level of regulation of functional CORT available for biological action in the stress response system. If not bound to CBG, CORT can easily move from blood into brain tissue across the blood-brain barrier (BBB), where it binds to 2 types of corticosteroid receptors; the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR).[24] MR has the highest affinity for CORT and is predominantly localised in the hippocampus, amygdala and prefrontal cortex, important corticolimbic brain regions in the appraisal of stress and initiation of a stress response if needed. Although GR is more widely distributed throughout the brain, its strength to bind CORT is about 10-fold lower compared to MR. CORT binding to MR and GR in the hippocampus and prefrontal cortex indirectly inhibits CRH biosynthesis in the hypothalamic paraventricular nucleus via interconnections of mostly GABA-ergic interneurons between these forebrain regions.[25] As a consequence, overall HPA activity decreases and reduces CORT synthesis and release. This type of autoregulation of CORT ensures termination of the stress response when the triggering stimulus is no longer there.

Under conditions of severe and chronic stress, the parvocellular CRH neurons in the paraventricular nucleus can produce even more arginine vasopressin, which acts as an additional stimulus for ACTH release via specific V1b receptors on corticotrophic cells in the anterior pituitary.[26,27] Sustained stress also compromises the inhibitory role of the hippocampus on the HPA cascade of neuroendocrine events via downregulation of MR and GR and in the long run reduced hippocampal neurogenesis.[28,29] Furthermore, stress triggers a CRH producing neuronal system outside the hypothalamic paraventricular nucleus (Figure 2A), where CRH accessibility to its neural receptors is regulated by CRH binding protein (CRH-BP).[22] Of particular importance to HPA regulation are the CRH projections from the central amygdala to the paraventricular nucleus, which stimulate hypothalamic CRH synthesis.[23,30] Yet CRH signalling is not limited to HPA regulation; CRH also influences other types of brain function as part of the overall stress response. CRH neuromodulatory effects in the hindbrain influence NE projections from the locus coeruleus to the forebrain resulting in enhanced arousal and vigilance (Figure 2A). If dysfunctional, this pathway can contribute to depression and anxiety. Similar local signalling effects of CRH on neurotransmission in amygdala and the midbrain ventral tegmental area and dorsal raphe nucleus are also implicated in anxiety behaviour and enhanced sympathetic nervous activity in response to stress.[22,23]

The meso-corticolimbic system

Connectivity between the midbrain and the limbic system in the forebrain is crucial for higher brain functions involved in the regulation of stress sensitive mood states. Distinct MCLS projections, as part of a larger monoamine neurotransmission system in the brain, include DA and 5HT pathways originating in the midbrain and projecting anteriorly (in a forward direction) to selected limbic and frontal cortical regions (Figure 1A; blue projections-DA/red projections-5HT). These projections typically function in those brain circuitries responsible for cognition, regulation of emotion, behaviour, motivation and reward besides movement.

The DA pathways within the MCLS of most interest to mood control are the meso-limbic and the meso-cortical projections from the ventral tegmental area in the midbrain. These either target the limbic network of interactions between the nucleus accumbens, amygdala, hippocampus and ventral striatum or extend further anterior to the prefrontal cortex among other frontal cortical regions involved in executive functioning (Figure 2B).[31] These specific DA projections play an important role in motivational behaviour and are thought to underpin the incentive, preparatory or acquisition aspects of reward based behaviours.[32-34] Reward deficit due to compromised function of the mesolimbic DA pathway has been associated with addiction disorders and may play a key role in *ahedonia* (the inability to experience pleasure), which is a diagnostic feature of depression.[20,23]

Biosynthesis of DA for MCLS neurotransmission takes place in the ventral tegmental area and relies on neuronal uptake of the amino acid precursor tyrosine, which is hydroxylated into dihydroxy-l-phenylalanine (DOPA) by tyrosine hydroxylase (TH). Final processing of DOPA by DOPA-decarboxylase (DDC) generates the neurotransmitter DA. Metabolism of DA within the presynaptic terminus is attained by monoamine oxidase A (MAO-A) activity, whereas catechol-oxymethyltransferase (COMT) metabolises DA mostly in the synaptic cleft. These catabolic enzymes are present in all forebrain target regions of the MCLS. They are also synthesised in the ventral tegmental area and feed forward projection loops exist from cortical regions like the prefrontal cortex (PFC) back to the midbrain.

Reuptake of DA as a means to regulate DA availability for neurotransmission is achieved by specific Na^+/Cl^- dependent DA transporters (DAT). A low density of DA transporters in the prefrontal cortex allows diffusion of synaptic DA into other cortical and subcorticical regions not directly targeted by the meso-corticolimbic DA projections.[35] Reception of DA at the post synaptic cleft relies on binding to specific DA receptors [D1-like receptors (DRD1, 5) and

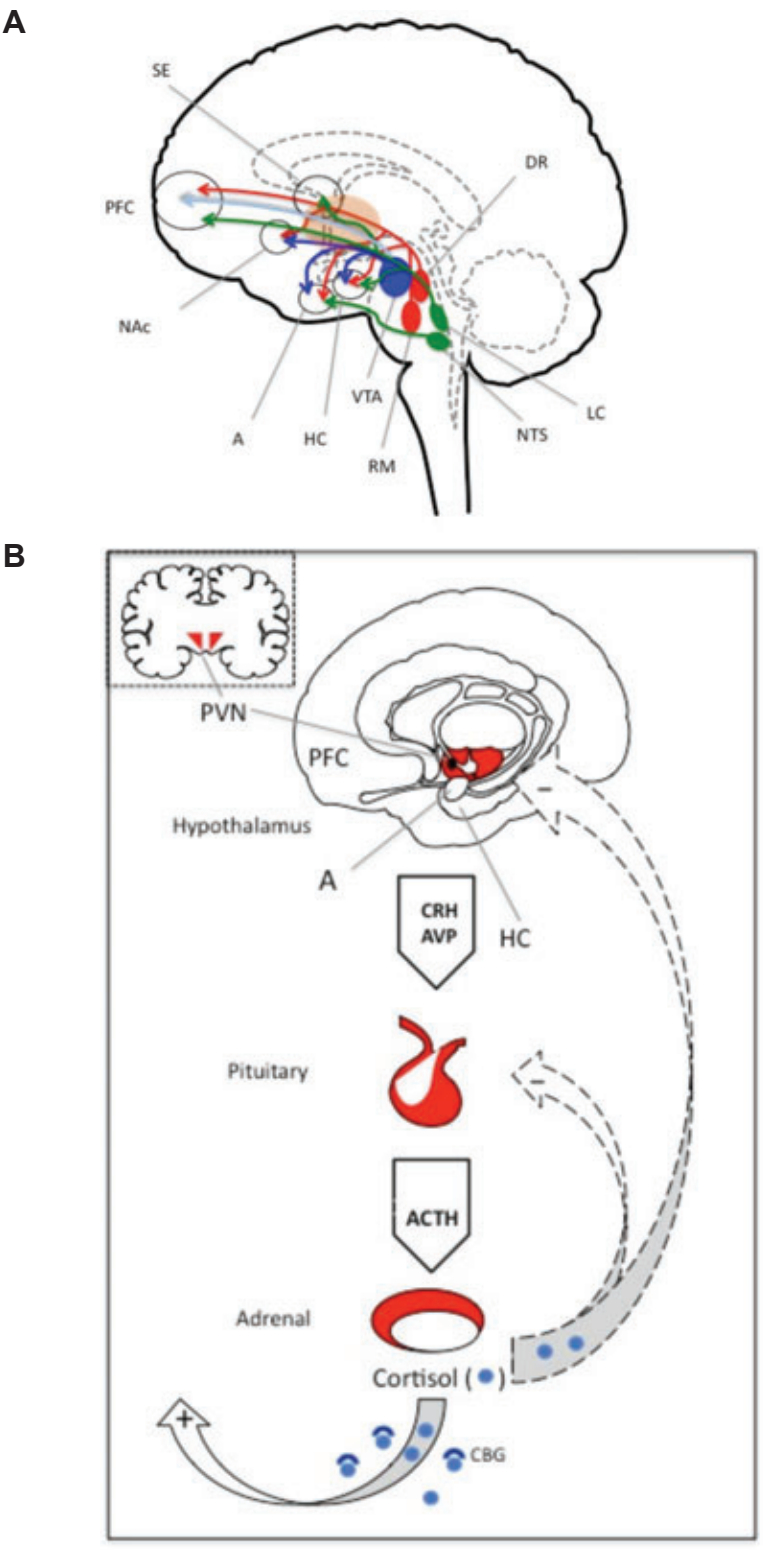

Figure 1. Signalling pathways responsible for stress responsiveness, emotional behaviour and adaptation. A) Forward (anterior) signalling pathways originating in the midbrain (light and dark blue: dopamine-ergic/red: serotonin-ergic) and the hindbrain (green: norepinephrine-ergic) and projecting to major corticolimbic forebrain regions. B) the stress-sensitive cascade of neuroendocrine events along the hypothalamic-pituitary-adrenal axis. The red areas represent the hypothalamus, the anterior pituitary and the adrenal cortex respectively from top to bottom. The insert in the top left corner represents a coronal view of the human brain at the level of the hypothalamus, where the paraventricular nucleus (red triangles) symmetrically flanks the 3rd ventricle at the base of the brain. Cortisol (blue dots), as the end product of the hypothalamic-pituitary-adrenal axis, is partly buffered in the circulation by corticosteroid binding globulin (dark blue caps).

D2-like receptors (DRD2, 3, 4)].[36] These G-protein coupled transmembrane receptors are either located: i) on postsynaptic terminals of interconnecting neurons that establish synaptic neurotransmission within functional corticolimbic networks, or ii) on a wider range of forebrain neurons sensitive to neuromodulation by DA. All types of DA receptors are present in corticolimbic DA target regions, albeit in different configurations per region; however, the meso-corticolimbic circuitry related to mood regulation does not seem to include specific autoreceptors (mostly D2-like in the rat brain) in the human midbrain ventral tegmental area region for direct feedback.

The 5HT pathways within the MCLS, important to mood control, arise in selected raphe nuclei (dorsal raphe and raphe magnus) of the midbrain and project to a series of limbic and cortical regions linked to regulation of emotion, memory and focussed attention (Figure 1A).[37,38]

Biosynthesis of 5HT is similar to DA; it is reliant on the neuronal uptake of tryptophan, which is converted to the intermediate metabolite 5-hydroxytryptophan by tryptophan hydroxylase (TrH) before being processed by l-aromatic acid decarboxylase to 5HT, the signalling factor specific to this neurotransmission system. Metabolism of 5HT is achieved by enzymatic activity of MAO-A, which metabolises the neurotransmitter into its inactive metabolite 5-hydroxyindolacetic acid (5HIAA) among others. Moreover, 5HT, like DA, has the ability to influence signal transduction in the corticolimbic system as: i) a neurotransmitter of interconnected neurons of a functional circuit, and ii) a neuromodulator via more widespread diffusion of 5HT in limbic and frontal brain regions linked to regulation of mood and motivation.

Reuptake of 5HT by the presynaptic neuron regulates the availability of 5HT in the synaptic cleft and is most specifically controlled by the 5HT transporter (5HTT) mediating presynaptic neuronal reuptake of 5HT. Genetic variance in the human *5HTT* gene in combination with environmental stimuli has been implicated in the development of depression.[39-41] In particular, *abuse in childhood* has been implicated in the development of depression in adulthood: individuals who are homozygous for the risk 5HTT allele and who also suffered abuse in childhood, have been reported to have a three-fold increase in their risk of major depression in adulthood (Caspi *et al*., 2003). Although alternative studies have confirmed this adverse gene x environment interaction effect and the finding remains supported by some,[39-42] it is disputed by others.[4]

Reception of 5HT is achieved by a family of transmembrane receptors characterized by diversity in both species and brain region specific isoforms (5-HT1A, B, 2A, 3-7). 5HT1

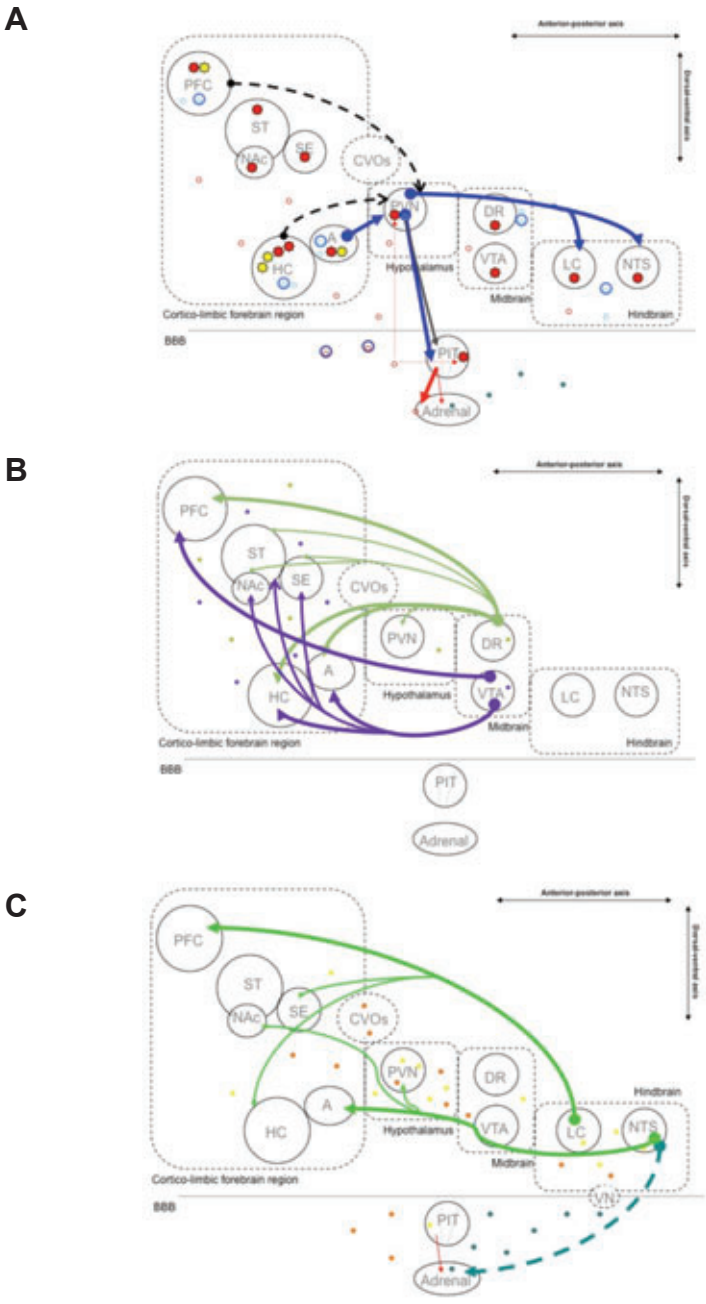

Figure 2. Schematic signalling pathways between various brain regions of the forebrain corticolimbic network, the forebrain hypothalamus, the midbrain and the hindbrain. These signalling pathways specifically relate to A) the neuroendocrine response to stress. Arrows: blue - corticotrophin releasing hormone, red - adrenocorticotrophic hormone, dark grey - arginine vasopressin, dashed red - cortisol negative feedback action, dashed black-various indirect neuromediators/solid stars: red - glucocorticoid receptor, yellow - mineralocorticoid receptor/open circles: red - cortisol, light blue - corticotrophin releasing hormone, large dark blue - corticosteroid binding globulin, large middle blue - corticotrophin releasing hormone BP/ solid dots: middle green - adrenalin. B) The catecholamine projections within the mesocorticolimbic circuitry. Arrows: purple - targeted dopamine projections, dark green - targeted serotonin projections/solid dots: purple - diffused dopamine, dark green - diffused serotonin. C) The hindbrain autonomic response system and the renin angiotensin system. Arrows: light green: targeted norepinephrine projections, red - adrenocorticotrophic hormone targeting the adrenal medulla, dashed middle green - indirect epinephrine-projection via the sympatho-adrenal medulla axis/solid dots: orange - peripheral angiotensin II, yellow - brain angiotensin II. The neurophysiological detail outlined in the conceptual flowcharts is predominantly based on fundamental studies performed in rodent models, which over time have been complemented by supportive clinical research and neuropharmacological studies in humans, underscoring the high degree of similarities in cognitive brain function underlying emotion and behaviour across species.

receptors typically mediate a suppressive effect on neuronal firing. Receptor type 5HT1A is predominantly postsynaptic and abundantly present in forebrain regions such as prefrontal cortex, hippocampus, amygdala and lateral septum. It also functions as an autoreceptor on 5HT synthesizing neurons in the dorsal raphe and raphe medium region of the midbrain. 5HT1B receptors have been identified in the rat brain but not in the human brain.[43] In contrast to 5HT1 receptors, 5HT2-7 receptors tend to mediate stimulatory 5HT influences on neuronal activity in the prefrontal cortex, hippocampus, amygdala, nucleus accumbens and the hypothalamus. It is worth noting though that 5HT target regions may contain neurons with similar numbers of suppressive (5HT1) and stimulatory (5HT2-7) receptors. Here, the polarisation state of the neuron at the time of 5HT signalling will determine its overall neuroactive impact via the various types of 5HT receptors on the same neuron.

The hindbrain autonomic regulatory system

Two essentially different types of projections within the HARS contribute significantly to stress-sensitive mood regulation: i) the NE projections from the hindbrain forward to forebrain, which take part in stress related alertness and memory processing, and ii) the E projections from hindbrain via the spinal cord out to the periphery, which are instrumental for the stress-induced fight-or-flight response (Figure 2C).

Part of the anterior NE projections from the locus coeruleus in the hindbrain to the prefrontal cortex, hippocampus and septum are functionally implicated in memory and anxiety-related behaviour. Others from the NE-rich A2 cell group of neurons in the hindbrain solitary tract nucleus strongly innervate the amygdala, resulting in distinct enhancement of memory processing of emotionally loaded events (Figure 2C).

Biosynthesis of NE occurs in specific catecholaminergic neurons, which synthesise DA but unlike DA-neurons are able to convert DA into NE in the presence of dopamine β-hydroxylase within specific intracellular vesicles of the catecholaminergic neuron.[44] Metabolism of NE is controlled by MAO-A and COMT in a similar manner to that of DA. Likewise, neuronal reuptake of NE is carried out by NE transporters (NET), which function like DATs.

Reception of NE is mediated via α and β-type adrenergic receptors, which have brain region specific expression patterns: α_1 and α_2-adrenergic receptors are detected in abundance in the PFC, while the HC, A and SE contain higher densities of β-adrenergic receptors. The significance of NE signalling via β-adrenergic receptors in memory processing is highlighted by the indication that β-adrenergic receptor blockers can play a therapeutic role specifically in posttraumatic stress disorder, which is characterised by traumatic memory retrieval followed by enhanced emotional responsiveness under non-threatening circumstances.[29] The action of β-blockers is believed to inhibit with NE neurotransmission in the basolateral amygdala to negatively impact on the consolidation of emotionally loaded memories of stressful events.

A significantly different group of catecholaminergic neurons within the HARS express the enzyme phenylethanolamine-N-methyltransferase (PNMT) in addition to all other components of catecholamine biosynthesis pathway. PNMT allows the conversion of NE into epinephrine. These neurons are part of the sympatho-adrenal medulla (SAM) axis, a series of interconnections from the hindbrain via the spinal cord to the adrenal medulla (Figure 2C).[25] The SAM axis represents the primary controller of adrenaline release from the adrenal medulla, which acts as the stress hormone of stress-induced cardiovascular function among other actions of the autonomic nervous system. The HPA axis mediator ACTH can also stimulate adrenaline release under stressful circumstances, albeit to a lesser degree (Figure 2C). Typically being a peripheral effecter of the SAM axis, adrenaline cannot easily cross the BBB. Yet indirectly, stress-induced adrenaline can enhance NE signalling from the hindbrain to the amygdala via stimulation of the vagus nerve and consequent solitary tract nucleus activation to contribute to emotionally loaded memory processing via the amygdala in response to stress (Figure 2C).[44]

The renin angiotensin system

The RAS is the least characterised neural signalling system of interest to mood regulation and is better known for its role in regulating blood pressure and water and salt retention through ANG II. Peripherally generated ANG II is known to influence the brain by crossing the BBB in the circumventricular organs, a selected group of brain regions containing *leaky* capillaries with compromised barrier function (Figure 2C);[45] however, there is growing evidence of the existence of a distinct brain RAS.

Biosynthesis of Ang II starts with enzymatic cleavage of its precursor angiotensinogen into ANG I by renin. Angiotensin converting enzyme (ACE) then processes ANG I into a shorter 8 amino acid long peptide ANG II (sometimes also referred to as ANG-(1-8)). Various aminopeptidases are able to convert ANG II into even shorter but still bioactive angiotensin peptides (ANG III, IV and ANG-(1-7)), each differing slightly in amino acid sequence. Metabolism of ANGs is catalysed by endopeptidases. Most evidence for brain ANG II biosynthesis and release has been derived from analysis of RAS activity in the hypothalamus.[46] Reception of brain ANG II is accomplished by ANG receptors (AT1 and AT2)[47] throughout the central nervous system. This distribution pattern includes strategic brain regions involved in cognition and emotion, like the hippocampus, amygdala, septum and prefrontal cortex.[48]

Whether brain ANG II and its shorter derivatives should be classified as neurotransmitters awaits confirmation on the intraneuronal expression and formation of all RAS components. Nonetheless, as neuromodulators, the influence of brain ANGs appears to go beyond its classical involvement in cardiovascular control through regulation of sympathetic nerve activity.[49] Recent studies have highlighted their functional contribution to the regulation of stress, emotional behaviour and aspects of cognitive functioning.[46,50-53] Moreover, the best angiotensinergic pathway described in the hypothalamic paraventricular nucleus (PVN) directly affects the functional circuitry underlying stress and adaptation (Figure 2C).[52,53] Recent evidence from animal studies supports a role for the stress-sensitive brain RAS in co-regulating hypothalamic CRH expression and in turn facilitating secretion of ACTH from the pituitary.[52,53]

Integrated networks: thinking across systems

Optimal neurotransmission within the MCLS and NE hindbrain projections to the forebrain require exposure of the brain to CORT levels within a normal range.[29] The wide-ranging influence of CORT on the control of cognition, emotion and behaviour is made possible by the presence of corticosteroid receptors (MR and GR) throughout the brain and within all major neurotransmission systems underlying mood regulation.[28,54] Likewise, the location of stress-sensitive CRH and ANG II synthesis, release and reception appears to overlap considerably with the MCLS and the HARS.

A sudden change from non-stressful to stressful conditions induced by acute stress triggers the corticolimbic forebrain system to appraise the stress *stimulus* and initiate an overall stress response. This sets in motion almost simultaneously a series of related events that involve all neurobiological systems described in section 1:

SAM axis activation within HARS stimulating adrenaline release to mediate the fight or flight response to stress;[25]

HPA axis activation resulting in enhanced availability of CORT in the periphery and

brain to stimulate the production of glucose as an energy source in the response to stress as well as to enhance cerebral alertness and appraisal of the stressor for an appropriate stress response;[28,54]

Extrahypothalamic CRH activation resulting in elevated CRH synthesis and release from the amygdala projections to the hypothalamic paraventricular nucleus to sustain stress-induced activation of the HPA axis;[55]

RAS activation to enhance renin secretion leading to more centrally available ANG II contributing to stress-induced activation of the HPA axis;[50]

MCLS activation to release additional DA and 5HT in the forebrain;[23,56]

HARS activation to enhance NE release in the forebrain.[57]

The overall stress response also includes powerful interactive actions in the brain to increase the impact of some of these stress-induced events:

Stress-induced SAM axis activation within HARS augments NE release in the amygdala via indirect stress-induced adrenaline effects on the solitary tract nucleus in the hindbrain;[25]

Stress-induced HPA axis activation boosts the NE effect on improving memory processing of emotionally loaded events by stress-induced CORT action in the amygdala;[29,54]

Stress-induced MCLS activation further increases diffusely available DA in the corticolimbic system providing more opportunity for NETs to internalise DA for deamination into NE,[58] which adds to NE neurotransmission in the forebrain already enhanced by stress;

Stress-induced HPA axis activation eventually terminates the stress response directly via CORT binding to GR within the HPA axis but also indirectly via CORT binding to GR throughout the hippocampus, prefrontal cortex and other GR-rich forebrain regions that contribute to the MCLS.[29,54]

Under healthy conditions, a successful stress response may therefore temporarily exceed the normal range of CORT and other stress mediators, but this is usually followed by an efficient return to baseline activity of all systems when the stressful stimulus no longer exists and has been dealt with. The overall initiation and termination of stress responsiveness in the brain seems primarily dependent on CORT's mode of action via a balanced ratio of the MR and GR present in the integrated network of neuroactive signalling systems underlying in mood regulation.[28,54,59] Persistent exposure to stress tends to reverse these effects, which can lead to damage in the brain beyond repair of which hippocampal degeneration is a well known example.[23,28,29] Psychological stress underlying mood disorders tends to be of chronic nature and has been shown to result in long term HPA axis activity exceeding the normal range (Holsboer and Ising, 2009. *Data not shown*). Evidently, such chronic exposure to relatively high CORT relates strongly to psychological dysregulation (*i.e.* the inability to control emotion, behaviour and cognition).[20] This suggests that impaired regulation of stress hormones may directly impair MCLS and HARS functioning, creating neurodeficiencies causally linked to stress-related psychopathology. Clearly, powerful interactions between the neuroendocrine stress response systems (the HPA and SAM axis, the RAS) and the MCLS and HARS underlying regulation of mood related cognition, emotion and behaviour, necessitate an integrated network approach to further entangle the complexity of depression and anxiety.

One important application of a systems biology approach is in theoretically grouping SNPs identified by genome-wide association studies of depressive symptoms into biologically meaningful polygenic profile scores that can be tested in genetic epidemiological designs. For example, 3 of 6 genes replicated in a meta-analysis of genetic predictors of major depressive disorder (MDD) are in stress response pathways as described: *DRD4*, *SCL6A3* and *SCL6A4*.[60] Additionally, 2 of 4 four genes identified in a 2011 systematic review of genes associated with MDD, and replicated using genome wide data from the Genetic Association Information Network study,[61] are also in stress response pathways as described: *SCL6A2, ACE*. To date there has been little attempt to piece these replicating findings together into larger polygenic mechanism. This is largely due to uncertainty about how to do so. A systems biology approach avoids the substantial multiple testing burden associated with *atheoretical* testing of combinations of SNPs in genome-wide design. It also provides a means of testing more complex polygenic mechanisms than the two-way interactions typically tested in genome-wide designs. In this way, a systems biology approach provides an important framework for putting the pieces delivered by GWAS together in an integrated (polygenic) way.

Principles for prioritising genes and gene networks

Neurobiological knowledge of the stress response systems can work in parallel with genome-wide approaches by providing another strategy to identifying (prioritising) genes or composites of genes for investigation within genetic association designs. In particular, neurobiological knowledge of the stress response could be used to prioritise genes for more in depth investigation than genome-wide marking can provide. This could extend to next generation sequencing of genes that play rate-limiting roles in neurosignalling within stress response pathways. It could also extend to other determinants of gene expression, including epigenetic regulators (see below for further discussion).

Based on a considered analysis of the biological architecture of the stress-response systems, we propose four considerations (or principles) for theoretical selection of genes with intrinsic variations that are likely to influence the risk for mood regulation disorders; however, we acknowledge that this is not exhaustive. In what follows we describe each principle for theoretical candidate gene prioritisation and provide examples. Notably, these principles highlight specific aspects of genes, in which polymorphisms can compromise the functionality of encoded signalling and other types of proteins that act within individual systems of the integrated network regulating the overall stress response. In turn, a polygenic risk for depressive symptoms can be the additive outcome of independent smaller genetic effects in any of the four neurosignalling systems of interest, or the result of direct gene x gene interactions underpinning compromised neurosignalling.

Genes controlling rate limiting steps in biosynthesis that have no known compensatory mechanism

Some rate limiting steps have compensatory mechanisms; hence, a functional genetic mutation in one system may be masked by genetic counterbalance in another. This phenomenon is referred to as gene redundancy in genetically manipulated mouse models, in which compensation of aberrant gene function masks the phenotypic outcome of the genetic manipulation of interest.[62] However, genes that function across defined neurosignaling pathways, generally lack functional *backups*, highlighting the need for sequence and functional integrity. A good example of such a pivotal gene is *TH* in the MCLS. TH regulates the rate-limiting step in the biosynthesis of DA. Although its pivotal role to hydroxylate tyrosine parallels the activity of TrH to hydroxylate tryptophan in the early steps of monoamine biosynthesis and within the MCLS, TH could replace TrH in the rare absence of TrH to prevent 5HT depletion in the brain, it has no known compensatory mechanism itself. This highlights a key role for TH in overall brain monoamine neurosignalling.

Genes for which the gene product is the target of effective medicinal treatment of mood disorders

Selective serotonin reuptake inhibitors (SSRIs), which operate on the MCLS, are

widely prescribed as anti-depressants, which operate on the MCLS. SSRIs target the 5HT signalling system at the level of 5HT reuptake by 5HTT at the presynaptic end of 5HT neurons in the brain to prevent any unnecessary loss of functional 5HT in the synaptic cleft. The importance of 5HTT in the regulation of 5HT availability for postsynaptic reception in the MCLS has been long recognised.[40] Similarly, MAO-A inhibitors are geared towards enhancement of functional 5HT for neurotransmission. Both *5HTT* and *MAO-A* have already been intensively studied as candidate genes for gene-environment interactions with early life stress in depression;[39,41] however, their capacity to individually act as biomarker for depression risk remains equivocal.[4,42] Another MCLS gene product targeted by mood-regulatory medication would be the 5HT1A receptor as a common target site for anxiolytic drugs.[37] Within the HARS system, candidate genes can be prioritised by their gene products being drug targets for the NE-selective tricyclic antidepressants, which serve as a blocker of NET activity in the reuptake of NE to prolong NE clearance from the synaptic cleft and reduce the availability of functional NE for neurotransmission. Within the RAS, ACE inhibitors and AT1 receptor antagonists among other anti-hypertensive drugs, appear to reverse cognitive decline and signs of depression in anxious patients with high blood pressure.[63] Antiglucocorticoid compounds acting as corticosteroid receptor antagonists within the HPA axis have been tested for their antidepressant activity specifically in psychiatric patients with elevated HPA activity and animal models of this condition.[24,26,64] Such therapy highlights the indispensable role of GR in mediating CORT effects on brain and neuroendocrine function. Some depressed or anxious patients with elevated HPA activity also appear to benefit from CRHR1 receptor antagonists blocking CRH action in the hypothalamus among other CRH positive brain regions.[26]

Genes that directly influence more than one neurobiological system relevant to mood regulation

The best example of a gene that expresses a neuronal component which functions across all systems underlying mood regulation is the *GR*. Its widespread presence throughout the brain regions of neurosignal origin and innervation in the MCLS, HARS, RAS, SAM axis and HPA axis creates an overall sensitivity to CORT, which can turn into an overall vulnerability to psychological stress in mood related depression and anxiety.[24,25,29] *CRH* expression in the hypothalamic paraventricular nucleus but also a wide range of extrahypothalamic brain regions involved in MCLS and HARS function allows the stress-sensitive neuropeptide CRH to regulate more than one mood related neurobiological system. Finally, NE-selective *NET*s within the HARS also appear capable of taking up DA in a NE neuron within the corticolimbic system for further processing into NE.[58] By being able to target one of the effectors of the MCLS, a dual role for NETs in forebrain target regions of the MCLS and the HARS is highlighted.

Genes for which selected polymorphisms contribute to enhanced risk for atypical mood regulation based on empirical considerations

Previous genetic association studies have reported specific polymorphisms in various genes encoding for key mediators of the integrated network described in this review, to raise the odds of exhibiting adverse mental health behaviour. A recent review of GWAS with relevant outcomes for a broad range of psychiatric disorders lists genes like *MAO-A*, *5HTT* and *SLC6A4* as candidates for involvement in the neurobiology underpinning depressive symptoms.[1] Functional studies with a focus on the impact and mechanism of action of individual gene variations on depression and anxiety provide evidence in support of a key role for genes like *COMT*, *5HTT*, *ACE*, *MR* and *GR*.[39,65-67] Based on previous study outcomes, such genes could continue to be prioritised in future candidate gene studies.

Demonstration of a systems biology approach

We have tested the potential value of *theoretical* and systems biology driven prioritisation of gene combinations in our recent study on the polygenic risk for stress-related traits of depression and anxiety in the Western Australian Pregnancy (Raine) cohort.[68] To trial the application of the proposed systems biology approach to candidate gene selection within the integrated neuro-network in the brain, we utilised the four principles previously described with emphasis on genetic predictors with the highest likely aetiological significance to mood disorders. We defined an early endophenotypic outcome based on the toddler temperament score (TTS);[69] assessed at one year of age and strongly associated with atypical HPA function in late adolescence. Significant associations were then demonstrated with SNPs in *NR3C1* (the gene encoding for GR) and *SLC6A4* (the gene encoding for 5HTT). These effects were limited to male participants in the Raine cohort. The magnitude of impact of the number of adverse alleles in the specific SNP in *NR3C1* on the toddler temperament sub-score for rigid and reactive behaviour was surprising, accounting for changes of up to two standard deviations in the TTS sub-score. Notably, a multi-gene profile scoring approach incorporating three specific polymorphisms in *NR3C1* and *SLC6A4* (coded for risk and adjusted for independent non-genetic factors) provided the strongest effect sizes with increasing number of adverse alleles being associated with adverse changes in the TTS sub-score of up to 3 standard deviations.[68] A similar use of polygenic risk analysis could incorporate another example of synergistic hormone action at the interface of the HPA axis and HARS within the integrated neuronetwork discussed. In the amygdala, stress-induced CORT binding to GR and NE-induced excitability of this specific corticolimbic brain region overlap.[70,71] Together, these events influence memory processing under stressful circumstances such that the joint effect appears to exceed normal memory processing able to cause malignant forms of memory underlying post-traumatic stress disorder. This type of stress-related mood disorder is characterised by enhanced emotional responsiveness triggered by stimuli which are non- threatening at the time of perception but relate to distinct memories of a traumatic event in the past.[25,29]

Environmental moderation of biological pathways

It is likely though that most of the genetic associations previously described are not solely caused by sequence variation in specific genes. First, evidence is accumulating that such associations can be modified by environmental exposures and life experiences to augment the genetic influence on mood behaviour and susceptibility to depressive disorders, albeit in an individual and gender specific manner.[1,28,39,72] Here, the ability of some interactions to counterbalance the genetic influence by mediating adaptive changes in neurobiology leading to resilience should not be overlooked in an effort to better understand the neurobiology of stress-related mood disorders.[72] In particular, investigation of systems biology driven polygenic risk mechanisms need not be restricted to genetic (DNA sequence variation) studies. Environmentally induced epigenetic changes to the genome can also regulate the functionality of gene products by modulating gene expression and in turn their protein levels, which would justify epigenetic analysis of theoretically prioritised candidate genes. Epigenetic analysis refers to the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence in isolation.[73,74] Examples include methylation of

the CpG dinucleotide of DNA and the covalent modification of DNA-packaging histones.[75,76] Together, epigenetic marks establish a macromolecular environment that controls the level of underlying gene expression. Perhaps most importantly, epigenetic marks appear reversible, tissue specific and can be affected by environmental changes (for example diet, infection, toxins, social environment).[77]

Unfortunately for psychiatric and behavioural genetic research, brain tissue is only available *post mortem*.[78,79] Nevertheless, many studies have utilized this tissue in epigenetic studies.[77,80,81] In addition, the applicability of alternative, more easily accessible peripheral tissues instead, such as blood or cheek swabs, is still under investigation and is likely to be gene-dependent.[82-85] A further consideration is that both genetic and epigenetic variation in concert are likely to contribute to disease. Therefore it may be appropriate to study genetic variation *and* epigenetic variation at the same locus in the same individuals.[67,86,87] This parallel approach accentuates the place for epigenetics alongside genetics in functional network or *systems biology* approaches to studying the molecular psychoneurobiology of depression.

Conclusions

In this paper we have described an approach to advancing research on genetic risk for depression and related disorders based on a detailed consideration of the neurobiology of stress responsiveness. We have described the physiological architecture of major neurobiological systems underlying the stress response: the *HPA axis*, the *MCLS,* the *HARS* and the *RAS*. We have suggested that knowledge of interaction between systems could be used to theoretically group SNPs identified by genome-wide association studies of depressive symptoms into biologically meaningful polygenic profile scores that can be tested in genetic epidemiological designs. We have further suggested that neurobiological knowledge of the stress response could be used to prioritise genes for more in depth investigation than genome-wide marking can provide. This could extend to next generation sequencing of genes that play rate-limiting roles in neurosignalling within stress response pathways. We have done this by describing four principles for candidate gene selection, which is based on the cumulative weight of evidence for the role of the gene products in each of these neurosignalling systems and as part of their interactions. Again, neurobiological knowledge of interaction between systems could be used to theoretically group findings from this more in depth search strategy. To illustrate a systems biology approach we have presented findings from a genetic investigation of temperamental risk factors for depression using data on child behaviour from the Western Australian Raine Study. This example also illustrates the importance of phenotypic precision in detection of polygenic risks, and the value of data from longitudinal study designs (with repeated measures of mood and behaviour) for improving phenotypic outcomes. Our examples further emphasised the potential importance of intermediate traits or endophenotypes based on distinction between specific complex disease symptoms within a patient population, pre-disease traits or peripheral biomarker dynamics in prospectively studied human cohorts. In the specific context of genetic epidemiological studies of stress-sensitive neurosignalling in depression and anxiety, the cortisol awakening response (as a reflection of an individual's stress reactivity) may be a relevant biological endophenotype.[88] Peripheral assessment of morning stress hormone dynamics could contribute to improved reproducibility within and between longitudinal cohort studies of an insightful biomarker-based intermediate phenotype in genetic causality studies. It is conceivable that more refined definitions of study outcomes in the form of endophenotypes will optimise the detection of genetic aspects to the etiology of psychopathology, identifying susceptibility to the disease before clinical diagnosis in those predisposed.

In summary, we have argued that insight into the functional neuroanatomy of the HPA axis, MCLS, HARS and RAS provides an important source of information for the prioritisation of genes of high significance to the psychoneurobiology underlying control of emotional and behaviour, and therefore to the aetiology of mental health problems. A theoretical neuroscience framework complements the hypothesis-free GWAS approach. It allows a high likelihood of identification of those genes that may be functionally related to the cause of disease. There is a pressing need to further develop and refine appropriate methods for investigating polygenic risk in genetic epidemiology much better.

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