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Neuro-behavioural impact of changes in hippocampal neural activity

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Abstract

Hippocampal metabolic hyperactivity and neural disinhibition, i.e. reduced GABAergic inhibition, have been associated with schizophrenia, although a causal link between disinhibition and metabolic hyperactivity remains to be demonstrated. Regional neural disinhibition might also disrupt neural activation in projection sites, such as the prefrontal cortex and striatum, which may contribute to cognitive impairments and positive symptoms characteristic of schizophrenia. To further examine the brain-wide impact of hippocampal disinhibition and the associated behavioural and cognitive changes, we combined ventral hippocampal infusion of the GABA-A antagonist picrotoxin with translational neural imaging and behavioural methods in rats.

First, we used a conditioned emotional response paradigm to assess the impact of hippocampal disinhibition on aversive conditioning and salience modulation in the form of latent inhibition (chapter 2), both of which have been reported to be disrupted in schizophrenia. These experiments demonstrated hippocampal disinhibition caused disrupted cue and contextual fear conditioning, whilst we found no evidence that hippocampal disinhibition affects salience modulation as reflected by latent inhibition of fear conditioning. The disruption of fear conditioning resembles aversive conditioning deficits reported in schizophrenia and may reflect disruption of neural processing at hippocampal projection sites.

Second, we used SPECT imaging to map changes in brain-wide activation patterns caused by hippocampal GABA dysfunction (chapter 3). SPECT experiments revealed increased neural activation around the infusion site in the ventral hippocampus, resembling metabolic hippocampal hyperactivity consistently reported in schizophrenia. In contrast, activation in the dorsal hippocampus was significantly reduced. This resembles the finding of anterior hippocampal hyperactivity coupled with reduced posterior hippocampal activation in patients with schizophrenia. Hippocampal disinhibition also caused marked extra-hippocampal activation changes in neocortical and subcortical sites, including sites implicated in fear learning and anxiety such as the medial prefrontal cortex (mPFC), septum, lateral hypothalamus and extended amygdala which may contribute to the disruption of fear conditioning demonstrated in chapter 2. Importantly, increased activation in the mPFC corresponds with previously reported prefrontal-dependent attentional deficits caused by hippocampal disinhibition.

Third, to complement these findings we used magnetic resonance spectroscopy (MRS) to determine the effects of hippocampal disinhibition on neuro-metabolites within the mPFC (chapter 4). Using MRS, we demonstrated that hippocampal disinhibition causes metabolic changes in the mPFC, reflected by increased *myo*-inositol and reduced GABA concentrations.

Overall, our results demonstrate ventral hippocampal disinhibition causes regional metabolic hyperactivity, supporting a causal role between GABA dysfunction and increased anterior hippocampal activity. In addition, hippocampal disinhibition causes activation and metabolic changes at distal sites, which may contribute to clinically relevant behavioural deficits, including impaired aversive conditioning, as demonstrated in our behavioural studies.

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Chapter 1: Introduction

Dysfunctional inhibitory neurotransmission has been linked to the pathophysiology of a number of psychiatric disorders including Alzheimer's disease, autism spectrum disorder and schizophrenia (Ambrad Giovannetti and Fuhrmann, 2019, Marin, 2012). Inhibitory gamma-aminobutyric acid (GABA) transmission plays a key role in balancing and controlling excitatory neurotransmission (Isaacson and Scanziani, 2011) and the processing of information requires precise spatio-temporal control of excitatory neurons (Letzkus et al., 2015, Panzeri et al., 2015). In addition, GABA input to excitatory pyramidal neurons play a significant role in the synchronisation of large scale neural networks (Cobb et al., 1995) which is important for behavioural and cognitive function (Uhlhaas et al., 2009). Specifically increased hippocampal neural disinhibition, i.e. reduced inhibitory GABA transmission, has emerged as a key characteristic of schizophrenia pathophysiology and has been linked to both cognitive impairments and psychosis (Heckers and Konradi, 2015). Hippocampal disinhibition has also been linked to age related cognitive decline (Nava-Mesa et al., 2014), although the evidence for the involvement of GABA dysfunction is more compelling in schizophrenia (Heckers and Konradi, 2015). Recent evidence has provided a causal link between hippocampal disinhibition and some of the characteristic cognitive impairments of schizophrenia including deficits in attention and everyday-type memory (McGarrity et al., 2017). Whilst hippocampal disinhibition has been linked to some

clinically relevant cognitive deficits, a more detailed understanding of the role hippocampal GABA dysfunction may play in the generation of psychosis and disruption of wider neural networks has yet to be established.

Hippocampal dysfunction in schizophrenia: regional hyperactivity and GABA dysfunction

The hippocampus has been implicated in schizophrenia for decades, with early post-mortem morphometric studies reporting reductions in hippocampal regional volume (Brown et al., 1986, Falkai and Bogerts, 1986). This evidence was later confirmed with the use of structural neuroimaging, where consistent hippocampal volume deficits have been reported (Roeske et al., 2020, Steen et al., 2006). Studies which have investigated hippocampal volume deficits in more anatomical detail have found volume reductions are more prominent in the anterior region of the hippocampus, as compared to the posterior section (Lieberman et al., 2001, Narr et al., 2004, Schobel et al., 2009a). The more pronounced volume reduction in anterior compared to posterior hippocampus is particularly evident in early stage schizophrenia. Comparison of hippocampal volume deficits in acute versus chronic schizophrenia demonstrate that acute patients have anterior but not posterior hippocampal volume reductions, whilst both anterior and posterior volume deficits are present in chronic schizophrenia (Ho et al., 2017, McHugo et al., 2018). Significantly, structural hippocampal changes are also correlated with cognitive

deficits. For example, patients with hippocampal grey matter loss demonstrate deficits in hippocampal-dependent spatial memory compared to healthy controls (Ledoux et al., 2014). A more recent study by Vargas et al. (2018) also showed that hippocampal volume reductions correlate with both visual learning and working memory deficits. In addition, studies utilising *in vivo* magnetic resonance spectroscopy (MRS) have reported consistent reductions in the concentration of hippocampal N-acetyl aspartate (NAA), which is associated with neurodegeneration and is therefore viewed as a marker of neuronal integrity (Brugger et al., 2011, Kraguljac et al., 2012). These findings are consistent with the prominent volume reductions reported in structural and morphometric analyses. Overall, hippocampal volume reductions are a key pathological feature of schizophrenia with deficits correlating with cognitive impairments, and changes in the anatomical locus of volume reductions linked to the duration of the disorder.

In addition to volume deficits in the hippocampus, the use of functional imaging techniques has revealed resting regional hyperactivity within the hippocampus in schizophrenia (Friston et al., 1992, Kawasaki et al., 1992, Liddle et al., 1992a, Molina et al., 2005). Importantly, increased cerebral blood flow is correlated with increased metabolic demand (Logothetis et al., 2001, Ogawa et al., 1990) and thus higher neuronal activity, linking increases in brain perfusion with increased excitatory neurotransmission. More recent, higher resolution imaging studies have also reported a differentiation

in activity between the anterior and posterior hippocampus, comparable to the volumetric changes. Hyperactivity of the anterior section of the hippocampus has been consistently reported in both chronic and acute schizophrenia (McHugo et al., 2019, Schobel et al., 2009b, Schobel et al., 2013, Talati et al., 2014). Schobel et al. (2013) also demonstrated that hyper-metabolism in the anterior hippocampus spatially coincides with anterior hippocampal atrophy in patients with schizophrenia. It has been hypothesised that neuronal hyperactivity driven by an excitation-inhibition imbalance, due to dysfunction of GABAergic inhibitory interneurons (see next paragraph), leads to the volume deficits reported in structural imaging studies (Heckers and Konradi, 2015, Lieberman et al., 2018). Hippocampal hyperactivity is also associated with the clinical symptoms of schizophrenia. A seminal study by Liddle et al. (1992b) reported a significant positive correlation between cerebral blood flow in the parahippocampal gyrus and reality distortion in a group of patients with schizophrenia. Subsequently, more recent studies have replicated this finding, whereby increased hippocampal activity correlates with positive symptom severity (Lahti et al., 2006, Molina et al., 2003, Schobel et al., 2013). Hippocampal hyperactivity is also associated with poor cognitive performance including on assays of attention and memory (Tregellas et al., 2014).

As outlined in the preceding paragraph, hippocampal hyperactivity is a significant feature of both acute and chronic schizophrenia and studies have suggested dysfunctional inhibitory GABA transmission is

a major contributing factor to hippocampal volume loss and hyperactivity. Early histological studies from the hippocampus of patients with schizophrenia revealed cellular abnormalities including reduced neuronal size (Arnold et al., 1995, Benes et al., 1991), although in many studies the overall number of neurons within the hippocampus was not reduced compared to controls (Dwork, 1997, Heckers et al., 1991, Walker et al., 2002). However a recent meta-analysis of post-mortem studies reported selective reductions in neuronal number in the left, but not right hippocampus, with no parallel differences in neuron density across the hippocampus (Roeske et al., 2020). The overall decreases in neuronal number appear to be driven by specific reductions in inhibitory neuron populations. An early study by Benes et al. (1998) investigating specific cell-type populations, reported reductions in the number of non-pyramidal cells in the CA2 and CA3 subfields of the hippocampus. Later analysis has shown this reduction in non-pyramidal cells in the hippocampus is restricted to selected subtypes of interneurons. Specifically, there is a reduction in the number of parvalbumin and somatostatin-positive neurons, but not calretinin positive neurons, in the hippocampus of schizophrenia patients (Konradi et al., 2011, Torrey et al., 2005, Zhang and Reynolds, 2002). In addition, Konradi et al. (2011) also reported reduced parvalbumin and somatostatin mRNA levels, whilst seeing no reduction of cells in the pyramidal cell layer. Significantly, parvalbumin-positive interneurons constitute approximately 20%, and somatostatin-positive neurons between 30-50% of all GABAergic neurons within the hippocampus (Freund and

Buzsaki, 1996). Parvalbumin and somatostatin-positive cells both play key roles in the control of pyramidal cell activity (Bartos et al., 2007, Viollet et al., 2008) and a reduction in the number of these inhibitory neurons could be responsible for the hyperactivity seen in the hippocampus of schizophrenia patients.

In addition to changes in the numbers and density of specific types of interneurons within the hippocampus, alterations in the genes involved in GABA transmission have also been reported in post-mortem studies. Studies examining the gene expression profile of hippocampal subfields using laser microdissection, have shown significant reductions in the expression of both GAD67 and GAD65 in CA3/2 and GAD67 in the CA1 hippocampal subfield. GAD67 and 65 are isoforms of the enzyme glutamate decarboxylase which is responsible for converting glutamate into GABA. Additionally, these studies also identified a number of genes which are implicated in synaptic transmission including genes involved in GABA and kainate receptors (Benes et al., 2007, Benes et al., 2008). Changes at the synaptic level itself have also been found in post-mortem studies of the hippocampus. Relatively consistent reductions in the presynaptic proteins synaptophysin, SNAP-25, and synapsin have been reported, with these reductions affecting both the level of protein and mRNA expression (Harrison, 2004). With dysfunctional inhibitory neurotransmission in the hippocampus being strongly implicated in the pathophysiology of schizophrenia, several studies have analysed the concentrations of the excitatory and inhibitory neurotransmitters

glutamate and GABA *in vivo* using MRS (although it should be noted that the glutamate and GABA pools measured by MRS do not only include the extracellular neurotransmitter pool, but also intracellular pools of the compounds, including pools stored in vesicles and involved in metabolic pathways), however the data from these studies is somewhat inconsistent (see chapter 4 for further discussion of the findings of clinical MRS studies). Increased hippocampal glx (combined glutamine and glutamate) has been reported in patients with schizophrenia, and this increase correlated with hippocampal volume reductions (Kraguljac et al., 2013). A study of first episode psychosis (FEP) however, found no increase in glx, although patients who had a long duration without treatment had significantly increased glx (Briend et al., 2020), but this did not correlate with changes in hippocampal volume. In addition, a study comparing individuals with a high risk of psychosis (UHR) and FEP patients found decreased hippocampal glx in UHR individuals but not in FEP (Shakory et al., 2018). A further study of FEP showed no significant changes in hippocampal glx concentrations, nonetheless higher levels of glx correlated with decreased hippocampal functional connectivity to regions of the default mode network (Nelson et al., 2020). Considering glutamate alone, a meta-analysis by Marsman et al. (2013) reported no change in glutamate in the hippocampus, however decreased glutamate has been reported in a later study (Stan et al., 2015). Currently the study by Stan et al. (2015) is the only clinical MRS study to have measured GABA in the hippocampus in schizophrenia, and this study reported no differences in

hippocampal GABA concentration in the patient sample compared to controls. Although the authors do note the measurements may lack the sensitivity to detect changes in GABA when those changes may be limited to specific subfields. Overall the evidence from clinical neuroimaging studies strongly implicates hippocampal hyperactivity, possibly due to cellular and molecular abnormalities of inhibitory neurons, as a key feature of schizophrenia, which has been linked to psychotic and cognitive symptoms.

Significantly, a number of rodent models of hippocampal hyperactivity provide evidence of a causal link between psychosis-related and cognitive impairments (Katzel et al., 2020). Electrical or chemical stimulation of the ventral hippocampus causes increases in striatal dopamine, which is a key feature of schizophrenia, and also causes locomotor hyper-activity seen as a putative rodent correlate of positive symptoms (Bast et al., 2001c, Legault and Wise, 1999, Taepavarapruk et al., 2000). In addition, disinhibiting, i.e. reducing inhibitory GABA transmission, the ventral hippocampus also induces hyper-locomotion (Bast et al., 2001a, McGarrity et al., 2017, Nguyen et al., 2014). Furthermore, deficits in pre-pulse inhibition (PPI), which is often used as a psychosis related index in rodent studies (Arguello and Gogos, 2006, Bast and Feldon, 2003), are caused by hippocampal hyperactivity either via chemical/electrical stimulation or chemogenetic disinhibition (Bast et al., 2001c, Howland et al., 2004, Nguyen et al., 2014). Although PPI deficits may be strain sensitive with picrotoxin induced disinhibition having contrasting effects in

different rat strains (Bast et al., 2001a, McGarrity et al., 2017). Hippocampal hyperactivity is also associated with cognitive deficits including attentional and memory deficits in rodent models. Specifically ventral hippocampal disinhibition, via pharmacological or chemo- and optogenetic inhibition of GABA transmission, disrupts sustained attention (McGarrity et al., 2017, Tan et al., 2018) and short term working memory (McGarrity et al., 2017, Nguyen et al., 2014). In addition, optogenetic activation of the ventral hippocampus includes hyper-locomotion and disrupts hippocampus dependent short term memory (Wolff et al., 2018). Neurodevelopmental models of schizophrenia which cause similar hippocampal pathology also provide evidence of disrupted cognition. Animals with a cyclin-D2 knockout exhibit working memory impairments and disrupted cognitive flexibility (Grimm et al., 2018). Whilst the gestational administration of methylazoxymethanol acetate (MAM) model demonstrates disrupted cognitive flexibility, reduced PPI and working memory impairments (Lodge and Grace, 2009). Overall these studies suggest that ventral hippocampal hyperactivity may contribute to both the positive symptoms and cognitive deficits characteristic of schizophrenia.

Importantly, tonic neural disinhibition may not only effect local activity within that region but could disrupt activity and cognitive functions at distal sites by aberrant drive of neuronal projections (Bast et al., 2017). Hippocampal hyperactivity has been linked to several features of schizophrenia symptoms, including cognitive

deficits and psychosis, and these behavioural deficits likely involve disrupted coordination of several brain regions. The following sections discuss the intrinsic and extrinsic connectivity of the hippocampus and the potential relevance of this connectivity to the contribution that hippocampal hyperactivity may have in the manifestation of psychosis and cognitive deficits.

Hippocampal function and connectivity

The hippocampus is a region conserved across mammalian species organised into three distinct subfields: the hippocampus proper (consisting of CA1, CA2 and CA3) the dentate gyrus and subiculum. The hippocampus has long been linked to the formation of declarative memories, since the study of patient H.M. who suffered significant memory impairments after resection of the hippocampus to treat severe epilepsy (Scoville and Milner, 1957). In addition, there has been a longstanding link between the hippocampus and the control of emotion, and these diverse behavioural functions are differentially associated with different parts of the hippocampus along its longitudinal axis (posterior-anterior in humans and non-human primates and dorso-ventral or septo-temporal in rodents) (Bannerman et al., 2004, Bast et al., 2009, Fanselow and Dong, 2010, Moser and Moser, 1998, Strange et al., 2014).

The major source of neocortical neuronal inputs to the hippocampus is from the entorhinal cortex (Kerr et al., 2007, Steward, 1976,

Swanson and Kohler, 1986, Tamamaki and Nojyo, 1995, Tamamaki, 1997, van Strien et al., 2009, Witter et al., 1988), which acts as a gateway for information transfer to the hippocampus. The entorhinal cortex receives monosynaptic input from multiple regions including the perirhinal, olfactory and gustatory cortices, amygdala and hypothalamus (Burwell, 2000, Burwell and Amaral, 1998, Pitkänen et al., 2000). Entorhinal inputs to the hippocampus are topographically ordered in a way such that dorsolateral areas of the entorhinal cortex provides input to the dorsal hippocampus and more ventromedial entorhinal cortex provides input to the ventral hippocampus (Dolorfo and Amaral, 1998, Insausti et al., 1997, Witter et al., 2000). Therefore the dorsal hippocampus largely receives information from visual and auditory cortices, relayed via the dorsolateral entorhinal cortex, while the ventral hippocampus receives information from the amygdala and hypothalamic nuclei via the ventromedial entorhinal cortex (Naber et al., 2001, Canto et al., 2008).

Information is passed through the hippocampus through the 'trisynaptic pathway' whereby the entorhinal cortex connects to the dentate gyrus through the perforant pathway, the dentate gyrus then connects to CA3 through mossy fibres, CA3 then projects to CA1 via Schaffer collaterals and subsequently CA1 projects to the subiculum. Although, a number of reciprocal projections have been reported within the hippocampal circuit. This includes projections from CA3 back to the dentate gyrus and CA1 projections back to CA3, however the inhibitory or excitatory nature of these connections is not well

understood (Amaral et al., 1991, Li et al., 1994, Swanson et al., 1978). The subiculum and CA1 subfields are the main output structures of the hippocampal formation and project back to the entorhinal cortex, in the same topographical manner as the input to the hippocampus, and also to numerous cortical and subcortical sites (Cenquizca and Swanson, 2007, Witter, 1993).

As previously mentioned the hippocampus can be organised across the longitudinal axis with dorsal and ventral efferent projections preferentially connected to distinct regions. The dorsal hippocampus sends strong efferent connections to the retrosplenial cortex, the anterior cingulate cortices, and anterior thalamic and mammillary nuclei (Cenquizca and Swanson, 2007, Ishizuka, 2001, Kishi et al., 2000, Van Groen and Wyss, 2003). These regions are heavily involved in the processing of visuospatial information, memory processes, environmental exploration and spatial navigation (Aggleton et al., 2010, Harker and Whishaw, 2004, Jones and Wilson, 2005), and these connections are consistent with the role of the dorsal hippocampus in spatial learning and memory functions (Bannerman et al., 2014, Bast, 2007, Fanselow and Dong, 2010, Moser and Moser, 1998, Strange et al., 2014). Conversely, the ventral hippocampus sends strong efferent connections to the amygdala, prefrontal cortex, hypothalamus and ventral striatum (Cenquizca and Swanson, 2006, Cenquizca and Swanson, 2007, Groenewegen et al., 1987, Hoover and Vertes, 2007, Petrovich et al., 2001), which is consistent with the role of the ventral hippocampus

in the regulation of emotional and affective behaviours (Bannerman et al., 2004, Bannerman et al., 2014). In rodents ventral hippocampal excitatory projections to the medial prefrontal cortex are unidirectional and strongly innervate the prelimbic, infralimbic and anterior cingulate cortices (Cenquizca and Swanson, 2007, Hoover and Vertes, 2007, Jay and Witter, 1991). This pattern of connectivity between the hippocampus and prefrontal cortex is also consistent in the primate brain, where anterior hippocampal neurons (homologous to ventral hippocampus) project to the anterior and medial prefrontal cortex (Aggleton, 2012, Barbas and Blatt, 1995, Roberts et al., 2007). The prefrontal termination points of ventral hippocampal projections are heterogeneous and are involved in numerous behaviours including working memory, cognitive flexibility and goal oriented learning (Euston et al., 2012, Kesner and Churchwell, 2011). In addition, the hippocampal-prefrontal circuit has been implicated in the control of spatial and working memory, decision making, conditioned fear and anxiety (Floresco et al., 1997, Padilla-Coreano et al., 2016, Sotres-Bayon et al., 2012, Spellman et al., 2015, Wallis et al., 2019, Wikenheiser et al., 2017, Zeredo et al., 2019). Significantly, disruption of this circuit has been proposed as a key feature of several psychiatric disorders (Godsil et al., 2013). In addition, the ventral hippocampus has strong bidirectional connectivity with the amygdala. Ventral hippocampal projections innervate the basolateral, basomedial and central amygdala (Kishi et al., 2006, Pitkänen et al., 2000), whilst the lateral and basolateral amygdala provide input to the ventral hippocampus. In addition,

some ventral hippocampal neurons projecting to the basolateral amygdala also project to the prefrontal cortex (Ishikawa and Nakamura, 2006). Importantly, the amygdala plays an essential role in the formation and expression of conditioned fear and anxiety (Tovote et al., 2015). Furthermore, the hippocampus also strongly innervates the lateral septum and hypothalamus (Kishi et al., 2000, Risold and Swanson, 1997, Swanson and Cowan, 1979) which have been proposed, alongside the amygdala and prefrontal cortex, to be part of the neural circuit mediating anxiety and conditioned fear (Adhikari et al., 2011, Anthony et al., 2014, Tovote et al., 2015).

In addition, ventral CA1 and subiculum efferents also innervate the caudomedial parts of the nucleus accumbens (Groenewegen et al., 1987, Voorn et al., 2004). The nucleus accumbens is a key region involved in reward processing, salience and novelty detection particularly when considering dopaminergic systems (Berridge, 2007, Legault and Wise, 2001, Lisman and Grace, 2005). Partly through the strong glutamatergic projections to the nucleus accumbens, the ventral hippocampus is part of a circuit which is able to modulate dopamine release within the striatum. It has been repeatedly shown that electrical and chemical stimulation of the ventral hippocampus induces dopamine release within the nucleus accumbens, which is mediated by increased activity of ventral tegmental area (VTA) dopamine neurons (Blaha et al., 1997, Brudzynski and Gibson, 1997, Floresco et al., 2001, Legault and Wise, 1999, Legault et al., 2000, Taepavarapruk et al., 2000). It is thought the ventral hippocampus

can modulate striatal dopamine release via a polysynaptic route. This involves glutamatergic input to the nucleus accumbens, subsequently increasing inhibitory GABA transmission to the ventral pallidum which removes the inhibitory effect of the ventral pallidum on dopamine neurons of the VTA (Floresco et al., 2001, Floresco et al., 2003, Grace et al., 2007, Legault and Wise, 2001).

However several other brain regions can influence the release of dopamine in the striatum by interacting with hippocampal-midbrain circuitry. This includes areas of the medial prefrontal cortex (infralimbic and prelimbic), midline thalamic nuclei (nucleus reuniens), amygdala and medial septum (Sonnenschein et al., 2020). For example, studies have shown that the infralimbic cortex can regulate the proportion of active VTA neurons, but this is dependent on activity within the hippocampus and nucleus reuniens (Zimmerman and Grace, 2016). In addition, the medial septum also exerts modulatory control over midbrain dopamine neuron activity which is again dependent on ventral hippocampal activity (Bortz and Grace, 2018).

Although there are strong similarities in hippocampal neural anatomy between rodents and primates there are apparent differences when considering midbrain connectivity. Rodents have a larger ventral striatum and VTA in comparison to primates, resulting in differences in the innervation of distinct striatal neuron populations (Joel and Weiner, 2000). It has been suggested that the pathway from the VTA

to the ventral striatum in rodents is partly homologous to the projections of midbrain dopamine neurons to the associative striatum in primates, a central region of the striatum encompassing parts of the caudate and putamen (Joel and Weiner, 2000, McCutcheon et al., 2019). In rodents medial VTA neurons innervate the ventral striatum and neurons projecting from the substantia nigra innervate the dorsal striatum (Ikemoto, 2007), whereas neurons that lie at the transition between the lateral VTA and substantia nigra (SN) innervate the associative striatum (Joel and Weiner, 2000). In primates, the proportionally smaller VTA, projects to the ventral striatum with the dorsal SN innervating the associative striatum and the ventral tier of the SN innervating the motor related striatum (Duzel et al., 2009, Joel and Weiner, 2000, Lynd-Balta and Haber, 1994).

Considering the anatomical connectivity between the hippocampus and midbrain dopamine circuits, it is possible that hippocampal disinhibition could lead to an increase in striatal dopamine release. Increased excitatory drive from the hippocampus to the striatum has been suggested as key contributor to the pathophysiology of schizophrenia (Grace, 2016). Increased excitatory activity is hypothesised to lead to increased striatal dopamine release, a key feature of schizophrenia (Howes et al., 2012), which subsequently leads to psychosis by causing an aberrant allocation of salience (Kapur, 2003, Katzel et al., 2020).

Aims of the PhD project

The extensive connectivity from the hippocampus to cortical and subcortical sites suggests that hippocampal disinhibition may disrupt a range of behavioural functions beyond the specific memory functions typically associated with the hippocampus by disrupting neural processing both within the hippocampus and at projection sites.

Therefore, to assess this idea, the aims of this project were to determine whether hippocampal disinhibition disrupts the modulation of salience and associative learning, in the form of aversive conditioning, and to assess the effect of hippocampal hyperactivity on brain-wide neural activity. To this end we used a combination of pharmacological manipulation, behavioural testing and translational *in vivo* neuroimaging. To model dysfunctional GABA transmission we utilised ventral hippocampal infusions of the GABA-A antagonist picrotoxin. Previous studies have shown that hippocampal picrotoxin infusions cause memory and attentional deficits (McGarrity et al., 2017).

Aim 1: Impact of hippocampal disinhibition on aversive conditioning and latent inhibition

To test the hypothesis that hippocampal hyperactivity disrupts aversive conditioning and salience modulation we used a conditioned emotional response latent inhibition paradigm (Nelson et al., 2011a) (chapter 2). Disrupted latent inhibition reflects the hyper-associability

of stimuli which would normally be deemed as irrelevant and thus is seen as a disruption of salience processing, similar to which is hypothesised to cause hallucinations and delusions (Kapur, 2003). Using an aversive CER latent inhibition paradigm also enabled us to explore the effects of ventral hippocampal disinhibition on the fear conditioning response which has been shown to be disrupted by hippocampal hyperactivity (Zhang et al., 2001). Importantly, aberrant aversive conditioning has been reported in schizophrenia, alongside impairments in salience modulation (Jensen et al., 2008). In addition, we also performed locomotor activity testing (LMA), as ventral hippocampal disinhibition causes robust increases in locomotion and thus provided us with a positive control of behavioural output (chapter 2). We hypothesised ventral hippocampal disinhibition would abolish latent inhibition, thus disrupting salience modulation, whilst also disrupting fear conditioning.

Aim 2: Brain-wide impact of hippocampal disinhibition as measured using SPECT

To answer the question of whether hippocampal disinhibition causes regional hyperactivity, which is a key feature of schizophrenia pathophysiology, and to examine the distal impact in extra-hippocampal sites, we combined local hippocampal picrotoxin infusions with functional whole brain SPECT imaging (Chapter 3). SPECT imaging allows the measurement of brain-wide patterns of neural activity in awake rats via imaging of cerebral blood flow. Briefly, this involves injection of a tracer in awake unrestrained

animals, which is subsequently mapped in anaesthetised animals, providing a measure of the spatial pattern of cerebral blood flow during the time of tracer injection (Oelschlegel and Goldschmidt, 2020). Using SPECT imaging we can measure the brain wide effects on neural activity caused by hippocampal disinhibition. Because SPECT measures of regional cerebral blood flow are similar to non-invasive imaging measures of brain activity used in clinical studies, we can also compare our data with the findings of human clinical imaging studies.

Aim 3: Impact of hippocampal disinhibition on prefrontal neurochemistry as assessed using MRS

We subsequently further investigated the effects of hippocampal disinhibition on neurochemistry in the medial prefrontal cortex, using proton magnetic resonance spectroscopy (^1H -MRS) in chapter 4. MRS is a non-invasive technique, which enables the measurement of a neurochemical profile consisting of brain metabolites, which have distinct functions including neurotransmission and energy and membrane metabolism (Duarte et al., 2012). In addition, MRS is seen as a key tool in bridging the translation of basic research and clinical applications. MRS studies have recently more widely been used to reveal aberrant neurometabolites concentrations, including GABA and glutamate, levels in schizophrenia in several brain regions, including the prefrontal cortex (Egerton et al., 2017b, Marsman et al., 2013). However, findings from these studies are complex and the mechanisms underlying neuro-metabolite changes require

clarification. Using this technique we assessed the effect of hippocampal disinhibition on the underlying neuro-metabolism in the medial prefrontal cortex. Significantly, disruption of this circuit is thought to contribute to some of the cognitive symptoms of schizophrenia (McGarrity et al., 2017).

Chapter 2: Hippocampal disinhibition reduces contextual and elemental fear conditioning whilst sparing the formation of latent inhibition

Introduction

Psychosis is a fundamental feature of psychiatric disorders, such as schizophrenia, and the characteristic symptoms of hallucinations and delusions are thought to be driven by aberrant salience processing (Gray et al., 1991, Kapur, 2003). Psychosis is hypothesised to be caused by elevated striatal dopamine (Howes and Kapur, 2009), which is a key feature of schizophrenia (Abi-Dargham et al., 2000, Howes et al., 2009, Laruelle and Abi-Dargham, 1999). Hippocampal hyperactivity, potentially caused by GABA dysfunction, is a key characteristic of the pathophysiology of schizophrenia (Lisman et al., 2008, Tamminga et al., 2010, Heckers and Konradi, 2015) and is correlated with the onset of psychosis (Schobel et al., 2009b, Schobel et al., 2013). It has been hypothesised that hippocampal hyperactivity could disrupt neural circuitry leading to an increase in striatal dopamine, thereby causing psychosis (Grace, 2012, Grace, 2016).

Dopamine dysregulation and aberrant salience processing

Schizophrenia patients demonstrate increased attention to irrelevant cues (Morris et al., 2012) and alterations in salience processing (Knolle et al., 2018). Importantly, dysregulated modulation of dopamine, which has long been understood to be a major pathophysiological feature of schizophrenia, is thought to contribute to the emergence of psychotic symptoms by disrupting salience processing (Davis et al., 1991, Heinz and Schlagenhauf, 2010, Howes and Kapur, 2009, Kapur, 2003). Early evidence of dysregulated dopamine came from the observation that antipsychotic treatments have affinity for dopamine receptors (Creese et al., 1976, Seeman and Lee, 1975), and that pharmacological agents that trigger the release of dopamine cause psychosis like symptoms (Angrist and Gershon, 1970, Lieberman et al., 1987). Subsequently, clinical imaging studies using positron emission tomography (PET) or single-photon emission computed tomography (SPECT) reported significantly elevated dopamine within the striatum of schizophrenia patients (Abi-Dargham et al., 1998, Laruelle and Abi-Dargham, 1999, Laruelle et al., 1996). Multiple studies have shown that striatal dopamine dysregulation is pre-synaptic which affects dopamine synthesis and baseline synaptic dopamine levels (Howes et al., 2012), and is associated with positive symptom severity and the onset of psychosis (Howes et al., 2011, Laruelle et al., 1999). More recent studies have shown the predominant dopamine increase to be within dorsal subdivisions of the striatum, with the greatest increase in the associative striatum (McCutcheon et al., 2018, McCutcheon et al.,

2019). Whilst the predominant dopamine abnormality seems to be localised to the associative striatum several studies have reported alterations in reward related ventral striatal activation. Specifically, patients with psychosis demonstrate hypo-activation of the ventral striatum during reward anticipation and reward prediction error processing (Corlett et al., 2007, Ermakova et al., 2018, Murray et al., 2008, Radua et al., 2015).

Significantly, the ventral hippocampus sends strong projections to the NAc (Groenewegen et al., 1987) and indirect connections to the VTA which influence the release of dopamine in the striatum and midbrain (Floresco et al., 2001). The ventral hippocampal formation also sends some sparse projections to the medial dorsal striatum (Finch, 1996, Groenewegen et al., 1987, Hunnicutt et al., 2016). Through these connections stimulation of the ventral hippocampus can influence striatal dopamine. Electrical stimulation or chemical manipulation of ventral hippocampal neurons increases neuronal firing in the VTA and subsequently increases dopamine levels in the VTA and NAc (Floresco et al., 2001, Legault and Wise, 1999, Legault et al., 2000). Enhanced glutamatergic drive from the hippocampus to the striatum resulting in increased activation of striatum projecting midbrain dopamine neurons is hypothesised to be an important mechanism in the pathophysiology of schizophrenia (Katzel et al., 2020, Sonnenschein et al., 2020).

Phasic dopamine release is thought to encode the difference between expected and obtained reward outcomes, known as reward prediction errors, and these prediction errors allow for the updating of expectations based on prior experience (Schultz et al., 1997). Phasic dopamine release is thus thought to represent the assignment of incentive salience to sensory stimuli and internal representations (Berridge, 2007), and it is thought that dysregulated striatal dopamine release can lead to delusions and hallucinations which are the hallmarks of psychosis. Aberrant striatal dopamine transmission could lead to stimulus-independent release of dopamine which subsequently leads to the aberrant assignment of motivational salience or attention to irrelevant information (Heinz and Schlagenhauf, 2010, Gray et al., 1991, Kapur, 2003). The aberrant assignment of salience to an otherwise irrelevant stimulus thus leads to an updating of prior beliefs or representations based on faulty information (Fletcher and Frith, 2009). The subsequent delusions and hallucinations caused by the abnormal formation of associations are therefore thought to be a mechanism to contextualise and rationalise these unusual experiences and as a consequence often reflect prior experiences and beliefs (Kapur, 2003).

To better understand the neuro-psychological abnormalities that underlie positive symptomology in schizophrenia, behavioural tasks have been adapted from earlier animal learning studies. One prominent behavioural paradigm that has been used for this purpose is latent inhibition (LI). LI refers to the effect of pre-exposing a

stimulus without consequence which is later established as a cue for an outcome. LI is demonstrated by retarded learning of a pre-exposed cue compared to a non-pre-exposed cue during a subsequent test of learning (Lubow and Moore, 1959). LI can therefore be seen as the effective filtering of irrelevant information as reflected by the reduction in association of a previously inconsequential cue. Importantly, attentional deficits are thought to be a key feature of schizophrenia (Nuechterlein and Dawson, 1984), and it has been suggested that LI reflects selective attentional processes. It has been proposed that prior exposure to a stimulus results in reduced attention to that stimulus, which leads to slower associative learning of that stimulus with an outcome compared to a non-pre-exposed stimulus (Lubow and Gewirtz, 1995, Pearce and Hall, 1980, Wagner and Rescorla, 1972).

In agreement with the notion that dysfunctional attentional processing is a key feature of schizophrenia, deficits in LI have been reported in schizophrenia patients. Deficits in LI are generally restricted to the acute phase of the disorder, with studies of chronic patients demonstrating similar or even enhanced LI compared to controls (Baruch et al., 1988, Gal et al., 2009, Gray et al., 1992, Gray et al., 1995a, Rascle et al., 2001, Vaitl et al., 2002). However, some studies have reported normal LI in both unmedicated and medicated acute patients (Williams et al., 1998, Swerdlow et al., 1996). Inconsistencies in findings may be explained by small sample sizes and differing behavioural paradigms used to assess LI (Schmidt-

Hansen and Le Pelley, 2012). Overall, it seems likely that LI is abnormal in schizophrenia, although, depending on the chronicity of the disorder, this may reflect either an attenuation or a potentiation of LI.

In the present study we used a latent inhibition paradigm to assess attentional allocation in a rodent model. Whilst a variety of behavioural paradigms are used to assess LI in rodents, they are all performed using the same basic procedure (Weiner and Arad, 2009). Initially animals are either pre-exposed to a stimulus or non-pre-exposed to the same stimulus. Both of these groups are subsequently conditioned using pairings of the pre-exposed stimulus and a reinforcer. After conditioning performance is measured using a behavioural index, where LI is reflected as reduced conditioning in the pre-exposed group (see: Weiner and Arad (2009)).

Hippocampal and dopaminergic involvement in LI

As discussed above, LI appears to be disrupted in acute schizophrenia patients and hippocampal hyperactivity is a key neurobiological feature of acute schizophrenia (Heckers and Konradi, 2015). However, it is unclear whether dysfunctional hippocampal activity contributes to these LI deficits. Early studies suggested hippocampal lesions disrupt LI (Kaye and Pearce, 1987, McFarland et al., 1978), but see (Clark et al., 1992). However later studies have demonstrated that LI is spared by more selective hippocampal lesions (Coutureau et al., 1999, Honey and Good, 1993, Reilly et al., 1993, Shohamy et

al., 2000) but see (Oswald et al., 2002). These contrasting findings are most likely due to the destruction of axons of regions outside of the hippocampus in earlier studies, as lesions of the retro-hippocampus and entorhinal cortex both disrupt LI (Coutureau et al., 1999, Yee et al., 1995). However, Honey and Good (1993) demonstrated that whilst LI is spared by selective cytotoxic hippocampal lesions, the contextual specificity of LI is disrupted by these lesions which suggests the hippocampus may play a specific role in the contextual modulation of LI. Interestingly, deactivation of the ventral subiculum during pre-exposure disrupts the formation of LI (Peterschmitt et al., 2005, Peterschmitt et al., 2008). In addition, analysis of neural activation patterns during an LI paradigm, using c-fos immunocytochemistry, reported increased activity in the subiculum in animals that were pre-exposed to the conditioned stimulus (Sotty et al., 1996).

Whilst evidence for the role of the hippocampus in LI is varied, projection sites of the hippocampus have been implicated in modulating LI, including the mesencephalic dopamine system, pre-frontal cortex and thalamic nuclei (Young et al., 2005, George et al., 2010, Perez-Diaz et al., 2017, Nelson et al., 2018). Significantly, animal and human experiments have shown dopamine plays a critical role in the formation of LI. Administration of the indirect dopamine agonist amphetamine abolishes LI in rats (Weiner et al., 1988) and healthy human volunteers (Kumari et al., 1999, Thornton et al., 1996). Conversely LI is potentiated by anti-psychotic medication,

which act at dopamine receptors, in both rats and humans (Weiner et al., 1997, Williams et al., 1997). Further investigations into the underlying neurobiology of latent inhibition have implicated striatal dopamine transmission as a key mediator in the regulation of LI. Infusion of amphetamine into the nucleus accumbens, specifically the core sub-region, can disrupt latent inhibition, while the disruptive effect of systemic amphetamine administration, as mentioned above, is blocked by infusion of the antipsychotic haloperidol into the nucleus accumbens (Joseph et al., 2000, Nelson et al., 2011a). In addition, lesions of the shell sub-region of the nucleus accumbens, causing destruction of dopaminergic neurons, potentiate LI, whilst core dopaminergic lesions have no impact upon LI (Nelson et al., 2011b). In addition, *in vivo* measurement of dopamine using voltammetry has shown stimulus pre-exposure reduces dopamine within the nucleus accumbens shell (Jeanblanc et al., 2002, Murphy et al., 2000). Significantly, it is the effect of pre-exposure at conditioning which seems to be modulated by dopaminergic mechanisms. Systemic amphetamine administered during pre-exposure does not disrupt LI (Weiner et al., 1984), but does disrupt LI when administered before both pre-exposure and conditioning (Gray et al., 1997, McAllister, 1997). Consistent with this finding, there is no change in striatal dopamine release during pre-exposure, however pre-exposure to the conditioned stimulus reduces the release of dopamine in the NAC during conditioning (Murphy et al., 2000, Young et al., 1993).

In addition, amphetamine infusions into the dorsal region of the striatum can disrupt LI (Ellenbroek et al., 1997), although an earlier study did not find any effect of dorsal striatal amphetamine on LI (Solomon and Staton, 1982). However, more recent studies have suggested a functional dissociation exists within the dorsal striatum with respect to the formation of LI. For example, a study using in vivo voltammetry by Jeanblanc et al. (2003) revealed reduced dopamine release in the non-pre-exposed group compared to the pre-exposed group within the anterior dorsal striatum, whereas there were no differences in dopamine release in the posterior dorsal striatum. In addition, lesions of the dorsolateral striatum disrupt LI (Perez-Diaz et al., 2017), and an earlier study by the same authors' demonstrated inactivation of the DLS impairs the retrieval of LI, while inactivation of the dorsomedial striatum has no effect on LI (Diaz et al., 2014). In addition, suppressing neuronal activity in the ventral subiculum with tetrodotoxin (TTX) alters LI related anterior dorsal striatal dopamine release (Peterschmitt et al., 2005). Altogether these findings raise the possibility that dopamine release in the striatum, which modulates LI, could be augmented by hippocampal GABA dysfunction and subsequently disrupt LI.

Additional hippocampal projection sites and LI

The hippocampus is also connected to the medial prefrontal and cingulate cortex (Conde et al., 1995, Jay and Witter, 1991), which in turn project to regions of the ventral and dorso-medial striatum (Guo et al., 2015, Hintiryan et al., 2016, Hunnicutt et al., 2016). Whilst

striatal dopamine has received significant attention for its role in LI, there is some evidence that prefrontal dopamine plays a modulatory role in LI, and ventral hippocampal NMDA stimulation can facilitate PFC dopamine transmission (Peleg-Raibstein et al., 2005). For example, dopaminergic lesions of the prelimbic cortex enhance LI (Nelson et al., 2010); whilst optogenetic activation of prelimbic dopaminergic axon terminals increases conditioned responding to a familiar CS (Morrens et al., 2020), which suggests prelimbic dopamine negatively modulates LI at conditioning. However, infusions of dopamine agonists and antagonists into the mPFC have been reported to have no impact on LI (Broersen et al., 1996, Ellenbroek et al., 1996, Lacroix et al., 2000a). These data suggest prefrontal dopamine projections may be involved in LI but this appears to be sub-region specific.

In addition to the role of dopamine in LI, studies investigating the electrophysiological properties of LI revealed pre-exposure to a CS attenuates CS-evoked neural responses in the cingulate cortex (Talk et al., 2005). In addition, inactivation of the infralimbic (IL) cortex, using the NMDA antagonist DLAP-5 or GABA agonist muscimol, was reported to disrupt the formation of LI when administered during PE (Lingawi et al., 2017, Lingawi et al., 2018), the authors suggested the disruption of LI acquisition is driven by a loss of the encoding of a CS-no event association which is stored in the IL. Hippocampal stimulation can evoke neuronal responses in the medial prefrontal cortex (mPFC) (Degenetais et al., 2003, Liu and Carter, 2018, Thierry

et al., 2000) and disrupt attentional processes, which are dependent on balanced PFC activity (McGarrity et al., 2017, Tan et al., 2018). This raises the possibility that prefrontal control of LI acquisition could be disrupted by hippocampal hyperactivity. Similar to the effect of prefrontal manipulations, entorhinal cortex inactivation using TTX also disrupts LI when administered at pre-exposure (Jeanblanc et al., 2004, Seillier et al., 2007). Overall, these studies suggest disruption of activity in hippocampal projection sites can disrupt the formation of LI, and thus hippocampal disinhibition may be expected to modulate LI.

Aversive conditioning deficits

In addition to deficits in attentional allocation seen in schizophrenia patients, as reflected by disrupted LI, deficits in the ability to process aversive stimuli have also been reported in schizophrenia patients (Jensen et al., 2008). Previous findings demonstrate that the ventral hippocampus plays an important role in aversive learning and this suggests ventral hippocampal disinhibition may disrupt aversive conditioning. This then needs to be considered when using aversive conditioning procedures to assess LI and the impact of ventral hippocampal disinhibition on LI. The ventral hippocampus is involved in anxiety and fear-related processes (Bannerman et al., 2004, Fanselow and Dong, 2010), with lesion and functional inactivation studies having implicated the ventral hippocampus in fear conditioning to both contextual and explicit cues (Bast et al., 2001b, Maren, 1999, Zhang et al., 2014). Additionally, NMDA stimulation of

the ventral hippocampus disrupts contextual and cue conditioning (Zhang et al., 2001), whilst blockade of neuronal activity, including the activity of fibres of passage, using TTX, also blocks fear conditioning to both context and tone (Bast et al., 2001b). However functional inhibition of neurons within the hippocampus using muscimol only disrupts contextual fear and not cue conditioning (Bast et al., 2001b, Zhang et al., 2014). Importantly, dopaminergic function in the nucleus accumbens seems to be involved in Pavlovian conditioning (Parkinson et al., 1999) and the ventral hippocampus has numerous reciprocal connections to the amygdala (Pitkänen et al., 2000), which is well known for its role in fear conditioning (Fanselow and LeDoux, 1999). These data suggest balanced hippocampal activity is necessary for the expression of fear conditioning. Therefore, GABA dysfunction, leading to hippocampal hyper-activity could disrupt the expression of fear conditioning in an aversive LI paradigm, and a similar mechanism may contribute to aberrant aversive conditioning seen in schizophrenia patients (Jensen et al., 2008).

Aims

We aimed to examine the effect of ventral hippocampal disinhibition, using the GABA-A antagonist picrotoxin (McGarrity et al., 2017) on aversive conditioning and LI. To assess these processes, we used a conditioned emotional response procedure designed to produce robust LI in controls (Nelson et al., 2011a). Considering enhanced dopamine during pre-exposure (PE) and conditioning disrupts LI we

first investigated whether ventral hippocampal picrotoxin prior to both PE and conditioning would disrupt the formation of LI as reflected by the diminution of the PE effect (experiment 1). Secondly, we examined whether disrupting ventral hippocampal GABA function during PE only would disrupt the formation of LI, due to afferent connections to regions which control information processing during PE (experiment 2).

In experiment 1 we expected that hippocampal picrotoxin would reduce LI, which could be driven by several mechanisms. This includes the potential of the hippocampus to influence dopamine release in the striatum and PFC during conditioning, and the effect of aberrant prefrontal cortex activity during PE as mentioned above. Given the implication of ventral hippocampal activity in the modulation of fear conditioning, we also expected that hippocampal picrotoxin would disrupt fear conditioning, although fear conditioning may be sufficiently spared to detect LI and any disruption of LI. However, hippocampal picrotoxin during conditioning completely abolished fear conditioning in both PE and non-pre-exposed rats, so we were unable to examine changes in LI. Therefore, in experiment 2, we focused on the effect that ventral hippocampal disinhibition might have on LI at the PE stage. We hypothesised that picrotoxin infusion during PE would disrupt LI, possibly by disrupting attentional processing controlled by the mPFC.

Methods

Animals

Animals were male Lister hooded rats (Charles River, UK) weighing 310-400g (9-12 weeks old) at start of experiments. In Experiment 1, 72 rats were used for latent inhibition and locomotor activity experiments. These rats were tested in 3 batches of 24 rats. In Experiment 2, 32 rats were used in a single batch for latent inhibition testing only. See experimental design for further detail and for a sample size justification.

Rats were housed in groups of 4 in 2 level "double decker" cages (462mm x 403mm x 404mm; Techniplast, UK) with temperature and humidity control (21 ± 1.5 °C, $50\% \pm 8\%$) and an alternating 12h light dark cycle (lights on at 0700). They had ad libitum access to food and water (Teklad global 18% protein diet, Harlan, UK). All rats were habituated to handling by experimenters for at least 5 days prior to any experimental procedure. All experimental procedures were conducted during the light phase. All procedures were conducted in accordance with the requirements of the United Kingdom (UK) Animals (Scientific Procedures) Act 1986.

Stereotaxic implantation of guide cannula into the ventral hippocampus

Rats were anaesthetised using isoflurane delivered in oxygen (induced with 5% and maintained at 1.5-3%; flow rate 1L/min) and then placed in a stereotaxic frame. A local anaesthetic (EMLA cream, AstraZeneca, UK) was applied to the ear bars to minimise discomfort.

A gel was used (Lubrithal; Dechra, UK) to prevent the eyes drying out during surgery. After incision of the scalp, bilateral infusion guide cannula (stainless steel, 26 gauge, 8.5mm below pedestal, Plastics One, USA) were implanted in small pre-drilled holes in the skull. The stereotaxic coordinates for the injection site were 5.2mm posterior, ± 4.8 mm lateral from the midline and 6.5mm ventral from the dura for infusions into the ventral hippocampus, as in McGarrity et al. (2017). Stainless steel stylets, complete with dust cap, were placed into the guide cannula, which protruded 0.5mm beyond the tips of the guide cannula to prevent occlusion. Dental acrylic (flowable composite; Henry Schein, Germany) and four stainless steel screws were used to fix guide cannulae to the skull. The scalp incision was stitched around the acrylic pedestal to reduce the open wound to a minimum. All rats were injected with perioperative analgesia (Rimadyl, Large Animal Solution; 1:9 dilution; 0.1ml/100g s.c). After surgery rats were injected with 1ml of saline (i.p) to prevent dehydration. Antibiotics were administered on the day of surgery and subsequently every 24h for the duration of the study (Synolux; 140mg amoxicillin, 35mg clavulanic acid/ml; 0.02ml/100g s.c, Pfizer). After surgery, rats were allowed at least 5 days of recovery before any further experimental procedures were carried out. During this period rats underwent daily health checks and were habituated to the manual restraint necessary for drug micro-infusions.

Intracerebral microinfusion procedure

Rats were manually restrained throughout the infusion process. Stylets were replaced with infusion injectors (stainless steel, 33-gauge, Plastics One, USA), which extended 0.5mm below the guide cannula tips into the ventral hippocampus. Injectors were connected via flexible polyethylene tubing to 5- μ l SGE micro-syringes mounted on a microinfusion pump (sp200IZ, World Precision Instruments). A volume of 0.5 μ l/side of either 0.9% sterile saline (control) or picrotoxin ($C_{30}H_{34}O_{13}$; Sigma Aldrich, UK) in saline (150ng/ μ l/side) was infused bilaterally over the course of 1 min, as used in previous studies (McGarrity et al., 2017). The movement of an air bubble, which was included in the tubing, was monitored to ensure the solution had been successfully injected into the brain. Injectors were removed and replaced by stylets 60s after the end of infusion to allow for tissue absorption of the infusion bolus. Picrotoxin solutions were prepared on the day from frozen aliquots containing 150ng/0.5 μ l. Picrotoxin, which acts as a GABA-A antagonist, has the potential to cause epileptiform activity in the hippocampus (Qaddoumi et al., 2014). Therefore, all rats receiving infusions were monitored carefully for behavioural signs potentially related to seizure development during and after infusion; development of any of these signs was recorded. These signs include: facial twitching, wet-dog shakes, clonic limb movement, motor convulsions and wild jumping (Luttjohann et al., 2009, Racine, 1972). During previous work the dose of picrotoxin (150ng/0.5 μ l/side) used did not cause the onset of seizure-related behavioural signs and did not cause

electrophysiological signs of hippocampal seizures in anaesthetised recording studies (McGarrity et al., 2017).

Verification of cannula placements

After behavioural experiments rats were overdosed with sodium pentobarbital (Dolethal, Vetoquinol, UK) and were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in saline. Subsequently brains were removed and stored in 4% PFA. Brains were sliced at 80µm thickness using a vibratome and placed on microscope slides, placements of infusion injectors were identified using light microscopy. Injector placements were mapped onto coronal sections of a rat brain atlas (Paxinos and Watson, 2006).

Conditioned emotional response procedure with a pre-exposure phase to measure aversive conditioning and its latent inhibition

The conditioned emotional response (CER) procedure used follows the task previously described by Nelson et al. (2011a). The basic procedure requires rats to be trained to robustly drink from a waterspout, which are subsequently either pre-exposed (PE) or non-pre-exposed (NPE) to a conditioned stimulus (CS). Rats are conditioned to the CS and then the degree of conditioning to the CS is assessed. A reduction in conditioned suppression in the PE but not the NPE group is indicative of latent inhibition.

Apparatus

Six identical fully automatic conditioning chambers comprised of sound attenuating cases and ventilation fans (Cambridge Cognition, UK) were used. The inner chambers consist of a plain steel box (25 x 25 x 22cm) with a Plexiglas door (27 x 21cm). The floor of the inner conditioning chamber comprises of a shock delivery system, consisting of 1cm spaced steel bars. These are positioned 1cm above the lip of a 7cm deep sawdust tray. Mounted 5cm above the grid floor is a waterspout connected to a lickometer supplied by a water pump. Licks were registered by breaking a photo beam within the spout which triggered water delivery of 0.05ml per lick. The spout was only illuminated when water was available. Three wall mounted lights and the house light flashing on (0.5s) and off (0.5s) for 5 s functioned as the conditioned stimulus (CS). Scrambled foot-shock of 1mA intensity for 1s provided the unconditioned stimulus. The shock was delivered through the grid floor by a constant current shock generator (pulsed voltage: output square wave 10ms on, 80 ms off, 370 V peak under no load conditions; MISAC Systems, UK). Stimulus control and data collection were recorded using an Acorn RISC computer programmed in basic with Arachnid extension (Cambridge Cognition, UK).

Behavioural procedure

One day prior to behavioural testing rats were water restricted for between 18-22h. Subsequently, they received 1 h and 15 min of ad libitum access to water in their home cages for the duration of the experiment once daily testing was completed and in addition to the

access to water in the experimental chambers. The conditioned emotional response procedure consisted of several stages (Fig. 1), which are detailed below.

Pre-training

Rats were shaped for 1 day until all rats drank from the waterspout and were assigned an individual experimental chamber for the duration of the procedure. Subsequently, rats were given a 15-min session (timed from first lick) per day for 5 days to drink from the waterspout. During the sessions, the waterspout was illuminated throughout but no other stimuli were present. Total number of licks was recorded during each session to assess any pre-existing differences in drinking prior to infusions.

Pre-exposure

The pre-exposed (PE) animals received 30 5s flashing light CS presentations with an average inter-stimulus interval of 60s. The non-pre-exposed (NPE) control animals were confined to the experimental chamber for an identical period of time (32mins) without receiving any CS presentations. Water was not available during the session and the waterspout was not illuminated.

Conditioning

One day after pre-exposure, rats were conditioned by two light-foot shock pairs, with the foot shock (1 mA/1s) delivered immediately following the termination of the flashing light (5s).

The first CS-UCS was presented after 5mins had elapsed and the second pairing 5-minutes after the first, followed by a further 5min in the chamber. Water was not available during the session and the waterspout was not illuminated for the duration of the session.

Reshaping

The day after conditioning, rats were reshaped using the same procedure as used during pre-training. This was to re-establish drinking behaviour after the conditioning session. Latency to first lick during reshaping was used as a measure of contextual fear conditioning to the conditioning chamber (Nelson et al., 2011a, Nelson et al., 2013).

Test

The day after re-shaping, rats underwent a test session to assess conditioning to the CS. During the session water was available throughout and the waterspout was illuminated. Once the rats had performed 50 licks the CS was presented continuously for 15 minutes. The time taken to complete 50 licks before CS presentation (excluding latency to first lick) provides a measure of individual baseline variation (A period). This time was compared to the time taken to complete 50 licks after CS presentation (B period). A suppression ratio ($A / (A+B)$) was used to assess the overall level of conditioning to the light CS, adjusted to individual variation in drinking.

Experimental design and data analysis

Both experiments 1 and 2 were run in a between subjects design with a target sample size for both experiments of 16-18 per group. This would give us a power of > 80% to detect effect sizes of $d=1$ for differences between groups (using between-subjects pairwise comparisons, two-tailed, with a significance threshold of $p<0.05$), which has been suggested to be appropriate for neurobiological studies of aversive conditioning (Carneiro et al., 2018). Experiment 1 was run in 3 identical series of 24 rats per series. Experiment 2 was planned as a sequential design, consisting of 2 identical series of experiments, each containing 32 rats. Experiment 2 was ended after the first series due to futility, as there was clearly no evidence that the target effect size the study was powered for could be achieved (Neumann et al., 2017).

Two of the four rats in each cage were randomly assigned to the saline and the other two to the picrotoxin infusion group, and subsequently one rat of each pair was randomly assigned to either pre-exposed or non-pre-exposed conditions. The experimenters were blinded with respect to the group allocation at the start of the experiment. In both experiments, several rats had to be excluded from the analysis of the whole experiment or some later stages of the experiment. During Experiment 1, 13 rats fell ill, with presumed meningitis, before testing, two further rats sustained blocked guide cannulae resulting in exclusion from the experiment, and one rat showed adverse effects, extended convulsive seizures, after

microtoxin infusion. During Experiment 2, one rat died during surgery and a further three rats fell ill, with presumed meningitis, before testing. The final sample sizes contributing to the analysis of performance measures at the different test stages in Experiment 1 and 2 are shown in Table 1.

	Experiment 1		Experiment 2	
Group	Reshaping	Test	Reshaping	Test
Saline NPE	14	14	7	7
Saline PE	13	10	8	8
PTX NPE	15	15	6	6
PTX PE	17	17	7	7

Table 1. Final number of animals included for data analysis per group for each stage of both latent inhibition experiments.

For Experiment 1 drug infusions took place before both pre-exposure and conditioning sessions, whereas during experiment 2 drug infusions took place before pre-exposure only (Fig. 1). Rats were infused in batches of two pairs, by two experimenters, with each pair including one rat to receive saline and one rat to receive microtoxin infusions. The two experimenters infused one pair, then the second pair, and testing started 10 min after the infusions for both rats of the second pair had been completed. This meant all rats had at least a 10 min period between the end of the infusion and the start of testing, but for the rats from the first pair (and often also one rat of the second pair) the period was slightly longer, by the time it took to complete the infusion of the second pair (typically about 2 min).

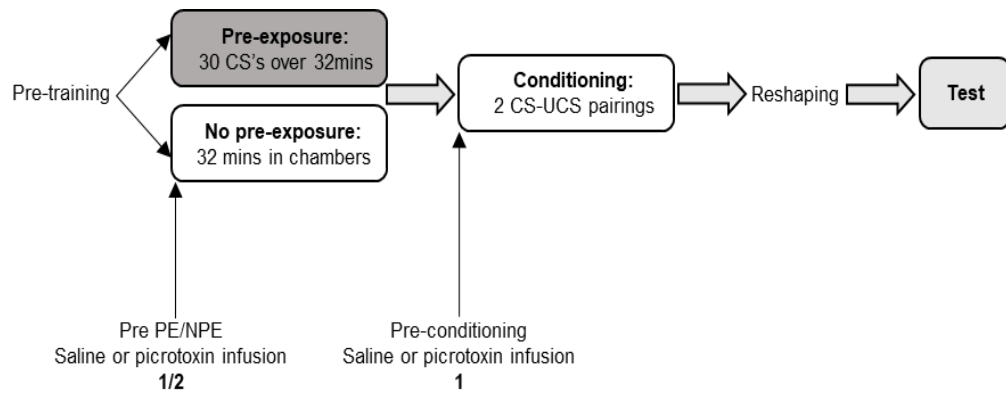


Figure 1. Task design for latent inhibition experiments. In Experiment 1, hippocampal infusions took place before both pre-exposure and conditioning. In Experiment 2, infusions took place only prior to pre-exposure.

Statistical analysis was performed using a 2x2 analysis of variance (ANOVA) with between subject factors of pre-exposure group (NPE/PE) and drug infusion (Saline/Picrotoxin). All statistical tests and graphs were completed using SPSS (version 23, IBM Corp, USA), JASP (JASP Team: version 0.12.2, 2020) and GraphPad prism (version 8, GraphPad software, USA). The accepted level of significance was $p < 0.05$. Raw latency data (time to first lick during reshaping) or time 'A' data (time to 50 licks during test) were log transformed, as they showed unequal variance (Levene's test, all $F > 5$, $p < 0.002$), to ensure a normal distribution and suitability for parametric analysis (Nelson et al., 2011; Nelson et al., 2012).

Locomotor activity testing

Locomotor activity was measured as in a previous study from the group (McGarrity et al., 2017), using 12 clear Perspex chambers (39.5 cm long × 23.5 cm wide × 24.5 cm deep) with metal grid lids

placed in a dimly lit (45-65 lux) chamber. The chambers were placed in frames containing 2 levels of a 4 × 8 photo beam configuration (Photobeam Activity System; San Diego Instruments, USA). All photocells were connected to a PC running the PAS software (version 2), two consecutive breaks of adjacent beams within the lower level of photo beams generated a locomotor count. To start a session, rats were placed into the centre of the chambers. Total locomotor counts were calculated for each 10 minute block of testing.

Baseline locomotor activity was assessed for 30 minutes without infusion to identify any differences between groups prior to infusion. The following day rats were placed in the boxes for 30 minutes to habituate, after this period rats were infused with either saline or picrotoxin and placed in the boxes for a further 60 minutes to measure any effects of drug treatment. We expected picrotoxin infusion into the ventral hippocampus to induce hyper locomotion (McGarrity et al., 2017), therefore a pre-infusion habituation period would allow any differences in locomotor activity caused by drug micro-infusions to not be occluded by high baseline activity.

Locomotor activity testing was conducted after the CER paradigm in experiment 1, as a positive control and for comparison with previous studies. Ventral hippocampal disinhibition has been previously shown to induce robust locomotor hyper-activity (Bast et al., 2001a, McGarrity et al., 2017) and therefore hyper-activity would reflect a behavioural impact of picrotoxin infusion. Rats were infused in pairs

simultaneously, and post-infusion testing began immediately after completion of the infusion. Rats were allocated to the same drug condition during LMA testing as they were during the previous infusions during the LI paradigm. Locomotor activity was measured in 1-min intervals and calculated in 10-minute bins pre and post infusion. Locomotor activity data was analysed using repeated measures ANOVA, with drug treatment as the between subject's factor and 10-min bins as the within subject's factor. Data was analysed using GraphPad prism (version 7) software.

Results

Verification of infusion cannula placements

Cannula tips were mapped onto coronal sections adapted from the Paxinos and Watson (2006) rat brain atlas. All cannula tips were located between 4.8 and 6.3mm posterior to bregma within the ventral hippocampus (Fig. 2).

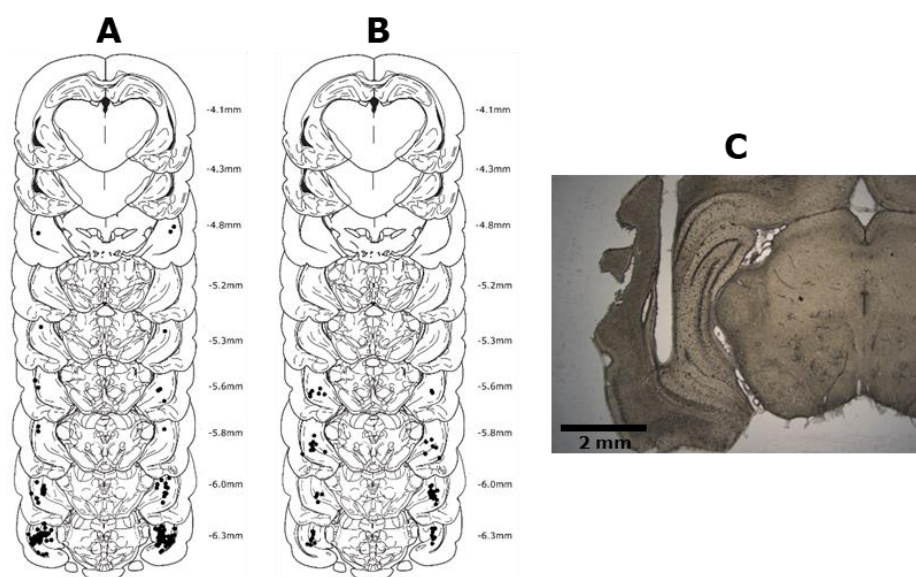


Figure 2. Approximate locations of infusion cannula tips (black dots) as verified by light microscopy of coronal brain slices (80 μ m) for rats in experiment 1 (**A**) and 2 (**B**). Placements are mapped onto coronal sections adapted from Paxinos and Watson rat brain atlas, number on the right indicate level relative to bregma. **C:** Illustrative coronal brain section showing placement of infusion cannula as seen by light microscopy.

Experiment 1: Effect of ventral hippocampal picrotoxin infusion at pre-exposure and conditioning stages of the CER

Pre-training

Analysis of latencies to lick at the end of pre-training, prior to pre-exposure, showed no overall effect of infusion group ($F < 1$) or of

conditioning group to be ($F < 1$) and there were no interactions of these factors ($F < 1$) (data not shown).

Reshaping

Ventral hippocampal picrotoxin, compared to saline, infusion reduced latency to first lick after reintroduction to the conditioning context during the reshaping session in the NPE group, which reflects reduced contextual fear conditioning; in the PE group, both saline and picrotoxin groups showed similarly low levels of contextual conditioning, as measured by low latencies to lick and reflecting that PE reduced contextual conditioning in the saline group (Fig. 3A). These observations were supported by a significant infusion x pre-exposure interaction: $F_{(1, 55)} = 4.736$, $p = 0.034$, effect size: $0.051 \omega^2$ (ω^2). Hippocampal picrotoxin reduced conditioning in the NPE group ($F_{(1, 55)} = 11.878$, $p = 0.001$), but this was not apparent in the PE group due to a floor effect where there is a low baseline of conditioning ($F_{(1, 55)} = 0.154$, $p = 0.697$). In addition, pre-exposure to the CS reduced conditioning in saline infused rats, this is reflected by a significant reduction in latency in the PE group as compared to the NPE group ($F = 9.006$, $p = 0.004$), this effect was not present in picrotoxin infused animals ($F_{(1, 55)} = 0.003$, $p = 0.956$), probably reflecting a floor effect, i.e. the already low latencies in the PE group.

Light Test

There was no difference in time to 50 licks (Time A) between infusion group and pre-exposure groups (Effect of infusion, pre-exposure and

interaction: All $F < 3$, $p > 0.09$ (data not shown). The group difference in latency to first lick that were evident at reshaping were not present during the test stage, probably reflecting extinguished contextual conditioning in the saline NPE group. The suppression ratios during the light test revealed that hippocampal disinhibition markedly disrupted conditioning to the light CS in the NPE group, but did not affect conditioning in the PE group, i.e. there was no evidence that hippocampal disinhibition would have affected salience modulation (Fig. 3B). In saline-infused rats, the suppression ratio was markedly increased in the PE compared to the NPE group, reflecting reduced conditioning, i.e. LI (Fig. 3B, left). This difference between PE and NPE groups was not apparent in the picrotoxin-infused rats (Fig. 3B, right). However, this was due to picrotoxin-infused NPE rats showing a markedly higher suppression ratio than saline-infused NPE rats, i.e. reduced conditioning to the light CS (compare white bars in Fig. 3B). In contrast, suppression ratios were similar in picrotoxin and saline-infused PE rats (compare grey bars in Fig. 3B), not supporting that hippocampal disinhibition reduces the impact of CS pre-exposure on conditioning. These observations were supported by a significant infusion x pre-exposure interaction ($F_{(1, 52)} = 4.142$, $p = 0.047$, effect size: $0.043 \omega^2$). Further examination of the interaction by simple main effects analysis revealed a main effect of infusion in the NPE group ($F_{(1, 52)} = 10.014$, $p = 0.003$) reflecting increased suppression, i.e. reduced conditioning, caused by picrotoxin, compared to saline, whereas there was no effect of infusion in the PE group ($F_{(1, 52)} = 0.028$, $p = 0.867$).

This resulted in the absence of a difference between PE and NPE in the picrotoxin infused rats ($F_{(1, 52)} = 0.878, p = 0.353$), i.e. no LI, whereas saline infused rats showed markedly higher suppression in the PE compared to the NPE group ($F_{(1, 52)} = 12.111, p = 0.001$).

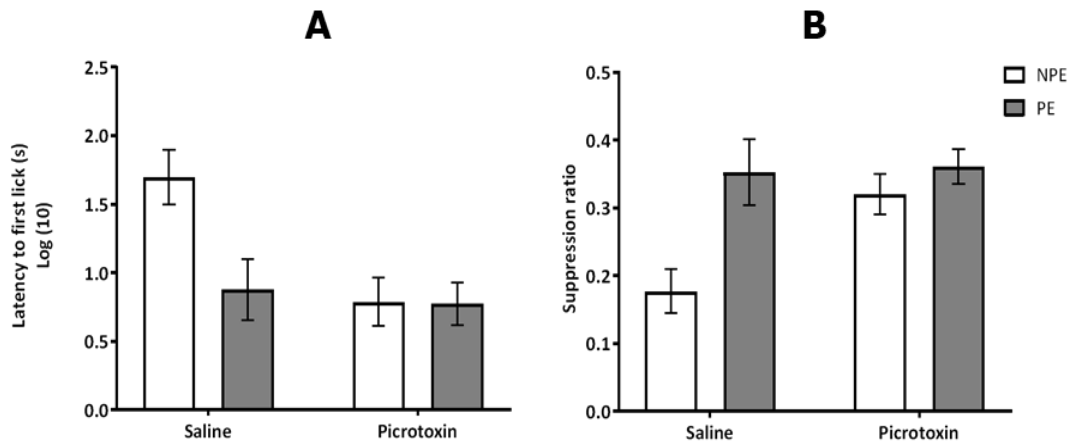


Figure 3. A: Mean (± SEM) latency to first lick values (s) (log transformed) in the conditioning chamber following the aversive conditioning session for non-pre-exposed (NPE: white bars) and pre-exposed (PE: grey bars) groups after saline or picrotoxin micro-infusions. Saline NPE animals show longer latencies compared to all other groups. **B:** Mean suppression ratio (±SEM) to the light conditioned stimulus for control (NPE: white) and PE (grey) groups that had previously had saline or picrotoxin infusions at pre-exposure and conditioning. Pre-exposure reduced fear responding to the CS in saline infused animals, indicated by reduced suppression ratio, this effect is abolished after picrotoxin infusion.

Experiment 2 – Effect of ventral hippocampal picrotoxin infusions at pre-exposure

Pre-training

Analysis of latencies to lick at the end of pre-training, prior to pre-exposure, showed no overall effect of infusion group ($F_{(1,24)} = 2.851,$

$p=0.104$) or of conditioning group to be ($F < 1$) and there were no interactions of these factors ($F < 1$) (data not shown).

Reshaping

Hippocampal picrotoxin infusion only at pre-exposure had no effect on conditioning to the context, as reflected by latencies to first lick during reshaping, and there was no difference between conditioning groups (Fig. 4A). The latter contrasts with the finding in Experiment 1, that pre-exposure reduced latencies to first lick in saline infused rats (Fig. 3A). Analysis of latency to first lick after reintroduction to the conditioning context revealed no effect of conditioning group ($F_{(1, 24)} = 0.17, p=0.68$), infusion ($F_{(1, 24)} = 1.71, p=0.20$) or interaction of these factors ($F_{(1, 24)} = 1.46, p=0.24$) (Fig. 4A).

Light test

There were no differences in the A period (time to 50 licks in the absence of light CS) between groups ($F_{(1, 24)} = 0.01, p=0.91$) or infusion condition ($F_{(1, 24)} = 1.12, p=0.30$) (data not shown). This indicates there was no difference between groups prior to the suppression test.

Figure 4B shows an effect of pre exposure in both drug infusion conditions, where pre-exposed groups have greater suppression ratios, therefore LI is present in both infusion groups. This is supported by ANOVA which shows an effect of conditioning group ($F_{(1, 24)} = 8.44, p=0.0078$, effect size: $0.221\omega^2$), but not of infusion ($F_{(1,$

$_{24}) = 0.13, p = 0.72$) and no interaction of these factors ($F_{(1, 24)} = 0.15, p = 0.70$).

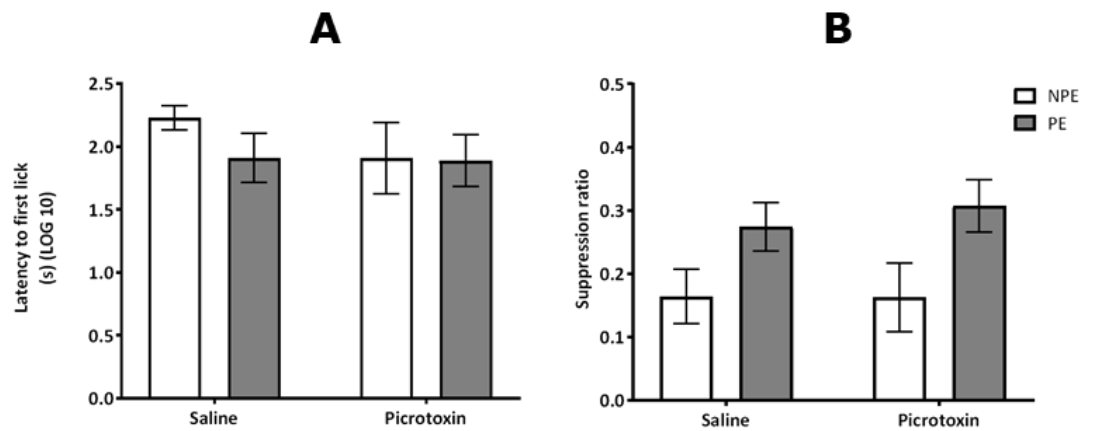


Figure 4. A: Mean (\pm SEM) latency to first lick values (s) (log transformed) in the conditioning chamber following the aversive conditioning session for non-pre-exposed (NPE: white bars) and pre-exposed (PE: grey bars) groups after saline or picROTOXIN micro-infusion at pre-exposure. All groups show similar levels of contextual conditioning, indicated by similar latency to first lick. **B:** Mean suppression ratio (\pm SEM) to the light conditioned stimulus for control (NPE: white) and PE (grey) groups that had previously had saline or picROTOXIN infusions at pre-exposure only. Pre-exposure reduced fear responding to the CS in both saline and picROTOXIN infused animals, indicated by reduced suppression ratio.

Hippocampal picROTOXIN infusion induces locomotor hyper-activity

There was no difference between the locomotor activity in saline and picROTOXIN groups during the first 30 min of testing prior to drug microinfusion (effect and interaction involving infusion: both $F < 1.5, p > 0.24$) only a significant reduction of locomotor activity over the three 10-min bins (main effect of 10-min bins: $F_{(2, 106)} = 189.9, p < 0.0001$), reflecting habituation to the activity boxes in both groups (Fig. 5, left). Consistent with previous findings (Bast et al., 2001a,

McGarrity et al., 2017), picrotoxin infusions into the ventral hippocampus markedly increased locomotor activity, especially during the first 20 minutes of the 60-min testing period post infusion (Fig. 5, right). This increase was reflected by a significant 10-min bin x infusion interaction ($F_{(5, 265)} = 17.61, p < 0.0001$).

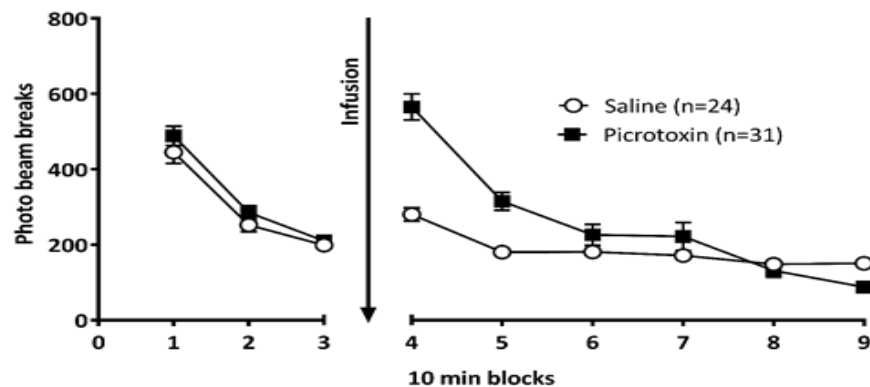


Figure 5. Hippocampal picrotoxin infusion causes increased locomotor activity. No change is observed in the 30min period prior to infusion in locomotor activity as represented by photo-beam breaks (\pm SEM). Ventral hippocampal picrotoxin (black squares) increased locomotor activity compared to saline (white circles).

Potential seizure-related behavioural effects of hippocampal picrotoxin

Although the dose of picrotoxin used did not show signs of seizure activity either behaviourally or using electrophysiology in previous work (McGarrity et al., 2017), in the present study we observed seizure-related behavioural effects in several rats following hippocampal picrotoxin infusion. In several rats receiving hippocampal picrotoxin infusions in Experiment 1 (23 out of 33 rats receiving picrotoxin) and 2 (6 out of 15 rats receiving picrotoxin) we observed seizure-related behavioural signs, including facial twitching,

wet-dog shakes and wild running, which can often be observed before full motor seizures (Luttjohann et al., 2009, Racine, 1972). These signs were only observed after infusions of picrotoxin and not after saline infusions and are detailed in table 2. Symptoms were observed within 5 min after the end of the picrotoxin infusion. Seizure-related behavioural signs were short-lived (30-45mins) and following these signs rats typically showed no persistent adverse effects of infusion, with the exception of one rat which showed long lasting seizure like behaviour and was culled.

Observed behaviour	First infusion	Second infusion	Third infusion
Facial twitching	1	2	3
Wet dog shakes	17	8	7
Wild running	9	3	5
Clonic limb movement	3	0	1

Table 2. Seizure-related behavioural signs observed after ventral hippocampal picrotoxin micro-infusions. Type of behaviour observed, and number of rats showing this sign is indicated. Number of incidences per infusion collated from both experiments 1 and 2 is shown.

The ventral hippocampus and subiculum show the earliest seizure activity in spontaneous model of seizures (Toyoda et al., 2013) and GABA network dysfunction of the limbic system is strongly implicated in the onset of seizures (Avoli and de Curtis, 2011). These behavioural changes initially provide good evidence that the hippocampal picrotoxin infusions have effectively disrupted GABA transmission and

suggest the local network has been disrupted. Interestingly population studies show epileptic patients have a higher risk of psychosis related illness than in the general population (Clancy et al., 2014, Clarke et al., 2012) suggesting a possible shared aetiology which could include hippocampal GABA dysfunction.

Discussion

In Experiment 1, ventral hippocampal picrotoxin infusion at pre-exposure and conditioning abolished fear conditioning to the light CS and, therefore, the reduction of fear conditioning in the PE compared to NPE group, which would reflect LI, could not be detected. Picrotoxin and saline infused rats in the PE group did not differ, showing similarly low conditioning, which does not support that hippocampal disinhibition affected salience modulation. In addition to disrupting conditioning to the light CS, ventral hippocampal infusion of picrotoxin also impaired contextual conditioning as reflected by reduced conditioning (lower latency to first lick) after reintroduction to the conditioning context. In Experiment 2, which specifically examined the impact of hippocampal disinhibition at the pre-exposure stage (and avoiding the disruption of aversive conditioning), there was no evidence for any impact on LI, with a pronounced CS pre-exposure effect present in both picrotoxin and saline infusion groups.

Robust LI in the CER paradigm

Using a between-subjects, off-baseline, CER procedure (Nelson et al., 2011a), we demonstrated robust LI in Lister hooded rats. The procedure was designed to support LI in vehicle control rats, whilst allowing for identification of suppressed LI. We observed robust LI in saline control rats in Experiment 1 and in both infusion groups in Experiment 2, reflected by a significant increase in suppression of licking behaviour in the NPE group as compared to the PE group. The reduced suppression after pre-exposure to the conditioned stimuli indicates

an intact LI effect, where these rats have not associated the previously inconsequential flashing lights with the aversive foot-shock applied to them in a subsequent conditioning session. This effect is in line with previous experiments using the same procedure in Wistar rats (Cassaday and Thur, 2020, Nelson et al., 2012, Nelson et al., 2011a).

PE-induced enhancement of contextual fear conditioning

Intriguingly there is a significant difference in latency to lick during reshaping between conditioning groups in the saline infused group in Experiment 1. The NPE rats have a significantly longer latency to lick compared to PE rats and therefore are more conditioned to the context compared to the PE group. The enhanced conditioning in the NPE group is somewhat unexpected. It may have been anticipated that rats that had not been pre-exposed to the CS would have paid less attention to the context, because this may have been overshadowed by the more salient CS (Odling-Smee, 1978). More specifically, due to the novelty of the flashing light stimulus during pre-exposure, rats may have been expected to pay increased attention to the highly salient light stimuli and consequently less attention to the general context. However, in this case, the opposite effect has occurred, whereby NPE rats were generally more fearful of the context. Such enhancement of contextual conditioning by pre-exposure to the light stimulus may reflect increased arousal due to the novelty of the light stimulus, which could lead to novelty enhanced memory formation (King and Williams, 2009). The added

novelty of the light CS in the NPE group during conditioning could also enhance hippocampal memory formation. Novelty induces dopamine release in the hippocampus (Moreno-Castilla et al., 2017), which contributes to memory encoding (Pezze and Bast, 2012), subsequently resulting in stronger context representation memory (Duszkiewicz et al., 2019, Lisman and Grace, 2005). Therefore, a novelty induced increase in arousal could lead to amplified association of the context during aversive conditioning and therefore an increase in contextual fear conditioning. However, the difference between NPE and PE groups in saline infused rats which was seen in Experiment 1 was not present in Experiment 2. This discrepancy could be explained, at least in part, by a ceiling effect in conditioning which masks any differences between NPE and PE group in Experiment 2. The level of contextual conditioning in Experiment 1 is substantially lower than during Experiment 2, which could be due to behavioural effects of the microinfusion procedure itself. More specifically, in contrast to Experiment 1, the rats in Experiment 2 were not subjected to drug infusions just before conditioning, which may have resulted in stronger conditioning and, thereby, may have prevented the detection of any PE-induced enhancement of contextual fear conditioning. In line with this explanation, previous studies reported a tendency for un-operated rats to show stronger fear conditioning than cannulated rats that received control infusions (see discussion in: (Zhang et al., 2001).

Ventral hippocampal disinhibition during PE and conditioning abolishes fear conditioning in the CER paradigm, but no evidence for an impact on salience modulation in the PE group

In Experiment 1, ventral hippocampal picrotoxin infused at both pre-exposure and conditioning eradicated fear conditioning to the light CS in the NPE group, resulting in similar levels of conditioning in both NPE and PE groups. Whilst disinhibition within the ventral hippocampus resulted in similar levels of conditioned suppression between NPE and PE groups, the effect is not mediated by the PE group and therefore is not due to aberrant salience allocation. Instead, the reduction in lick suppression in the NPE group indicates reduced conditioning to the CS (see discussion below). Similar to the present study, Pouzet et al. (2004), using a similar LI paradigm, demonstrated ventral hippocampal NMDA infusions reduced conditioned suppression in the NPE group, although there was also some evidence for disrupted LI with a trend towards greater conditioned suppression in PE compared to NPE rats. Overall, the results from Experiment 1 suggest the reduction of conditioned suppression in the NPE group reflects reduced associative learning of the CS-US pairing, which subsequently masks the expression of LI. In addition to reduced conditioning to the light CS, hippocampal picrotoxin infusion in Experiment 1 also reduced behavioural suppression, as reflected by reduced latencies to lick, when rats were re-exposed to the conditioning context during the reshaping session. The readiness to lick in the picrotoxin group suggests these rats were less fearful of the conditioning chamber context after aversive

conditioning and therefore, that ventral hippocampal picrotoxin infusions reduced conditioning to the context.

Disruption of elemental and contextual fear conditioning by ventral hippocampal disinhibition might reflect disruption of regional and distal processing

These results are consistent with the role of the ventral hippocampus in both conditioning to context and explicit auditory or visual cues. Lesions, inactivation (via sodium channel blockers), functional inhibition and stimulation of the ventral hippocampus have been found to disrupt both contextual and cue conditioning (Bast et al., 2001b, Maren, 1999, Kjelstrup et al., 2002, Zhang et al., 2001). However, manipulation of inhibitory neurons using the GABA-A agonist muscimol only disrupts contextual but not cue conditioning (Bast et al., 2001b, Maren and Holt, 2004, Zhang et al., 2014), whereas antagonism of hippocampal GABA-A receptors in this study disrupted conditioning to both cue and context. The difference in effects caused by opposing GABA manipulations could be due to the local and distal effects of these manipulations. Using measurements of cerebral blood flow to examine brain wide activation changes caused by ventral hippocampal disinhibition, we identified prominent changes in neural activity in regions implicated in fear conditioning, including the dorsal hippocampus, septum, hypothalamus and extended amygdala (see chapter 3). Furthermore, reduced ventral hippocampal GABA transmission could disrupt intra-hippocampal connections across the longitudinal dorso-ventral axis (Sik et al.,

1994, Sik et al., 1997), which could impair dorsal hippocampus information processing. Correct functioning of the dorsal hippocampus is necessary for the acquisition and expression of contextual conditioning (Matus-Amat et al., 2004), and it has been suggested that the dorsal hippocampus produces a contextual representation, which subserves contextual conditioning (Hunsaker and Kesner, 2008). In addition, ventral hippocampal disinhibition disrupts one trial place learning in the delayed-matching-to-place watermaze (McGarrity et al., 2017) and, therefore, disruption of contextual conditioning could be driven by impaired spatial memory, whereby visual cues are not processed, and a representation of the context is not established.

In addition to disruptions of hippocampal mechanisms of contextual fear, deficits in conditioning could be caused by disruptions in information processing in distal extra-hippocampal sites. Pavlovian fear conditioning is controlled by a circuit including the ventral hippocampus, amygdala and mPFC (Tovote et al., 2015). The ventral hippocampus has strong reciprocal projections to the basal amygdala (Petrovich et al., 2001, Pitkänen et al., 2000) and weaker projections to the central amygdala (Cenquizca and Swanson, 2007, Kishi et al., 2006), while also sending projections to the medial prefrontal cortex (Hoover and Vertes, 2007). The amygdala plays a central role in both the acquisition and expression of contextual and cue conditioned fear (LeDoux, 2000, Maren, 2001). Amygdala dependent fear expression can also be modulated by the prelimbic cortex, as well as by ventral

hippocampus inputs (Sierra-Mercado et al., 2011). The ventral hippocampus can modulate the input of information into the amygdala directly or via the mPFC. Projections from prelimbic cortex innervate the basal amygdala (Vertes, 2004) and lesions of the prelimbic cortex reduce fear expression (Corcoran and Quirk, 2007), while input from prelimbic cortex and ventral hippocampus to the basal amygdala have been shown to mediate contextual fear extinction (Orsini et al., 2011). Additionally, double projecting neurons to the amygdala and mPFC from the ventral hippocampus are activated during encoding and retrieval of context fear (Kim and Cho, 2017). Therefore, it is possible GABA dysfunction of the ventral hippocampus causes excitation of projections to the amygdala and mPFC which subsequently disrupts processing of cue and contextual information leading to disrupted fear conditioning in the CER paradigm.

Disrupted fear conditioning could also be driven by ventral hippocampal connectivity to other efferent brain regions. The lateral septum, a key output structure of the ventral hippocampus (Risold and Swanson, 1997) has long been implicated in anxiety and related behaviours (Sheehan et al., 2004). The septum receives strong connections from the hippocampus and is connected to the amygdala, mesolimbic dopamine system and hypothalamus (Deller et al., 1994, Risold and Swanson, 1997, Swanson and Cowan, 1979). The septum is therefore a potential modulator of hippocampal fear conditioning effects and glutamatergic transmission between the hippocampus

and septum has been shown to be important for the expression of cue and contextual conditioning (Calandreau et al., 2010, Vouimba et al., 1998). Therefore, aberrant septal activity caused by disinhibited ventral hippocampal firing could disrupt information processing in the septum and disrupt fear conditioning.

Whilst ventral hippocampal picrotoxin infusions disrupt the acquisition of cue and contextual fear conditioning, one factor that needs to be taken into account is state dependent learning i.e. information learned in one neural state can, in some cases, only be retrieved in the same state (Overton, 1964). In our case ventral hippocampal infusions take place during pre-exposure and conditioning but not during reshaping and test, thus the ventral hippocampus is in a different state during the expression of contextual and cue conditioning. To confirm the disruption of fear conditioning was not due to state dependence, animals would need to receive picrotoxin infusions before the test session and subsequently continue to show reduced conditioned fear as demonstrated with pre-conditioning infusions. However, pre-expression inactivation of the ventral hippocampus during a classical Pavlovian paradigm disrupts the expression of conditioned fear (Sierra-Mercado et al., 2011). Therefore, perturbation of ventral hippocampal activity during test may disrupt conditioned fear independent of state dependence, which would make assessing any state dependent effects of fear expression problematic. However, several studies have shown that state dependent learning does not account for the conditioning deficits of

specific local microinfusions, these include infusions of dopamine agonists or antagonists in the PFC (Pezze et al., 2003) and amygdala (Guarraci et al., 2000), and dorsal hippocampal infusion of NMDA (Bast et al., 2003). In addition, previous experiments using a similar 3 stage fear conditioning paradigm designed to elucidate drug effects on LI found no evidence for state-dependent effects (Barad et al., 2004). Altogether the reduced conditioned suppression driven by hippocampal picrotoxin infusions prior to conditioning seem to reflect actual deficits in associative processes that underlie the formation of fear memory.

Hippocampal disinhibition during PE has no effect on the formation of LI

Whilst conditioning is thought to be the critical stage of the effect of dopamine in LI (Morrens et al., 2020, Young et al., 2005), disruption of the acquisition of LI due to other neural mechanisms have been described (Hitchcock et al., 1997, Lacroix et al., 2000b). This includes disrupted LI due to aberrant activity in the mPFC, a prominent hippocampal projection site, during the pre-exposure stage (Lingawi et al., 2017, Lingawi et al., 2018). Intra-hippocampal infusion of picrotoxin at the pre-exposure stage of the CER paradigm had no observable effect on LI at test. Therefore, decreased GABA transmission in the ventral hippocampus during PE had no effect on the acquisition of LI, not supporting that this manipulation disrupts salience modulation mechanisms during PE.

It has been hypothesised (Maren and Holt, 2000) that the hippocampus is involved in the CS-no event association which is then subsequently stored at a distal site. Our data suggest that GABA dysfunction in the ventral hippocampus does not disrupt the formation of a CS-no event association as LI is spared which reflects the recollection of such an association. Whilst the formation of LI appears to be dependent on balanced neural activity in the infralimbic cortex (Lingawi et al., 2017, Lingawi et al., 2018), and the ventral hippocampus can modulate PFC activity (Degenetais et al., 2003), ventral hippocampal disinhibition during pre-exposure did not affect LI formation. This suggests any changes in neural activity in the mPFC, driven by ventral hippocampal disinhibition (see chapter 3), are not sufficient to disrupt the formation LI. This could be explained by the relationship between neural activity and behavioural function which can have distinct characteristics (Bast et al., 2017). The disruption of a CS-no event association memory in the infralimbic cortex reported in the aforementioned studies is driven by reduced neural activity and this may reflect a monotonic relationship whereby reduced activity disrupts LI but increased activity has no effect on behaviour.

Whereas the present experiments did not provide evidence that ventral hippocampal disinhibition during pre-exposure affects LI, previous experiments have shown that activity within the ventral subiculum plays a role in the formation of LI. Specifically, deactivation of the ventral subiculum deactivation using TTX during pre-exposure

disrupts LI as reflected by reduced conditioning in the PE group using a conditioned taste aversion (CTA) paradigm (Peterschmitt et al., 2005, Peterschmitt et al., 2008). This data implies the ventral hippocampal formation is important in encoding the information related to the CS–no event association. Considering the results of our study showing that ventral hippocampal disinhibition during PE does not affect the formation of LI, whilst LI is disrupted by inactivation of the hippocampus suggests that hippocampal activity is necessary for LI. However, hippocampal hyperactivity has a less detrimental effect on behaviour and therefore balanced activity during PE within the hippocampus is not required for the formation of LI.

However, the divergent associative paradigms used in these experiments does need to be taken into account when comparing these findings. There are apparent differences in the effects of dopaminergic manipulations when comparing between the CER and CTA paradigms. Nucleus accumbens shell lesions disrupt LI when using the CER paradigm (Tai et al., 1995) but conversely potentiate LI using a CTA paradigm (Pothuizen et al., 2006). In addition amphetamine administration at PE is enough to disrupt LI using CTA (Bethus et al., 2006) whereas this is not the case for CER (Young et al., 2005). In accordance with this, during the CER paradigm changes in dopamine efflux between PE and NPE groups are confined to the NAc shell (Murphy et al., 2000), whereas using CTA changes are seen in both shell and core regions of the NAc (Jeanblanc et al., 2002). Therefore, the disruption of LI by hippocampal manipulations could

be dependent on the type of associative paradigm used, possibly due to the conflicting effects on the dopamine system.

Any role for ventral hippocampal disinhibition in clinically relevant LI deficits?

The absence of any effects of ventral hippocampal picrotoxin infusion on the formation of LI in the present study does not support that dysfunctional ventral hippocampal GABA transmission contributes to the LI deficits seen in acute schizophrenia patients (Gray et al., 1995b, Raschle et al., 2001). However, LI is known to be sensitive to changes in context, reflected by attenuated LI when pre-exposure and conditioning take place in different contexts (Hall and Channell, 1986, Westbrook et al., 2000), and the hippocampus is important in the processing of contextual cues (Hobin et al., 2006, Maren and Holt, 2000). Complete hippocampal lesions remove the context specificity of LI, which is reflected by the transfer of LI across different contexts (Honey and Good, 1993). In addition, dorsal hippocampal inactivation using muscimol during the retrieval phase disrupts the contextual gating of LI (Holt and Maren, 1999), whilst ventral subiculum lesions also disrupt the context specificity of LI (Quintero et al., 2011). Therefore, hippocampal disinhibition might disrupt the expression of LI by disrupting the recall of contextual memory during retrieval. However, because ventral hippocampal disinhibition disrupts cued fear conditioning (experiment 1), it is difficult to assess this possibility using a CER paradigm. This is because infusions during the test phase could disrupt the expression of cued fear rather than disrupting

contextual gating of LI per se, therefore deficits in LI could be masked by deficits in conditioned fear. Future experiments investigating the impact of hippocampal disinhibition on the expression and contextual gating of LI would be important to understand if contextual processing deficits caused by ventral hippocampal disinhibition also impact on LI. Therefore, providing clarity on the behavioural impact of the characteristic hippocampal hyperactivity phenotype seen in schizophrenia.

Clinical relevance

Using the CER paradigm, we show ventral hippocampal disinhibition causes both elemental and contextual fear conditioning deficits. These deficits in associative processing resemble data from schizophrenia patients where participants showed abnormal learning in a Pavlovian conditioning paradigm (Jensen et al., 2008). Specifically, schizophrenia patients did not distinguish between the aversive CS+ (aversive audible stimuli) and non-aversive CS-, where they showed increased tolerance to the CS+ compared to controls. Similarly, deficits in discriminative aversive conditioning in patients with schizophrenia (Hofer et al., 2001), and abnormal hippocampal BOLD responses during fear conditioning compared to controls have been reported (Holt et al., 2012). These combined clinical studies suggest a similar mechanism could be responsible for abnormal conditioning seen in our data and in human studies, whereby there is a dysfunctional emotional processing circuit, caused by increased

hippocampal drive to projection sites, however the neural basis for this remains to be determined.

Conclusion

In conclusion, ventral hippocampal disinhibition caused contextual and elemental fear conditioning deficits, whilst the present study did not provide evidence that this manipulation affects the acquisition of LI. The conditioning deficits seen in this study demonstrate ventral hippocampal GABA dysfunction causes aberrant associative processing of aversive stimuli. This data suggests ventral hippocampal GABA dysfunction could underlie associative processing abnormalities in schizophrenia patients. However, disruptions of LI may not be driven by hippocampal disinhibition as the formation of LI, at least in this paradigm, is unaffected by GABA dysfunction in the hippocampus.

Chapter 3: SPECT imaging: Brain-wide activation changes caused by hippocampal neural disinhibition

Introduction

Hippocampal metabolic hyperactivity and neural disinhibition have been strongly associated with psychiatric disorders, including early stage schizophrenia and age-related cognitive decline (Bakker et al., 2012, Heckers and Konradi, 2015, Weiss et al., 2004). This aberrant hippocampal activity could drive cognitive impairments and psychological symptoms of these disorders, partly through the array of connections to other brain regions (Bast, 2011, Bast et al., 2017, Lisman et al., 2008, Small et al., 2011). One prominent idea is that hyperactivity of the hippocampus is part of a mechanism which drives increased dopaminergic firing, which leads to the characteristic hyper-responsiveness of the striatal dopamine system found in schizophrenia and is thought to underlie positive symptoms (Grace, 2010, Grace, 2012, Mitchell et al., 2000). In addition, the hippocampus has significant efferent connection to the PFC (Cenquizca and Swanson, 2007, Jay and Witter, 1991) and the PFC is implicated in important clinically relevant cognitive functions, including sustained attention (Pezze et al., 2014) and behavioural flexibility (Birrell and Brown, 2000, Floresco et al., 2008, McAlonan and Brown, 2003). Significantly, disruption of connectivity between

the hippocampus and PFC has been found in schizophrenia patients (Benetti et al., 2009), and this could contribute to the cognitive deficits seen in schizophrenia (Lesh et al., 2011). A significant body of work over the last few decades has implicated hippocampal hyperactivity at rest as a key feature of both schizophrenia and mild cognitive impairment (MCI), including studies of glucose metabolism, regional cerebral blood flow (rCBF) and BOLD (blood-oxygen-level-dependent) measurements (Heckers and Konradi, 2015). In the early 1990s, PET studies of glucose metabolism identified markedly increased basal hippocampal activity in patients with schizophrenia (Liddle et al., 1992a, Tamminga et al., 1992). Since then, a significant number of studies have shown consistent increases in basal hippocampal activity in patients with schizophrenia, using SPECT, PET and fMRI imaging respectively (Malaspina et al., 2004, Medoff et al., 2001, Scheef et al., 2010, Talati et al., 2016). In addition, hippocampal hyperactivity has been observed in patients with MCI compared to aged control participants without cognitive impairments (Dickerson et al., 2005, Putcha et al., 2011).

Thanks to more sophisticated MRI technology and analysis techniques (Ekstrom et al., 2009, Malykhin et al., 2010), recent studies have found evidence that there are differences in activity across the different cytoarchitectural subfields of the hippocampus, where increases in cerebral blood volume (CBV) have been identified specifically within the CA1 region of the hippocampus (Schobel et al., 2009b, Talati et al., 2014). Interestingly, CA1 hyperactivity appears

to correlate with progression to schizophrenia from high risk groups and with positive symptoms of the disorder (Schobel et al., 2009b, Schobel et al., 2013). In addition, Tregellas et al. (2014) found increased basal hippocampal activity is associated with a number of cognitive deficits and negative symptoms observed in schizophrenia patients. Subsequently several studies have also shown an increase in hippocampal perfusion in populations who are at high risk of developing schizophrenia, including high schizotypy individuals (Modinos et al., 2018a) and in individuals who hold delusional beliefs (Wolthusen et al., 2018). In addition to the many studies showing increased hippocampal resting activity, task-related activation of the hippocampus has also typically been found to be reduced (Heckers et al., 1998). Reduced recruitment of the anterior hippocampus during memory task has been identified in multiple studies in early psychosis and chronic schizophrenia (McHugo et al., 2019, Jessen et al., 2003, Ragland et al., 2001, Weiss et al., 2004).

To complement human imaging methods, which mostly reveal correlations but not causal relationships, between aberrant hippocampal activity and other neuro-behavioural measures, pre-clinical animal models of hippocampal dysfunction can help to characterise the functional deficits caused by aberrant hippocampal activity. Acute reduction of inhibitory GABA transmission within the ventral hippocampus, by blockade of GABA receptors or pharmacogenetic inhibition of GABAergic neurons causes behavioural alterations related to schizophrenia, such as hyper-locomotor activity

and sensorimotor gating deficits, although these deficits may be strain sensitive (Bast et al., 2001a, McGarrity et al., 2017, Nguyen et al., 2014). In addition acute blockade of ventral hippocampal GABA-A receptors can also cause attentional deficits usually mediated by prefrontal circuits and impairments in hippocampal dependent memory (McGarrity et al., 2017). These data suggest that regional disinhibition could cause aberrant drive of projections, leading to disrupted information processing at distal sites, thereby causing cognitive deficits (Bast, 2011, Bast et al., 2017).

Aims

Hippocampal hyperactivity and GABA dysfunction have been heavily associated with psychiatric disorders (Heckers and Konradi, 2015), but a causal link between disinhibition and metabolic hyperactivity within the hippocampus remains to be demonstrated. Moreover, the impact of hippocampal neural disinhibition on hippocampal projection sites remains to be determined. Therefore, we utilised high resolution multi-pinhole SPECT (Kolodziej et al., 2014, Oelschlegel and Goldschmidt, 2020) combined with ventral hippocampal picrotoxin (GABA-A antagonist) infusions (McGarrity et al., 2017) to examine changes in metabolic activation within the hippocampus and its projection sites caused by hippocampal GABA dysfunction. Increases in cerebral blood flow are correlated with increased metabolic demand and thus higher neuronal activity in that region (Attwell et al., 2010). Therefore, changes in regional cerebral blood flow (rCBF) are

indicative of changes in neuronal activity. The multi-pinhole SPECT method has several advantages over more widely used *in vivo* imaging modalities, including BOLD-fMRI and PET (Oelschlegel and Goldschmidt, 2020). Firstly, fMRI requires animals to be anaesthetised or severely restrained (Steward et al., 2005) and, secondly, PET has a typically lower spatial resolution when using small fields of view, as are used in rodent studies (Rodriguez-Villafuerte et al., 2014), which makes this method less attractive for measuring spatial patterns of neuronal activity in rodents. Using multi-pinhole SPECT permits sub-millimetre spatial resolution (Kolodziej et al., 2014, van der Have et al., 2009), allowing for more accurate spatial representation of neuronal activity. Using this method allowed us to map whole brain wide activity patterns caused by ventral hippocampal disinhibition to understand if and how metabolism within the hippocampus and in distal sites is affected by hippocampal GABA neuronal disinhibition. This includes regions, which not only receive direct unilateral projections, such as the PFC, but also those with reciprocal connections and those sites which have been indirectly linked to hippocampal activity. We hypothesised that picrotoxin infusions cause changes in rCBF both within the hippocampus and in distal sites, which may also explain some of the cognitive and behavioural changes caused by acute hippocampal disinhibition (Bast et al., 2017, McGarrity et al., 2017).

Methods

Subjects

12 male Lister hooded rats (Charles River, Germany, weighing 260-370g, 9-11 weeks old) were used for SPECT studies (see experimental design for a justification of sample size). Rats were housed in groups of 4 in translucent laboratory cages, type IV (Techniplast Deutschland GmbH, Germany) with temperature and humidity control (22 ± 2 °C, $55 \pm 5\%$) and an alternating 12h light/dark cycle (lights on at 0600). Animals had ad libitum access to food and water. All rats were habituated to handling by experimenters for at least 5 days prior to any experimental procedure. All experimental procedures were conducted during the light phase. The experiments were performed according to EU Directive 2010/63/EU for animal experiments and approved by the local ethical committee. All SPECT experiments were carried out at the Leibniz institute for neurobiology, Magdeburg, Germany.

Implantation of guide cannula and drug microinfusion into the ventral hippocampus

Stereotaxic implantation of guide cannula into the ventral hippocampus was performed as in chapter 2, with the exception of a different analgesia used (Metacam, 5mg/ml, 0.02/100g s.c., Boehringer Ingelheim, Germany). After surgery, rats were allowed at least between 4-5 days of recovery before jugular vein catheterisation. During this period rats underwent daily health checks

and were habituated to the manual restraint necessary for drug micro-infusions.

Jugular vein catheterisation

4 or 5 days after ventral hippocampal cannulae implantation, rats were implanted with a silicon catheter (Gaudig Laborfachhandel GbR, Germany; OD: 1.3 mm, ID: 0.5 mm, total catheter length 11 cm into the right external jugular vein under isoflurane anaesthesia as described previously for rodents (Mannewitz et al., 2018, Vincenz et al., 2017). The catheter was filled with catheter lock solution (Cath-Loc HGS, SAI Infusion Technologies, USA) to prevent clogging. After implantation, animals were given at least two days to recover from surgery before they received intra-hippocampal microinfusions followed by SPECT scans.

Intracerebral microinfusion procedure

Drug microinfusions were performed as described previously (chapter 2) and all animals were monitored for behaviour that might be related to seizure development. After hippocampal microinfusion of picrotoxin, but not of saline, we observed wet-dog shakes in each of the 12 animals used in this study within 5mins after the end of infusion, but no other seizure-related behavioural signs (as detailed in chapter 2). These effects were short lived (<10mins) and all rats showed no persistent adverse effects of infusions. For the timing of hippocampal microinfusions in relation to the tracer injection, see Experimental Design.

Preparation of ^{99m}Tc -HMPAO

The ^{99m}Tc -HMPAO injection solutions were prepared from commercially available kit preparations (Ceretek™, GE Healthcare, Germany). 30 mins before the experiments HMPAO aliquots were thawed. Five to ten min before use, the ^{99m}Tc -HMPAO solution was prepared by adding 270 MBq ^{99m}Tc -pertechnetate to the HMPAO-aliquots. 25 μl of a 200 μM SnCl_2 solution was added just after mixing the ^{99m}Tc -pertechnetate solution with the HMPAO-aliquots. The 200 μM SnCl_2 -solution was prepared just before use by adding 50 mg $\text{SnCl}_2 \times 2\text{H}_2\text{O}$ (Sigma-Aldrich, purissimum grade) to 1 L double-distilled H_2O and stirring for 30 sec.

Intravenous injection of ^{99m}Tc -HMPAO

For tracer injection, a Teflon tube (CS-Chromatographie Service GmbH, Germany, OD: 1/16-inch, ID: 0.5 mm), 40 cm in length and filled with 0.9% NaCl, was connected to the jugular vein catheter. A syringe filled with 270 MBq ^{99m}Tc -HMPAO (volume 400-500 μl) was connected to the Teflon tube and placed in a syringe infusion pump (Harvard Instruments, USA). Injections were made at flow rates of 80 μl per min for 7 min. After ^{99m}Tc -HMPAO injection, the Teflon tube and the catheter were cleared with 0.9% NaCl solution for 3 min.

SPECT/CT imaging

Immediately after the completion of intravenous injection of the flow tracer, rats were briefly induced with 4% isoflurane and transferred to the SPECT/CT-scanner and scanned under inhalation anaesthesia (2.0% isoflurane in 2:1 $\text{O}_2:\text{N}_2\text{O}$ volume ratio). A four head

NanoSPECT/CT™ scanner (Mediso/Hungary) was used for co-registered SPECT/CT imaging. SPECT scans of the rats' head were made using nine-pinhole apertures with 1.5mm pinhole diameter and a nominal resolution of < 1.2mm over a scan time of 2 hours. The Axial field of view (FOV) was 38.9 mm. Photopeaks were set to the default values of the NanoSPECT/CT (140 keV +/- 5%) for ^{99m}Tc. SPECT images were reconstructed using the manufacturer's software (HiSPECT™, SCIVIS, Germany) at an isotropic voxel size of 500µm. CT scans were acquired from the same FOV as SPECT scans (55 kVp, 360 projections, 500 ms per projection) and reconstructed with the manufacturer's software (InVivoScope™ 1.43, Bioscan Inc, USA) at an isotropic voxel-size of 200 µm. Each rat underwent two CT scans per imaging session, one before and one after the SPECT scan to control for movement during SPECT scans. Rats were excluded if motion artefacts were detected in the CT images. The CT scans were also used to confirm the cannula placements within the ventral hippocampus.

Experimental design and data analysis

The experiment was run in a within-subjects crossover design, with two counterbalanced series of measurements performed, where possible, resulting in four hippocampal infusions, two of picrotoxin and two of saline, followed by SPECT measurements for each rat. A period of at least 48 hours between SPECT measurements, in combination with drug micro-infusion, was observed for each rat to allow for Technetium-99m decay and for recovery from anaesthesia. The 10 min intravenous tracer injection began 10 min after the end

of the hippocampal microinfusion. The timing of tracer injection was based on our previous electrophysiological measurements to capture the peak effect of the hippocampal picrotoxin infusion on hippocampal neuron firing, which occurs between 10 and 30 min following infusion (McGarrity et al., 2017). A sample size of $n=12$ was chosen to give $>80\%$ power to detect an effect size of $d=1$ at a significance threshold of $p=0.05$ using two-tailed paired t-tests to compare saline versus picrotoxin infusions (power analysis conducted with GPower 3.1, (Faul et al., 2007)). Data analysis was performed using pooled data from the two scans in each infusion condition (saline or picrotoxin), if rats had completed all four scans. 10 rats completed both series of measurements, and for these 10 rats the mean of the two measurements in each infusion condition (picrotoxin or saline) was used for analysis. Two animals completed only one series of measurements, one after picrotoxin and one after saline infusion, due to blockage of the jugular catheters. For the two rats who only completed one series of measurements, the single values from the first series of measurements were used.

SPECT/CT-images were aligned to an MR template based on skull landmarks and the best fit of CT- and MR images for each rat using the MPI-Tool™ software (Advanced Tomo Vision, Germany). The MR template used was a high-resolution (isotropic resolution of 0.15 mm) MR-rat brain data set based on 8 male Lister hooded rats, weighing approximately 300g (Prior et al, 2019 (under revision)). The alignments were based solely on the best fit of CT- and MR images. SPECT-brain data were cut out of the overall SPECT-data using a

whole-brain VOI (volume of interest) with Osirix™ software (Pixmeo, Switzerland) and intensity normalised to the same global mean with the MPI-Tool™ software (Multi-Purpose Image Tool V6.42, ATV GmbH, Kerpen, Germany). For visualisation of the spatial patterns of the average blood flow for each drug condition, global mean normalized SPECT images of all animals in each drug infusion group were combined. For statistical analysis voxel-wise paired t-tests were performed (MagnAn-software, version 2.4, BioCom GbR, Germany) comparing mean tracer uptake between saline and picrotoxin infusions. In accordance with previous small animal radionuclide imaging uncorrected p values are given, and $p < 0.05$ was used as the significance threshold (Endepols et al., 2010, Thanos et al., 2013). SPECT/MR fusions images were produced using Osirix™ software and arranged for illustration using Photoshop™ software (version CS6, Adobe, US).

Results

The CT images averaged over all rats and overlaid on the MR brain template confirmed that the infusion cannula were all placed within the ventral hippocampus (Fig. 6A)

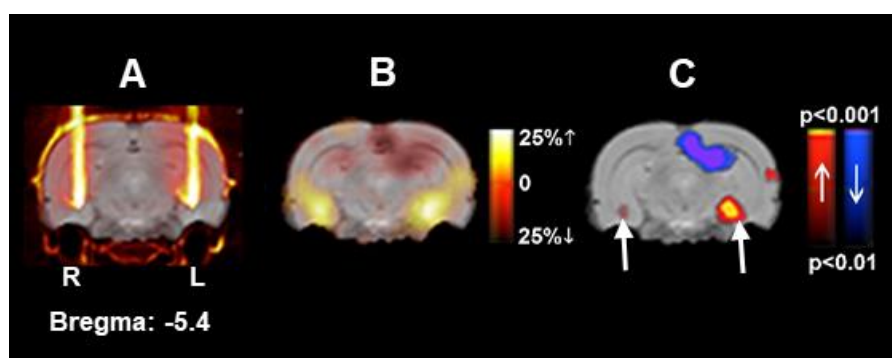


Figure 6. Increased rCBF at the site of neural disinhibition in the ventral hippocampus. **A:** CT overlaid onto a Lister hooded rat MR template indicating cannulae positions and infusion sites in the ventral hippocampus; co-ordinates relative to bregma (mm) are indicated below the CT/MR fusion image. (R/L) indicate right or left hemispheres. **B:** Mean differences in tracer uptake between saline and picrotoxin conditions at the indicated bregma position (-5.4) overlaid on an MR template. Mean increases are represented in yellow (up to 25%) and decreases in red/black (up to 25%). **C:** Shown is a map indicating significant changes in tracer uptake in the picrotoxin infusion condition versus saline infusion (paired *t*-test) overlaid on an MR template. Significant decreases in tracer uptake are represented as blue-purple ($p < 0.01-0.001$) with significant increases shown as red-yellow ($p < 0.01-0.001$). Significant increases in tracer uptake were found bilaterally around the infusion site within the ventral hippocampus (white arrows in C).

Increased rCBF in the ventral hippocampus

Infusions of the GABA-A antagonist picrotoxin as compared to saline infusion, caused an increase in ^{99m}Tc -HMPAO uptake around the infusion site in the hippocampus (Fig. 6B), which reached the

statistical significance threshold ($p < 0.01$) in many voxels bilaterally (Fig. 6C). The increase was somewhat more pronounced in the left hippocampus, where a larger number of voxels showed increases that were significant at $p < 0.01$. The increased rCBF observed at the site of drug infusion was contained within the ventral to intermediate hippocampus, showing no spread to more dorsal parts.

Reduced rCBF in the dorsal hippocampus

In contrast to the metabolic activation in the ventral hippocampus a striking deactivation, which was significant at $p < 0.01$ for several voxels, was observed in the dorsal hippocampus (Fig. 7I), with the deactivation in the left dorsal hippocampus extending further posterior than in the right dorsal hippocampus (e.g., see Fig. 6C where deactivation is evident in left dorsal hippocampus).

Extra-hippocampal activation changes caused by hippocampal picrotoxin infusion

SPECT data also revealed significant activation changes in multiple regions beyond the hippocampus. This included both activations and deactivations across both cortical and subcortical sites. Picrotoxin infusion into the ventral hippocampus caused CBF changes in several extra-hippocampal regions including: ventromedial prefrontal cortex, anterior cingulate cortex, lateral/medial septum, lateral hypothalamus, nucleus reuniens, dorsal raphe nuclei, entorhinal cortex and piriform cortices, anterior olfactory nucleus, olfactory bulb and extended amygdala.

Medial prefrontal cortex

We observed increases in blood flow that reached the $p > 0.05$ significance level in the anterior cingulate cortex (Fig. 7D). These significant activations were found ipsilateral to the more prominent activation in the left ventral hippocampus. In addition, there was also an increase in activation which reached the $p < 0.05$ significance level in the most ventral part of the medial prefrontal cortex, which corresponds to the dorsal tenia tecta and dorsal peduncular cortex (Fig. 7C).

Medial and lateral septum

Ventral hippocampal picrotoxin infusion caused a very marked activation in the septum, including both medial and lateral parts, which extended from the most anterior to most posterior portions of the septum and was significant at $p < 0.01$ in many voxels (Fig. 7D and G).

Hypothalamus and thalamus

We also detected significant activations of the nucleus reuniens and lateral hypothalamus at the $p < 0.05$ and $p < 0.01$ significance threshold (Fig. 7E and H), with the most robust activation of the lateral hypothalamus (significant at $p < 0.01$ for some voxels, Fig. 7H) contralateral to the more pronounced activation in the left ventral hippocampus.

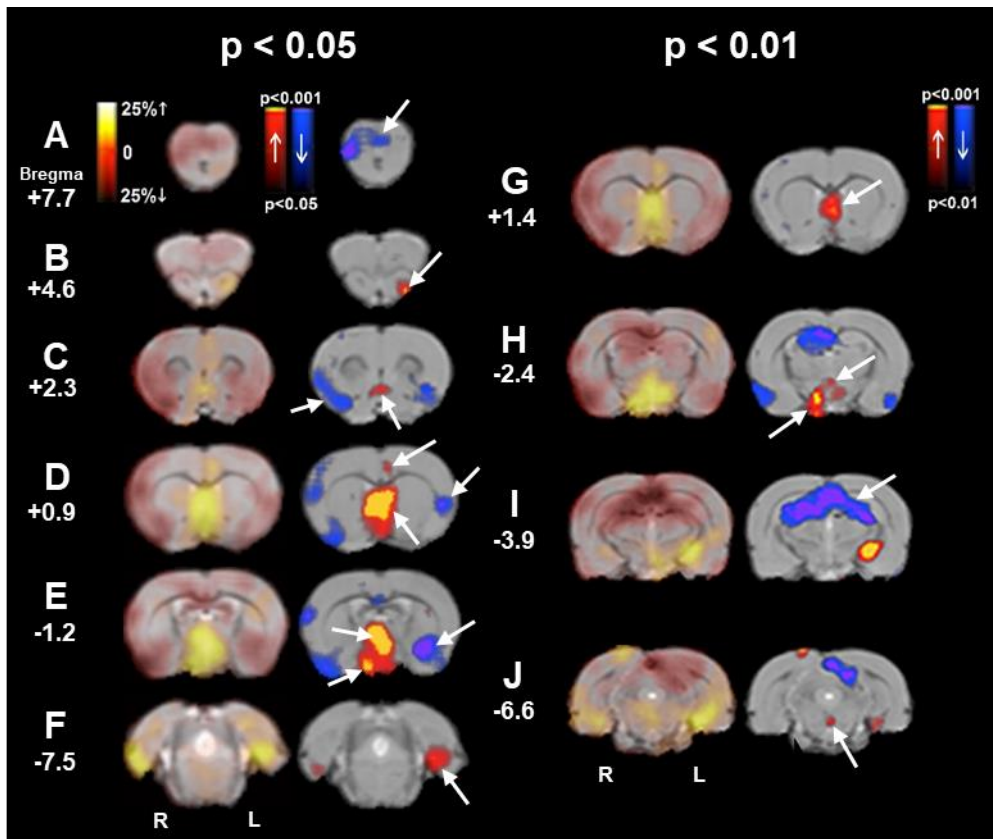


Figure 7. Brain-wide activation changes caused by ventral hippocampal disinhibition. Mean difference and significance maps illustrating differences in tracer uptake between picrotoxin and saline infusion conditions across the brain. Difference maps indicate increased tracer uptake in yellow (up to 25%), and decreased uptake in red/black (up to 25%). Significant increases (paired *t*-test) in tracer uptake in the picrotoxin compared to saline infusion condition are shown in yellow/red with significant decreases shown in blue/purple. Selected p-map sections are shown at either $p < 0.05$ (left) or $p < 0.01$ (right). Sections are arranged from anterior to posterior. Numbers on the left of the sections indicate position relative to bregma (mm); (R) right, (L) left. Significant increases in tracer uptake are seen in the anterior olfactory nucleus (arrow in B), most ventromedial prefrontal cortex (dorsal tenia tecta and dorsal peduncular cortex) (arrow in C), anterior cingulate cortex (arrow in D), lateral septum (arrows in D/G), medial septum (arrow in G), medial thalamus (arrows in E/H), lateral hypothalamus (arrows in E/H), entorhinal cortex (arrow in F) and dorsal raphe nuclei (arrow in J). Significantly decreased tracer uptake is seen in the olfactory bulb (arrow in A), piriform cortex (arrows in C), gustatory cortex (arrows in D), extended amygdala (arrow in E) and dorsal hippocampus (I).

Entorhinal cortex

Hippocampal disinhibition also caused increased bilateral activation in the entorhinal cortex with voxels exceeding the $p < 0.05$ significance threshold (Fig. 7F). Activation was centred on the medial entorhinal cortex and extending into parts of the lateral entorhinal cortex. Activation of the entorhinal cortex was more prominent in the left hemisphere with a greater extent of voxels exceeding the significance threshold.

Amygdala and extended amygdala

Ventral hippocampal picrotoxin also lead to deactivation of a region centred on the interstitial nucleus of the posterior limb of the anterior commissure (IPAC) and extended into adjacent regions of the central amygdala and extended amygdala, reflected by voxels exceeding the $p < 0.05$ significance threshold (Fig. 7E). The deactivation centred on the IPAC was prominent in the left IPAC and extended amygdala, which coincides with the robust activation in the left ventral hippocampus.

Olfactory regions

We also observed significant changes in olfactory regions including a marked bilateral deactivation in the piriform cortex which ranged from the anterior to posterior limits of the structure (Fig. 7C, E, and H). Tracer content in the piriform cortex decreased at the $p < 0.05$ level with peaks at $p < 0.01$ (Fig. 7H). Reductions in tracer content also extend dorsally to the gustatory/granular cortex (Fig. 7D). In addition, we also observed significantly decreased tracer uptake in

the medial and right olfactory bulb ($p < 0.05$) (Fig. 7A) and increased activation centred in the dorso-lateral part of the anterior olfactory nucleus, ipsilateral to the more marked activation in the left ventral hippocampus (Fig. 7B).

Dorsal raphe nuclei

Increased activation at the $P < 0.05$ significance level was also seen in the midline brainstem centred on the dorsal raphe nuclei (Fig. 7J).

Discussion

We used SPECT imaging to reveal brain-wide activation changes caused by hippocampal disinhibition in freely moving rats. Ventral hippocampal disinhibition markedly increased blood flow around the infusion site within the ventral hippocampus, whereas activation within the dorsal hippocampus was markedly decreased. In addition to activation changes within the hippocampus, we observed changes within cortical and subcortical sites. Within medial prefrontal cortical sites, we observed increases in rCBF in areas of the ventromedial prefrontal cortex, anterior cingulate cortex and entorhinal cortex. At subcortical sites, ventral hippocampal disinhibition caused a prominent increase in activation within the septum, the nucleus reuniens, lateral hypothalamic and dorsal raphe nuclei. In addition we observed significant activation changes in olfactory regions, including activation of the anterior olfactory nucleus and deactivation of the olfactory bulb and piriform cortex. In addition, we also observed significant deactivation of the extended amygdala.

Activation in ventral hippocampus and deactivation in dorsal hippocampus

Picrotoxin infusions into the ventral hippocampus caused a significant increase in rCBF around the infusion site in this region of the hippocampus. This finding is consistent with the increased hippocampal burst firing caused by local picrotoxin infusion (McGarrity et al., 2017), although it was not a foregone conclusion

that increased bursting corresponds to increased CBF as revealed by the SPECT findings. This significant increase in activity is localised to the area around the infusion tip, which indicates the infused drug is contained in the ventral region of the hippocampus. The metabolic hyperactivity in the ventral hippocampus caused by disinhibition in the present study resembles clinical findings of hippocampal hyperactivity in schizophrenia and age-related cognitive decline (Bakker et al., 2012, Heckers and Konradi, 2015). This finding is consistent with the idea the hippocampal GABA dysfunction may underlie the characteristic hippocampal hyperactivity seen in these disorders.

Interestingly, in contrast to a significant increase in activity in the ventral hippocampus after picrotoxin infusion, there was a marked deactivation of the dorsal hippocampus. The deactivation in the dorsal hippocampus is seen bilaterally across the entire dorsal hippocampus. This differentiation in activity could be driven by increased feedforward inhibition driven by interneurons that longitudinally project from the ventral to dorsal hippocampus (Sik et al., 1997). In line with the existence of feedforward inhibition from the ventral to dorsal hippocampus, Bast et al. (2009) demonstrated that cytotoxic lesions to the ventral hippocampus increased excitability in the dorsal hippocampus, as reflected by increases in evoked potential in the dorsal dentate gyrus. The contrasting activation changes between poles of the hippocampus observed in this study resemble different activation changes along the hippocampal longitudinal axis that have

been observed in schizophrenia. More specifically, hippocampal hyperactivity is mainly limited to the anterior hippocampus, which corresponds to rodent ventral hippocampus (Schobel et al., 2009b). In contrast, reduced activation was reported in the posterior hippocampus, analogous to dorsal hippocampus in rats, in a cohort of patients with schizophrenia, whereas anterior hippocampal activity was markedly increased in this cohort, reflecting an activity differentiation gradient across the hippocampal structure (Ragland et al., 2017). Our findings suggest the pattern of anterior hyperactivity, alongside posterior deactivation, in the hippocampus of patients with schizophrenia could be driven by disinhibition of the anterior hippocampus.

The differentiation in activation changes across the hippocampal dorso-ventral axis could have important functional significance. The dorsal hippocampus is important for the encoding of accurate place information and of learning about spatial contexts (Bast, 2007, Kjelstrup et al., 2008, Pothuizen et al., 2004, Moser and Moser, 1998). Notably, inhibition of the dorsal hippocampus causes contextual fear conditioning deficits (Esclassan et al., 2009, Wang et al., 2012, Zhang et al., 2014), and this may reflect disruption of the formation of contextual representations (Rudy and O'Reilly, 2001, Matus-Amat et al., 2004). On the other hand, inactivation and activation of the ventral hippocampus disrupts both contextual and cued fear conditioning (see chapter 2 and (Bast et al., 2001b, Maren, 1999, Zhang et al., 2001). In addition, both the dorsal and ventral

hippocampus are necessary for rapid place learning (Bast et al., 2009), and disinhibition of the ventral hippocampus also disrupts rapid place learning (McGarrity et al., 2017). The finding of reduced dorsal hippocampal activity caused by ventral disinhibition raises the possibility that disruption of rapid place learning and contextual conditioning caused by ventral hippocampal manipulations could be in part due to aberrant dorsal hippocampal mechanisms of encoding spatial representations.

Extra-hippocampal changes

Medial prefrontal cortex changes

In addition to activation changes in the ventral and dorsal hippocampus, significant changes in rCBF were identified in cortical and subcortical sites. This included medial prefrontal activations, which exceeded the significance threshold in the anterior cingulate cortex (ACC). These findings are supported by the anatomical connections that run between the hippocampus and prefrontal cortex (Jay and Witter, 1991), which include ventral hippocampal projections to the prelimbic (PL), infralimbic (IL) and ACC (Hoover and Vertes, 2007, Jay et al., 1989). The prefrontal cortex plays an important role in the control of several behavioural functions, including decision making, goal directed learning, behavioural flexibility and sustained attention (Birrell and Brown, 2000, Chudasama et al., 2003, Dalton et al., 2016, Euston et al., 2012, Floresco et al., 2008, Passetti et al., 2002, Pezze et al., 2014).

Significantly, the ACC has been shown to play a key role in mediating cost-benefit decision making in rats (Hillman and Bilkey, 2010, Walton et al., 2003) and in addition, ACC lesions reduce accuracy on the 5 choice serial reaction time task (5CSRT) (Chudasama et al., 2003).

Ventral hippocampal disinhibition also caused activation changes in ventromedial regions of the prefrontal cortex (vmPFC). Specifically, an increase in activation was seen in a region centred on the dorsal peduncular cortex and dorsal tenia tecta (DPP/DTT). Ventral hippocampus projections to the DPP/DTT have been identified (Arszovszki et al., 2014, Cenquizca and Swanson, 2007), but the functional significance of these connections remains unclear. Ventral mPFC lesions, including DP/DTT, cause response errors on the 5CSRT task whilst dorsal lesions caused omissions, which suggests a possible role for the vmPFC in some aspects of cognitive function (Maddux and Holland, 2011). Although, the lesions performed in this study also affected the infralimbic cortex. A recent study suggests the DP/DTT is involved in the sympathetic and behavioural response to stress and possibly integrates emotional and stress information, which is transmitted to the dorsomedial hypothalamus (Kataoka et al., 2020). Further studies are required to characterise hippocampal inputs to these regions and to understand the physiological and behavioural effects of these connections.

Overall, the increase in activation in prefrontal regions observed in the present study could provide a mechanism to explain the deficits in sustained attention caused by hippocampal disinhibition (McGarrity et al., 2017). Therefore, raising the possibility that imbalanced activity in the PFC, caused by hyperactivity in the hippocampus, could contribute to the cognitive deficits seen in psychiatric disorders such as schizophrenia.

Medial and lateral septum activation

A striking area of activation caused by hippocampal picrotoxin was observed across the whole septum, including both the medial and lateral areas, with increases of up to 25% compared to controls. This consistently significant ($p < 0.05-0.01$) increase in activation across the whole structure of the septum contrasts with other regions such as the mPFC, where statistically significant increases were only seen in sections of specific regions. The septum is a key nucleus which receives connections from the hippocampus, amygdala and thalamus, and subsequently projects to the hippocampus, thalamus and ventral tegmental area amongst other regions (Risold and Swanson, 1997). Recent neuroanatomical evidence from mice shows that hippocampal efferents to the lateral septum are significantly denser than those projecting to cortical sites (Tingley and Buzsaki, 2018). Interestingly, a hippocampal dorso-ventral gradient of inputs to the septum exists, whereby more ventral regions of the hippocampus project to the lateral septum, whilst in contrast only sparse dorsal hippocampal

projections to the lateral septum exist (Risold and Swanson, 1996, Risold and Swanson, 1997). The reciprocal connections between the hippocampus and septum are important for maintaining theta rhythms (Hangya et al., 2009), which are implicated in the memory and navigation functions of the hippocampus (Buzsaki and Moser, 2013). This reciprocity provides a key pathway for the hippocampal modulation of sensorimotor functions including locomotor activity (Bender et al., 2015). Locomotor activity is increased by hippocampal disinhibition (Bast et al., 2001a, McGarrity et al., 2017) and hippocampal stimulation by other means (Bast and Feldon, 2003), and our present findings support that this could be driven via the hippocampo-septal pathway. Reducing septal activity by muscimol infusion causes a reduction in the onset of locomotor activity (Wang et al., 2015), and septal connections to the raphe nucleus and VTA might control the onset and speed of locomotion (Fuhrmann et al., 2015). Thus, hyperactivity of the hippocampus could drive increased locomotor activity via the septum and is possibly an important pathway for the expression of other sensorimotor processes.

Hypothalamus and thalamus activation

The increased activation within the lateral hypothalamus following ventral hippocampal disinhibition is consistent with the strong projections from the ventral CA1 region of the hippocampus to the hypothalamic nuclei (Cenquizca and Swanson, 2006, Hahn and Swanson, 2015). Specifically, the ventral CA1 region of the

hippocampus provides the strongest hippocampal projection to the lateral hypothalamus (Cenquizca and Swanson, 2006), whilst the medial hypothalamus is strongly innervated by projections originating across the septo-temporal axis of the subiculum (Kishi et al., 2000). In addition, the substantial increase seen in the lateral hypothalamus could also partly be driven via the lateral septum. Our findings show a marked increase in activity in the lateral septum and the lateral septum has strong bidirectional projections to the lateral hypothalamus (Risold et al., 1997). The significant interconnections between, the hippocampus, septum and lateral hypothalamus produces a neural circuitry that can drive several aspects of behaviour including arousal and motivation (Hahn and Swanson, 2010, Hahn and Swanson, 2012). Recent findings suggest ventral hippocampus-lateral hypothalamus connections can modulate some anxiety related behaviours (Jimenez et al., 2018) and therefore aberrant hippocampal activity could affect the response to anxiogenic stimuli. Interestingly, the prevalence of anxiety related disorders is higher in schizophrenia than the general population (Buckley et al., 2009). The hippocampus, appears to modulate activity of the hypothalamic-pituitary-adrenal axis (HPA) (Herman et al., 2005), a key stress response system, and therefore hippocampal disinhibition could disrupt the response to stressors. Significantly, dysfunctional HPA axis function has been linked to schizophrenia pathophysiology (Walker et al., 2008).

In addition to the strong activation present in hypothalamic areas, we also observed significant activation of the nucleus reuniens (NR)

which is part of the midline thalamic region. The NR is a significant hub between the limbic system and cortical regions, it receives significant afferents from the mPFC (PL, IL, ACC) and ventral hippocampus (CA1 and subiculum) whilst also sending projections to both the hippocampus and mPFC (Bokor et al., 2002, McKenna and Vertes, 2004, Reep et al., 1996, Varela et al., 2014, Wouterlood et al., 1990). Additionally the NR provides a relay for PFC regions to the ventral hippocampus as there are no direct connections between the PFC and ventral hippocampus (Hoover and Vertes, 2012, Vertes, 2002, Vertes et al., 2007). The NR is also innervated by afferents from the hypothalamus, lateral septum and amygdala (McKenna and Vertes, 2004), which may contribute to its hyper-activation in the present study. Studies have shown the NR is critical in working memory tasks which are dependent on both the hippocampus and PFC. Inactivation or lesion of the NR disrupts performance on the delayed-non-match to position task (DNMTP) and radial arm maze task, which are dependent on hippocampal and PFC function (Hembrook and Mair, 2011, Hembrook et al., 2012). Inactivation of the NR can also disrupt behavioural flexibility; infusions of muscimol into the NR lead to increased perseverative spatial responding on a delayed non-match to sample spatial alternation T-maze task (Viena et al., 2018). Alongside the role the NR plays in memory and attention, it has also been shown to be required for hippocampal dependent contextual fear memory (Ramanathan et al., 2018). Considering the behavioural effects of aberrant NR activity and the extensive hippocampal-mPFC-NR circuitry, aberrant NR activation

caused by hippocampal disinhibition may contribute to impairments in important clinically relevant cognitive function, including spatial and working memory and behavioural flexibility. This could include deficits in behavioural flexibility and reversal learning which can be mediated by the NR (Linley et al., 2016), and impairments in these cognitive functions have been identified in schizophrenia patients (Prentice et al., 2008). In addition, work by Zimmerman and Grace (2016) showed that increased activity in the NR led to an increase in VTA neuron activity and enhanced amphetamine induced locomotion, this increase in VTA population activity is also dependent on ventral subiculum activity. Therefore, this suggests the circuitry between the ventral hippocampus and NR is important for the regulation of dopamine neuron firing in the VTA and hyperactivity within this circuit could lead to dopaminergic dysregulation which is a key feature of schizophrenia (Howes and Kapur, 2009). However, we found no evidence for activation of the VTA, reflected by increased rCBF, caused by hippocampal disinhibition in this study. Although, this does not necessarily exclude an effect of ventral hippocampal hyperactivity on VTA dopaminergic neuron activity as the relationship between dopaminergic neuronal activity and hemodynamic responses is debated. Specific activation of midbrain dopamine neurons has only minor effects on neurovascular responses within the mesolimbic dopamine system compared to less non-cell-type specific electrical stimulation (Brocka et al., 2018). Therefore, it is thought neurovascular changes caused by un-specific stimulation of the VTA are mostly mediated by glutamatergic mechanisms, with dopamine

playing a modulatory role (Brocka et al., 2018, Helbing et al., 2016). Consequently, the use of cerebral blood flow measurements in this study may not reveal changes specifically related to midbrain dopamine systems.

Activation changes in the entorhinal cortex

In addition to activation changes seen within the hippocampus, picrotoxin infusions also caused a significant increase in rCBF in both the medial (MEC) and lateral entorhinal cortex (LEC), with highly significant increases more prominent in the medial aspect. The increases in neural activation, as reflected by increased rCBF, are consistent with the strong functional and anatomical connectivity between the hippocampus and entorhinal cortex. Output connections from the CA1 subfield and subiculum provide the strongest innervation of the entorhinal cortex from the hippocampal formation, with the bulk of these connections innervating deep entorhinal layers (Nilssen et al., 2019, Tamamaki and Nojyo, 1995), although there are connections from the hippocampus to the superficial layers of the entorhinal cortex (van Strien et al., 2009). The connections from the hippocampus and subiculum to the entorhinal cortex follow a topographical arrangement whereby ventral hippocampal outputs project to the ventro-medial portion of the entorhinal cortex (Cenquizca and Swanson, 2007, Naber et al., 2001, van Strien et al., 2009, Witter et al., 2000), with the ventral hippocampus preferentially projecting to the medial band of the MEC (Kerr et al.,

2007). The entorhinal cortex is the key interface between the neocortex and hippocampus and plays a substantial role in memory, learning and the assimilation of spatial information (Eichenbaum et al., 2007, Moser and Moser, 2013). There is a general consensus that the MEC predominantly provides visuospatial information input to the hippocampal formation, whilst the LEC provides non-spatial environmental information (McNaughton et al., 2006, Navawongse and Eichenbaum, 2013, Yoganarasimha et al., 2011). However, it has been suggested that this information is integrated within the entorhinal cortex itself (Van Cauter et al., 2013, van Strien et al., 2009). Therefore, aberrant activity in the entorhinal cortex, caused by ventral hippocampal GABA dysfunction, could disrupt the flow of spatial and non-spatial information which could disturb the unified representation of information required for accurate episodic memory formation.

Changes in the amygdala and extended amygdala

Whilst ventral hippocampal disinhibition caused increases in neural activity across a range of brain regions, reduced rCBF was observed in a region of the extended amygdala centred on the IPAC. The IPAC is a dopamine rich region extending from the central amygdala, which forms part of the extended amygdala complex (Alheid et al., 1999, Shammah-Lagnado et al., 2001) and is directly innervated by the ventral hippocampus and subiculum (Groenewegen et al., 1987, Shammah-Lagnado et al., 1999). The IPAC and extended amygdala

have been suggested to be involved in motivational and affective behaviours (Otake and Nakamura, 2003), although the functional role of the IPAC is not well understood. However, neurons in the extended amygdala, including the IPAC, have previously been shown to be activated by the systemic administration of both typical and atypical antipsychotics and antidepressant drugs (Morelli et al., 1999, Pinna and Morelli, 1999, Pinna et al., 2019). In addition, neurons of the IPAC are activated by stress, and this activation is potentially mediated by D1 expressing neurons in the mPFC (Numa et al., 2019). These studies suggest the extended amygdala circuitry plays a role in the mediation of affective disturbances and aberrant activity of the extended amygdala driven by ventral hippocampal disinhibition could be a mechanism which contributes to these deficits.

Activation changes in olfactory regions

We also observed robust activation changes across the olfactory system including deactivation of the piriform cortex across the anterior-posterior axis, increased activation of anterior olfactory nucleus (AON) and deactivation of the olfactory bulb (OB) itself. Ventral hippocampal CA1 axons project to all layers of piriform cortex and dorsal endopiriform cortex through the longitudinal association bundle (Cenquizca and Swanson, 2007), with particularly prominent connections to the posterior piriform cortex (Wang et al., 2020). The piriform cortex is connected to multiple brain regions including the olfactory bulb and amygdala and is seen as 'sensory association like'

cortex (Johnson et al., 2000). The piriform cortex is a 3 layered cortical structure consisting of both GABAergic interneurons and pyramidal cells (Haberly and Bower, 1989, Kapur et al., 1997). GABAergic circuitry has a strong influence on excitatory neurons in piriform cortex (Luna and Schoppa, 2008), and reduction of activation in piriform cortex could be caused by increased local GABAergic influence over pyramidal cells. In addition, the piriform cortex also has significant reciprocal connections to the lateral entorhinal cortex (LEC) and medial entorhinal cortex (MEC) (Agster and Burwell, 2009, Burwell and Amaral, 1998). The entorhinal cortex is strongly connected to the ventral hippocampus and is a gateway for olfactory information to the hippocampus (Kerr et al., 2007). In addition, the LEC provides strong modulation of the piriform cortex; inactivation of the LEC causes increased spontaneous firing in the piriform cortex which consequently disrupts odour discrimination (Chapuis et al., 2013). Therefore, hippocampal disinhibition could disrupt piriform cortex activity through direct innervation, or via modulation of entorhinal-piriform circuitry.

In addition to the deactivation of the piriform cortex, we see a robust deactivation of the anterior region of the olfactory bulb, whilst observing increases in activity in the contralateral anterior olfactory nucleus (AON) caused by hippocampal disinhibition. The hippocampus sends dense projections to the ipsilateral AON (Agrabawi et al., 2016, van Groen and Wyss, 1990) and these projections have a topographical gradient across the ventral-

intermediate hippocampal axis, whereby the most ventral outputs innervate the more medial AON and intermediate outputs terminate at the more lateral areas of the AON (Aqrabawi and Kim, 2018). The AON's location between the olfactory bulb and piriform cortex has led to suggestions it is a key olfactory processing region, with reciprocal inputs from both the piriform cortex and olfactory bulb (Brunjes et al., 2005). Recent studies of the AON have highlighted its role in the processing of olfactory stimuli, where chemogenetic inhibition of the AON enhances olfactory sensitivity and the performance of olfaction-dependent behaviours whereas, AON activation produces the opposite effect (Aqrabawi et al., 2016). In addition, the same study found optogenetic stimulation of ventral hippocampal inputs to the AON impair olfaction-dependent behaviours, which demonstrates a functional-behavioural circuit between the hippocampus and olfactory regions (Aqrabawi et al., 2016).

In addition to hippocampal-AON connectivity, lateral regions of the ventral hippocampus (CA1) innervate the olfactory bulb (Gulyas et al., 1998, van Groen and Wyss, 1990), although this connectivity only equates to roughly 1% of connections to the olfactory bulb (Padmanabhan et al., 2018). In addition, the vast majority of connections to the olfactory bulb come from AON (63%), which includes 58% from the ipsilateral side and 5% from the contralateral AON. The present findings of AON activation and contralateral olfactory bulb deactivation could be driven by glutamatergic projections from AON to olfactory bulb interneurons, or by direct innervation of the olfactory bulb by the ventral hippocampus. AON

projections to the olfactory bulb make connections with OB inhibitory cells and these connections can modulate activity of olfactory mitral cells (Markopoulos et al., 2012). Therefore, the deactivation of olfactory bulb we see in this study could be explained by a mechanism where ventral hippocampal hyperactivity causes increased glutamatergic activity in the AON, which subsequently causes increased inhibitory transmission in the olfactory bulb through strong connectivity between the AON and olfactory bulb interneurons. Additionally, hippocampal glutamatergic projections could connect directly to olfactory bulb interneurons and inhibit neural activity directly, as efferents from the ventral hippocampus appear to terminate in the granule cell layer which is mostly occupied by inhibitory neurons (Cenquizca and Swanson, 2007, Nagayama et al., 2014). These results suggest hyperactivity in ventral hippocampus, caused by GABA dysfunction, can cause downstream effects in olfactory regions due to the connectivity between olfactory bulb, AON, piriform cortex and ventral hippocampus, which in turn could affect contextual information processing associated with sensory information.

In addition to blood flow changes within olfactory regions a decrease in rCBF is seen bilaterally in the gustatory cortex. This region is poorly researched and, in comparison to other sensory systems, such as the olfactory and visual regions, has received little attention. The posterior region of the gustatory cortex receives light input from ventral hippocampal CA1 through the longitudinal association bundle

(Cenquizca and Swanson, 2007) and therefore these connections could be responsible for activation changes seen in the gustatory cortex. As discussed in relation to the olfactory system, hippocampal disinhibition could disrupt gustatory functions which are integral for the representation of taste (Yiannakas and Rosenblum, 2017), although the role of hippocampal-gustatory cortex circuitry remains to be clarified.

Importantly olfactory deficits have been observed in schizophrenia, epilepsy and Alzheimer's disease (Burns, 2000, Kohler et al., 2001, Mathur et al., 2019, Masurkar and Devanand, 2014, Moberg et al., 1999), which have all been associated with hippocampal dysfunction (Nakahara et al., 2018). Furthermore, olfactory hallucinations have been described in psychiatric disorders, including schizophrenia and bipolar disorder; such hallucinations have been relatively overlooked compared to auditory and visual hallucinations, although olfactory-gustatory hallucinations are strongly associated with delusions in patients with bipolar disorder and schizophrenia (Baethge et al., 2005). The robust activation changes we observe in the piriform cortex, anterior olfactory nucleus and olfactory bulb suggests hyperactivity in the ventral hippocampus caused by GABA dysfunction could affect the processing of olfactory information and this could contribute to the olfactory pathology which has been associated with some psychiatric disorders.

Dorsal raphe nuclei activation

Hippocampal disinhibition also caused a significant increase in activation across the dorsal raphe nuclei (DRN). The DRN is a major constituent part of the serotonergic system, containing up to half of the serotonin neurons in the rat brain (Descarries et al., 1982). It is however a heterogeneous region and in addition to 5HT neurons a number of neuronal types are present including; GABA, dopamine and peptidergic neurons (Jacobs and Azmitia, 1992, Kirby et al., 2003, Michelsen et al., 2007). The DRN is not innervated by the hippocampus directly, but strong lateral hypothalamus projections to the raphe nuclei exist alongside inputs from orbital, cingulate, infralimbic and insular cortices (Peyron et al., 1997). The input from the prefrontal cortex is organised topographically whereby more ventral parts of the medial PFC provide stronger input to the DRN than the dorsal regions of the medial PFC (Goncalves et al., 2009). In addition, lateral hypothalamic neurons provide dense innervation of serotonin, rather than GABA, dorsal raphe neurons (Ogawa et al., 2014). The increase in DRN activation after hippocampal picrotoxin infusion we see in this study could be driven through a poly-synaptic route via the lateral hypothalamus. Ventral hippocampal disinhibition causes robust increases in lateral hypothalamic activity and through the dense innervation of the DRN by the lateral hypothalamus this could result in increased DRN activity. The dorsal raphe has been shown to be involved in reward processing and aversive information processing (Li et al., 2013, Nakamura, 2013) and dopaminergic neurons within this structure contribute to behavioural modulation

and salience induced arousal (Cho et al., 2017). Dysregulated aversive conditioning and reward processing have both been identified as features of psychiatric disorders such as schizophrenia (Jensen et al., 2008, Strauss et al., 2014, Waltz et al., 2009) and dysfunctional activity within the dorsal raphe nuclei could contribute to these cognitive impairments.

Conclusion

The present results show that hippocampal disinhibition causes widespread neural activity changes, both within and beyond the hippocampus. The changes in activation within and beyond the hippocampus could help to explain some of the behavioural and cognitive deficits caused by hippocampal disinhibition, including attentional impairments, locomotor hyperactivity and fear conditioning deficits (see chapter 2; (Bast, 2011, McGarrity et al., 2017). The present study suggests ventral hippocampal disinhibition could lead to impairments in clinically relevant functions, due to changes in neural activation at projection sites, such as behavioural flexibility and olfactory discrimination which could be tested in future studies.

Chapter 4: Effect of ventral hippocampal disinhibition on the neurochemical profile of the medial prefrontal cortex as revealed by 1^{H} MRS

Introduction

In the previous chapter, we showed that ventral hippocampal disinhibition increased neural activation, reflected by increased blood flow, in several projection sites of the ventral hippocampus. This included activation changes in distal sites such as the septum, thalamus and medial prefrontal cortex. Importantly, dysfunctional hippocampo-prefrontal connectivity has been linked to cognitive deficits seen in schizophrenia patients (Godsil et al., 2013, Meyer-Lindenberg et al., 2005). In addition, a previous study in rats has shown that hippocampal disinhibition disrupts attentional performance on the 5CSRT test, which is highly dependent on the medial prefrontal cortex but does not normally require the hippocampus (McGarrity et al., 2017). Therefore, understanding the relationship between hippocampal GABA dysfunction and prefrontal cortex activity may help to explain the underlying mechanisms, which cause the manifestation of cognitive symptoms relevant to schizophrenia. To further explore the relationship between hippocampal disinhibition and mPFC function we used *in vivo* proton

magnetic resonance spectroscopy (^1H MRS), which allows for the non-invasive quantification of neurotransmitters and metabolites. The neurochemical profile measured using MRS contains compounds that may act as neurotransmitters, or are components of neurotransmitter metabolic pathways, including glutamate, glutamine and GABA, and compounds involved in energy turnover such as lactate and glucose (Duarte et al., 2012). MRS therefore allows more detailed analysis of the neurochemical mechanisms which underlie changes in neural activity.

Importantly, MRS may serve as a translational bridge between findings in the rat model of hippocampal disinhibition and neurochemical findings in human patients. Localised MRS has been widely used in recent years to investigate changes in neuro-metabolite concentrations in psychiatric disorders, such as schizophrenia (Poels et al., 2014, Wijtenburg et al., 2015). Studies of glutamine and glutamate concentrations in the prefrontal cortex of unmedicated schizophrenia patients have generally reported increases in glutamatergic metabolites (Kegeles et al., 2012, Theberge et al., 2007, Theberge et al., 2002, van Elst et al., 2005). However, a recent meta-analysis identified increased glutamate and glutamine in high risk individuals, but not in first episode psychosis or chronic patients (Merritt et al., 2016). In addition, Reid et al. (2019) reported decreased anterior cingulate cortex glutamate in first episode schizophrenia, although these patients were treated with antipsychotic medication. Notably, increased glutamatergic

metabolites in the anterior cingulate cortex were found to correlate with increased symptom severity (Egerton et al., 2018, Egerton et al., 2012), psychotic symptoms (Bustillo et al., 2014) and treatment resistance (Demjaha et al., 2014, Iwata et al., 2019, Mouchlianitis et al., 2016). Furthermore, an elevated glutamine/glutamate ratio has been observed in the prefrontal cortex of patients with schizophrenia (Bustillo et al., 2010, Wijtenburg et al., 2017), which suggests an increase in excitatory neurotransmission as increased levels of glutamine have been suggested to reflect increased neurotransmission (Marsman et al., 2013). Conversely decreased concentrations of glutamate have been observed in patients with chronic schizophrenia (Kumar et al., 2020, Liemburg et al., 2016, Natsubori et al., 2014, Rowland et al., 2013, Theberge et al., 2007). Reduced glutamate concentrations in chronic patients could be caused by longitudinal brain changes reflecting an excitotoxic mechanism (Miller et al., 2009) or the continued use of antipsychotic medication which has been associated with reduced brain glutamate (Egerton et al., 2017a).

In addition to studies of glutamatergic metabolites, multiple studies have investigated the levels of GABA in the prefrontal cortex of patients with schizophrenia, although these findings have been inconsistent. Some studies reported increased GABA concentrations (Kegeles et al., 2012, Ongur et al., 2010), whilst others reported decreased or similar GABA levels compared to controls in both patient and ultra-high-risk populations (Marsman et al., 2014, Rowland et

al., 2012, Wenneberg et al., 2020) (for review see Egerton et al. (2017b). However, Modinos et al. (2018b) reported increased medial prefrontal GABA concentrations which correlated with increased hippocampal perfusion in individuals who develop psychosis. This suggests hippocampal hyperactivity could mediate alterations in neurotransmitter concentrations in the mPFC and that this may have functional consequences.

In recent years, several studies in rodents have been performed to probe the effects of both systemic pharmacological manipulations and peripheral nerve stimulation on metabolite concentrations using MRS (Crofts et al., 2020, Just et al., 2013, Seuwen et al., 2015, Sonnay et al., 2017). Notably, Iltis et al. (2009) reported an increased glutamine/glutamate ratio in the mPFC after systemic administration of the NMDA receptor antagonist phencyclidine (PCP). In addition, a transient increase in glucose and corresponding decrease in lactate concentration was reported. The study by Iltis et al. (2009) suggests manipulations of excitatory neurotransmission can lead to measurable changes in the neurochemical profile of the mPFC. Similarly, systemic administration of the NMDA receptor antagonist ketamine, at sub anaesthetic doses, also increased glutamine within the PFC (Napolitano et al., 2014). This study also revealed a ketamine induced reduction in PFC GABA concentrations in socially isolated but not normally reared rats, which is a putative developmental model of schizophrenia. Importantly, systemic NMDA receptor antagonism may induce disinhibition and increase neural activation in several

brain regions, including the hippocampus and prefrontal cortex (Grunze et al., 1996, Homayoun and Moghaddam, 2007).

Aims

Here, we use MRS to examine the neurochemical impact of a brain site-specific manipulation using intra-cerebral drug microinfusion (hippocampal disinhibition by picrotoxin infusion) in a distal brain region (mPFC). We aimed to investigate the effect of ventral hippocampal disinhibition on the neurochemical profile of the mPFC. To do this we combined disinhibition of the ventral hippocampus by local picrotoxin infusion (as in previous chapters) and *in vivo* ^1H MRS at 7 Tesla to measure neuro-metabolite concentrations in the mPFC. We hypothesised that ventral hippocampal disinhibition would increase metabolite concentrations that would reflect increased neurotransmission, including changes in the concentration of glutamate, glutamine and GABA or in the ratio of these metabolites.

Methods

Animals

16 male Lister hooded rats (Charles River UK), weighing 270-360g prior to surgery (9-11 weeks old) were used in the present study, of which 14 contributed to the MRS data (for a sample size justification and reasons for exclusion of two rats, please see experimental design). Animal housing and husbandry were as described previously in chapter 2.

Cannula implantation and verification of placements

Ventral hippocampal cannula implantation was performed as described previously in chapter 2, but using MR compatible infusion guide cannula (PEEK, 26 gauge, 8.5mm below pedestal; Plastics One USA) and MR compatible nylon screws (Plastics One, USA). After surgery, rats were allowed at least 5 days of recovery before any further procedures were carried out. During this period, rats underwent daily health checks. At the end of the MRS experiments, rats were transcardially perfused with saline followed by 4% PFA and brains were extracted for verification of cannula placements.

In vivo magnetic resonance spectroscopy combined with hippocampal microinfusions

Animal preparation

Rats were anaesthetised using isoflurane (5%) in a mixture of O₂ and N₂O (ratio: 0.33:0.66L/min). Rats were then transferred to a pre-

warmed cradle and their heads were immobilised using a tooth bar and ear bars. Eye lubricant was used to prevent ocular drying during scanning, and temperature was controlled by a rectal thermal probe and maintained at $37\pm 1^\circ\text{C}$ body temperature via a water-pump system connected to tubing embedded in the base of the rat cradle. Respiratory rate was monitored to provide an index of anaesthetic depth, using an ERT control/gating module (Model 1032, SA Instruments, Stony Brook, USA) and isoflurane concentration was adjusted (1-2.5%) to maintain a stable respiration rate (60 ± 10 breaths per minute).

Intracerebral microinfusion

Stylets were replaced with MR compatible injectors (PEEK, 33-gauge; Plastics One, USA), which extended 0.5mm below the guide cannula tips into the ventral hippocampus. 1- μL syringes (Hamilton, USA) were connected to the infusion injector via flexible Teflon tubing (0.65mm OD x 0.12mm ID; BASi, UK). Injector and infusion tubing were filled with picrotoxin solution or saline before the injector was inserted into the guide cannula. To prevent leakage and drug diffusion before the infusion, the piston of the syringe was pulled back to draw up a 0.25- μL air "plug", separating the infusion solution from the brain's extracellular space. The movement of an air bubble, which was included in the tubing at the end where it was connected to the syringe, was monitored to ensure the solution had been successfully injected into the brain. The air plug (0.25 μl), plus a volume of 0.5 μl /side of either 0.9% sterile saline (control) or picrotoxin

(C₃₀H₃₄O₁₃; Sigma Aldrich, UK) in saline (150ng/μl/side) was infused bilaterally over the course of approximately 90secs. For further details of the timings of microinfusions in relation to the MRS measurements, see experimental design.

¹H MRS acquisition

All experiments were undertaken using a Bruker Biospec 70/30 USR 7T horizontal bore small animal scanner (Bruker, Germany). A surface reception coil (consisting of 4 coils) was placed over the prefrontal cortex. A quick gradient echo acquisition scan was performed to ensure the correct positioning of the area of interest, containing the medial prefrontal cortex, in the centre of the magnet (TE=6ms, TR=100ms). Anatomical reference images were obtained in three orthogonal planes using a rapid acquisition with relaxation enhancement (RARE) sequence (Hennig et al., 1986) (TR=5s, TE=25ms, TE_{eff}=50ms, rare factor=8, flip angle: 180, matrix 256x256 over 40x40mm) with a slice thickness of 1mm sagittal/dorso-ventral, 0.5mm coronal. A voxel of 2 x 2 x 2mm³ was positioned in the medial prefrontal cortex (mPFC) for the acquisition of MRS data (Fig. 8A). Subsequently, a phase difference map was calculated using a gradient echo sequence to map the homogeneity of the overall magnetic field which was used to enhance B₀ homogeneity. Local magnetic field homogeneity was adjusted within the region of interest by applying FASTMAP (Gruetter, 1993). The average water line width for all spectra was 8.72 (±0.51) Hz (full-width half maximum) of the unsuppressed water peak across the

spectroscopy voxel. Localised proton MR spectroscopy was obtained using a Point Resolved Spectroscopy (PRESS) sequence (Bottomley, 1987) over 34 minutes (acquisition parameters: TE= 13.5ms, TR=2000ms, spectral width of 4000Hz with 2048 points and 1024 averages with eddy current compensation) after water suppression with variable power and optimized relaxation delays (VAPOR) (Tkáč et al., 1999).

Processing of MRS data

Quantitative analysis of the ^1H MRS data was carried out using LCModel software (Provencher, 1993) in a fully automated pipeline (Fig. 8B demonstrates an example *in vivo* ^1H MRS spectrum acquired from the voxel positioned within the mPFC). The average signal to noise ratio of the spectra as reported by LCModel was 8.93 ± 1.46 , and the analysis window chosen was 0.2-4.0 ppm. Data were fitted to a basis set containing 21 metabolites: alanine (Ala), aspartate (Asp), creatine (Cr), phosphocreatine (PCr), gamma-Aminobutyric acid (GABA), glucose (Glc), glutamine (Gln), glutamate (Glu), glycerophosphorylcholine (GPC), phosphorylcholine (PCh), glutathione (GSH), *myo*-inositol (*m*-Ins), lactate (Lac), n-acetyl aspartate (NAA), n-acetyl aspartate glutamate (NAAG), *scyllo*-inositol (Scyllo), taurine (Tau), total creatine (Cr+PCr), total choline (GPC+PCh), NAA+NAAG (tNAA) and glutamate + glutamine (Glx). Metabolite concentrations were determined as a ratio to total creatine (Cr+PCr). Cramér-Rao lower bounds (CRLB) was used as a measure of reliability of the metabolite quantification. For the majority of

metabolites a CRLB value under 20% was deemed to indicate reliable spectral fitting (Oz et al., 2014). For some metabolites that exist at low concentrations (Glc, Lac, Ala, Asp), measurements were included if the CRLB value did not exceed 50% (Just et al., 2013, Kreis, 2016), metabolites exceeding this range were excluded from further analysis.

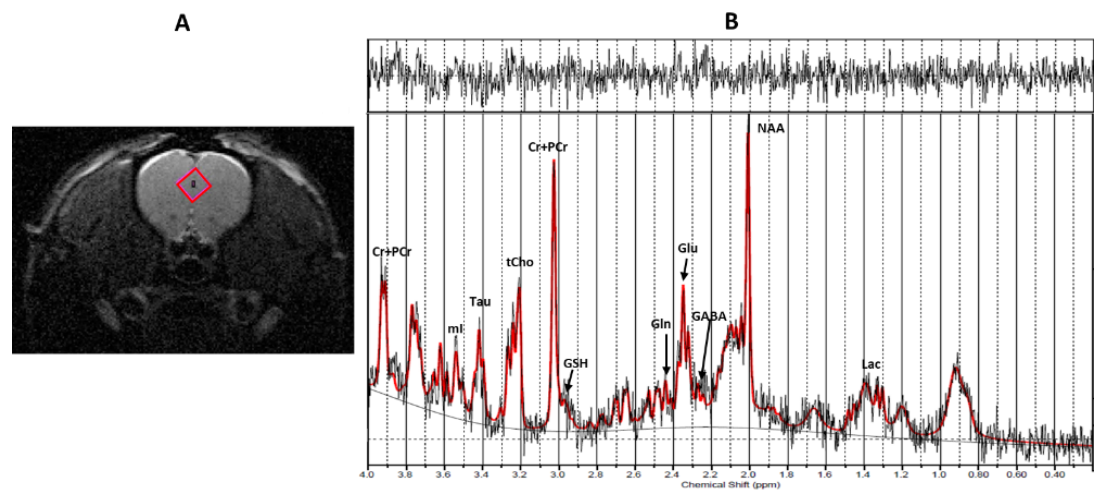


Figure 8. A: Localisation of the voxel of interest ($2 \times 2 \times 2\text{mm}^3$) in the medial prefrontal cortex (red box) used for quantification of relative metabolite concentrations.

B: Example spectrum generated with LCmodel. The bottom panel shows the original spectrum data, represented by the black line, and the LCmodel fitted spectrum shown in red. The top panel shows the residual error obtained after LCModel fitting (original data minus the fitted data). Labelled peaks indicate the following metabolites: Creatine + phosphocreatine (Cr+PCr), Glutamate (Glu), Glutamine (Gln), *myo*-inositol (mI), Taurine (Tau), Total Choline (tCho), gamma-Aminobutyric acid (GABA), Glutathione (GSH), N-acetylaspartate (NAA), Lactate (Lac).

In most acquired spectra *scyllo*-inositol, GPC and NAAG were below the detection threshold (CRLB >20%) and excluded from analysis, whilst lactate was not fitted in twelve individual spectra, glucose in twelve spectra, alanine in eleven spectra, aspartate in one spectrum

and phosphocholine in six spectra. If the concentration of any metabolite was not measurable in any of the four spectra taken in the same rat (i.e. baseline and post infusion in both drug conditions), this rat was then excluded from analysis for this metabolite. The final numbers of rats contributing to the measurements, are indicated for all metabolites measured in table 3.

Experimental design and analysis

The effects of picrotoxin and saline infusions on MRS measurements were compared in a within-subjects design, where each rat received two hippocampal infusions, one with picrotoxin and one with saline, on different days and the testing order was counterbalanced using a cross-over design. Pre-infusion baseline spectra were acquired from the voxel positioned in the medial prefrontal cortex prior to intra hippocampal drug microinfusions. Intra-hippocampal drug infusions took place immediately following the completion of the baseline scan. Post infusion MRS acquisition was performed 10min following the end of hippocampal infusion; the timing of spectral acquisition was chosen based on electrophysiological measures to capture the peak effect of picrotoxin infusion (McGarrity et al., 2017). We aimed for a sample size of at least $n=12$ to give >80% power to detect an effect size of $d=1$ at a significance threshold of $p=0.05$, using pairwise comparisons (power analysis conducted with GPower 3.1 (Faul et al., 2007)). Two rats were lost prior to MRS acquisition due to loss of the cement/cannula head cap, before we reached the target sample size of $n=12$. As we house our rats in groups of 4, we added another batch

of 4 rats, resulting in a final sample size of n=14. Metabolite concentrations were analysed using a 2X2 repeated measures ANOVA with drug infusion condition (saline or picrotoxin) and time (pre-infusion or post-infusion period) as within-subjects factors. In addition, we calculated difference scores, indicating the difference between pre-infusion and post-infusion concentrations in the saline and the picrotoxin condition. The difference score was produced by subtracting the concentration obtained from the pre-infusion baseline spectrum from the post-infusion concentration for each quantified metabolite. Statistical analysis of difference scores for each metabolite was performed using 2-tailed paired t-tests. Statistical significance was considered as $p < 0.05$. All statistical analyses and graphs were produced using GraphPad prism (version 7, GraphPad software, USA) and JASP (JASP Team, 2020).

Results

Cannulae tips were mapped onto coronal sections adapted from the Paxinos and Watson (2006) rat brain atlas. All cannulae tips were located between 4.8 and 6.3mm posterior to bregma within the ventral hippocampus (Fig. 9).

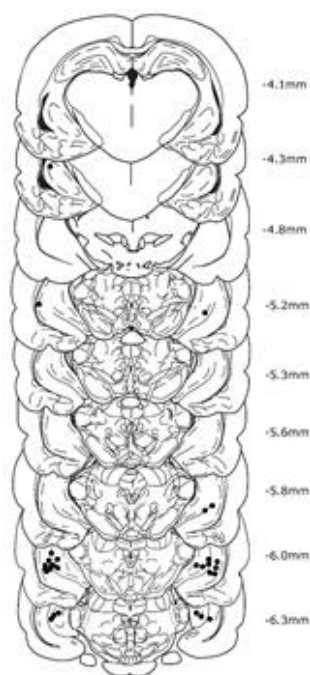


Figure 9. Approximate position of infusion cannulae tips as verified by light microscopy. Placements were mapped onto coronal sections modified from the Paxinos and Watson rat brain atlas. Numbers on the right indicate distance from bregma and black dots indicate cannulae tip location.

Table 1 depicts the average concentration and corresponding CRLB values for the 17 metabolites quantified in this study before and after intra-hippocampal infusion of saline and picrotoxin. Most metabolites (Glu, Gln, Glx, *m*-ins, tCho, NAA, tNAA, tau, Cr, PCr) were quantified with a CRLB value of less than 15%, although glutathione, GABA and phosphocholine had CRLB values between 15 and 20%, which indicates reliable metabolite quantification (Cavassila et al., 2000, Cavassila et al., 2001, Oz et al., 2014). The quantification of alanine, aspartate, glucose and lactate was less reliable with a CRLB greater than 20%.

	Saline Pre-infusion		Saline Post-infusion		Picrotoxin Pre-infusion		Picrotoxin Post-infusion	
	Conc/tCr	CRLB (%)	Conc/tCr	CRLB (%)	Conc/tCr	CRLB (%)	Conc/tCr	CRLB (%)
Ala n=5	0.16±0.03	30.4±6.8	0.17±0.04	32.2±8.0	0.13±0.04	36.8±13.1	0.17±0.03	29.4±5.9
Asp n=13	0.43±0.06	21.3±3.9	0.39±0.09	24.7±8.5	0.43±0.08	20.6±7.0	0.36±0.08	25.8±9.8
GABA n=14	0.29±0.05	18.6±3.8	0.33±0.06	16.4±3.0	0.34±0.06	15.5±3.7	0.31±0.05	16.4±3.7
Glc n=4	0.32±0.11	21.3±9.0	0.28±0.11	26.0±12.8	0.40±0.08	16.8±7.6	0.30±0.10	24.5±13.4
Gln n=14	0.60±0.11	11.5±2.7	0.58±0.08	11.9±2.6	0.63±0.10	10.6±3.1	0.57±0.12	11.7±3.4
Glu n=14	1.76±0.24	3.9±0.6	1.74±0.23	4.0±0.8	1.73±0.29	4.0±0.7	1.71±0.25	3.9±0.7
Glx n=14	2.35±0.31	4.1±0.6	2.32±0.27	4.1±0.7	2.36±0.37	3.9±0.8	2.28±0.34	4.0±0.7
GSH n=14	0.23±0.05	15.0±5.00	0.23±0.06	15.9±5.6	0.24±0.07	15.0±6.7	0.23±0.05	14.3±5.6
Lac n=5	0.25±0.07	29.6±10.4	0.31±0.07	21.6±4.2	0.18±0.02	33.4±9.3	0.33±0.06	19.0±2.0
m-Ins n=14	0.72±0.06	7.4±1.7	0.71±0.09	7.7±1.9	0.74±0.08	6.9±2.3	0.79±0.09	6.5±1.9
PCh n=8	0.18±0.03	18.8±13.0	0.17±0.03	23.6±16.2	0.17±0.03	17.0±14.7	0.17±0.02	19.5±10.1
tCho n=14	0.23±0.03	5.3±1.0	0.22±0.03	5.2±0.8	0.24±0.03	7.2±8.4	0.23±0.02	6.5±5.6
NAA n= 14	1.02±0.07	4.1±0.5	1.03±0.07	4.4±0.7	1.00±0.09	4.1±0.9	1.01±0.10	4.1±0.9
tNAA n=14	1.15±0.09	4.5±0.7	1.16±0.09	4.4±0.5	1.15±0.13	5.1±3.1	1.17±0.09	5.3±2.8
Tau n=14	0.94±0.11	5.7±1.3	0.91±0.07	5.9±1.1	0.94±0.10	5.7±1.8	0.94±0.08	5.2±1.3
PCr n=14	0.52±0.06	12.7±1.6	0.51±0.09	13.6±6.3	0.55±0.10	12.6±6.1	0.56±0.05	11.4±3.4
Cr n=14	0.48±0.06	13.5±3.4	0.49±0.08	12.9±3.0	0.45±0.10	13.1±6.4	0.44±0.05	13.5±6.0

Table 3. Average (mean ±SEM) concentration as a ratio to total creatine (phosphocreatine and creatine) and corresponding CRLB values for each metabolite measured using ¹H spectroscopy for both saline and picrotoxin infusion groups before and after drug infusion.

Figure 10 shows the calculated difference score (post-infusion minus pre-infusion) for each of the metabolites measured in this study in both infusion conditions (saline vs. picrotoxin). Analysis of the difference scores revealed a significant decrease in GABA ($t=2.845$, $df=13$, $p=0.0138$) and a significant increase in *myo*-inositol ($t=2.442$, $df=13$, $p=0.0297$) in the mPFC caused by ventral

hippocampal picrotoxin infusion compared to saline infusion. No further significant differences between saline and picrotoxin conditions were observed for the differences scores of any of the other quantified metabolites (glutamate, glutamine, glutathione, glucose, lactate, N-acetylaspartate, phosphocholine, taurine, alanine, creatine, phosphocreatine, and aspartate) or the glutamine/glutamate ratio (All: $t < 1.78$, $p > 0.149$).

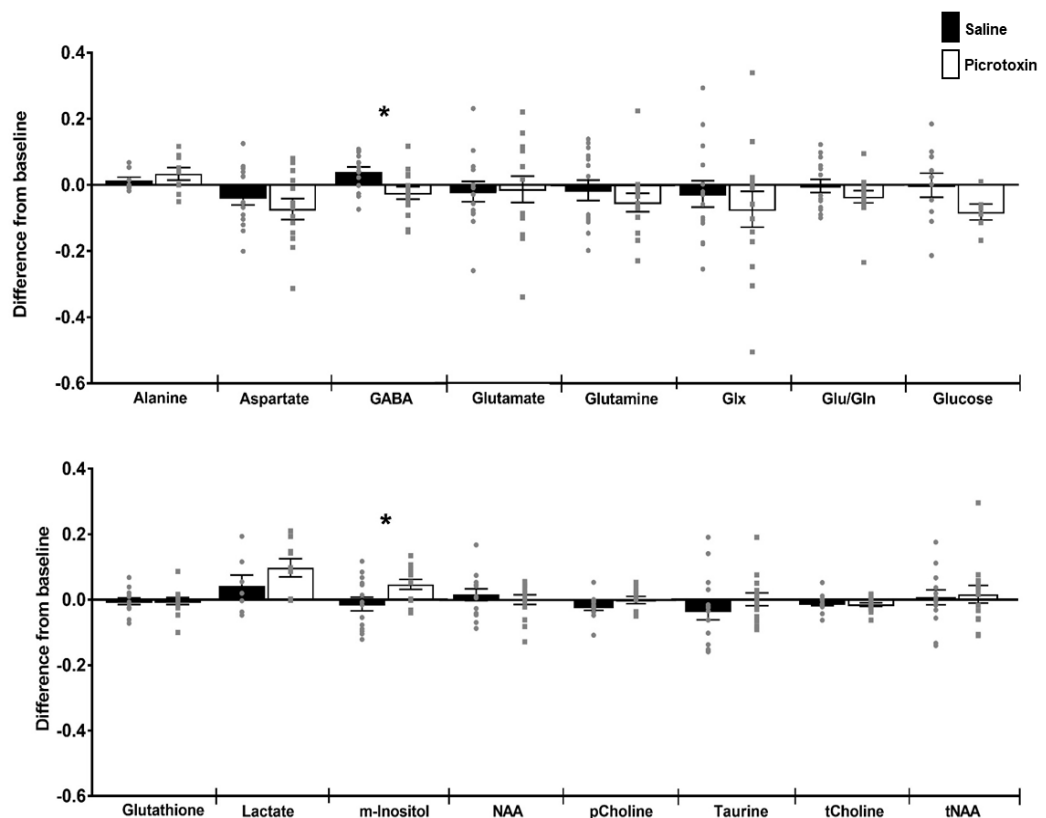


Figure 10. Changes in mPFC neuro-metabolite concentrations induced by ventral hippocampal picrotoxin and saline infusion. Infusion-induced changes are expressed as difference scores, i.e. the difference between the pre-infusion concentrations (as a ratio to total creatine) and the post-infusion concentrations. Black bars represent the difference between pre and post saline infusion, white bars represent the difference between pre and post picrotoxin infusion. Data are depicted as mean (\pm SEM) of $n=14$. There was a significant difference in mPFC GABA and *myo*-inositol concentration between saline and picrotoxin groups (* indicates significant difference between saline and picrotoxin conditions, $p < 0.05$ paired *t*-test).

We further investigated the changes in metabolite concentrations using a 2x2 (drug infusion x time point) ANOVA of metabolite/Cr+PCr concentrations before and after infusion in the saline and the picrotoxin infusion conditions.

Consistent with the analysis of differences scores, this analysis revealed hippocampal picrotoxin significantly decreased mPFC GABA concentrations (Fig. 11A) and increased mPFC *myo*-inositol concentrations (Fig. 11B). The 2x2 ANOVA of GABA concentrations during the pre-infusion baseline and the post-infusion period revealed a significant interaction of drug infusion and time point ($F_{(1, 13)} = 8.094, p = 0.014, \omega^2 = 0.118$). This interaction reflected that GABA concentration increased from pre-infusion baseline to post-infusion scan in the saline condition ($F_{(1, 13)} = 6.842, p = 0.021$), whereas there was no significant change between pre-infusion baseline and post-infusion scan in the picrotoxin condition ($F_{(1, 13)} = 1.672, p = 0.218$). In other words, hippocampal picrotoxin infusions attenuated the increase of GABA from pre-infusion baseline to post-infusion scans that was evident in the saline infusion condition.

The 2X2 ANOVA of *myo*-inositol concentrations revealed a significant interaction of infusion condition and time ($F_{(1, 13)} = 5.962, p = 0.030, \omega^2 = 0.041$). Simple main effect analysis demonstrates that *myo*-inositol concentration significantly increased from pre-infusion baseline to post-infusion scan in the picrotoxin condition ($F_{(1, 13)} = 9.535, p = 0.009$), whereas there was no significant difference

between pre-infusion and post-infusion *myo*-inositol concentrations in the saline condition ($F_{(1, 13)} < 1$, $p > 0.55$). The 2X2 ANOVA of all of the other metabolite concentrations or of the glutamine/glutamate ratio did not reveal any additional interactions or main effects involving infusion condition (all $F < 3.2$, $p > 0.15$).

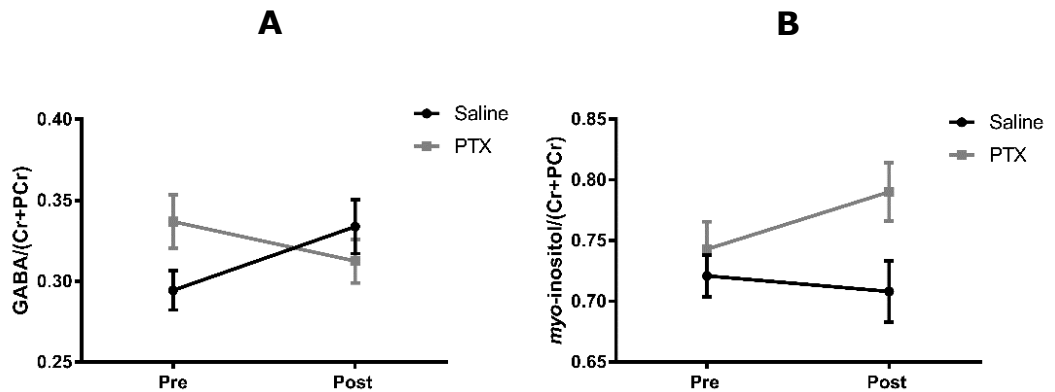


Figure 11. Concentrations (mean \pm SEM) of GABA (**A**) and *myo*-inositol (**B**) as a ratio to total creatine (Cr+PCr) for both drug infusion conditions, saline (black) and picrotoxin (grey), measured before and after hippocampal drug infusion. GABA concentrations increased between pre and post infusion in the saline condition but reduced in the picrotoxin group after infusion. The concentration of *myo*-inositol remained stable after saline infusion, whilst increasing in the picrotoxin group after infusion.

The 2x2 ANOVA also revealed significant overall differences between baseline and post infusion measurements, regardless of infusion conditions, for several metabolites. This included a significant increase in lactate in the post-infusion compared to pre-infusions measurements (Fig. 12A), reflected by a significant effect of time ($F_{(1, 4)} = 26.20$, $p = 0.007$) but no effect of drug infusion condition or interaction of these factors (Drug x Time interaction: $F_{(1, 4)} = 3.176$,

$p = 0.149$, drug effect: $F_{(1, 4)} = 0.45$, $p = 0.539$). It should be noted that it was not possible to fit lactate from all of the acquired spectra: data from nine rats CRLB values for lactate above 50% and were therefore excluded (four from the saline and five from the picrotoxin condition).

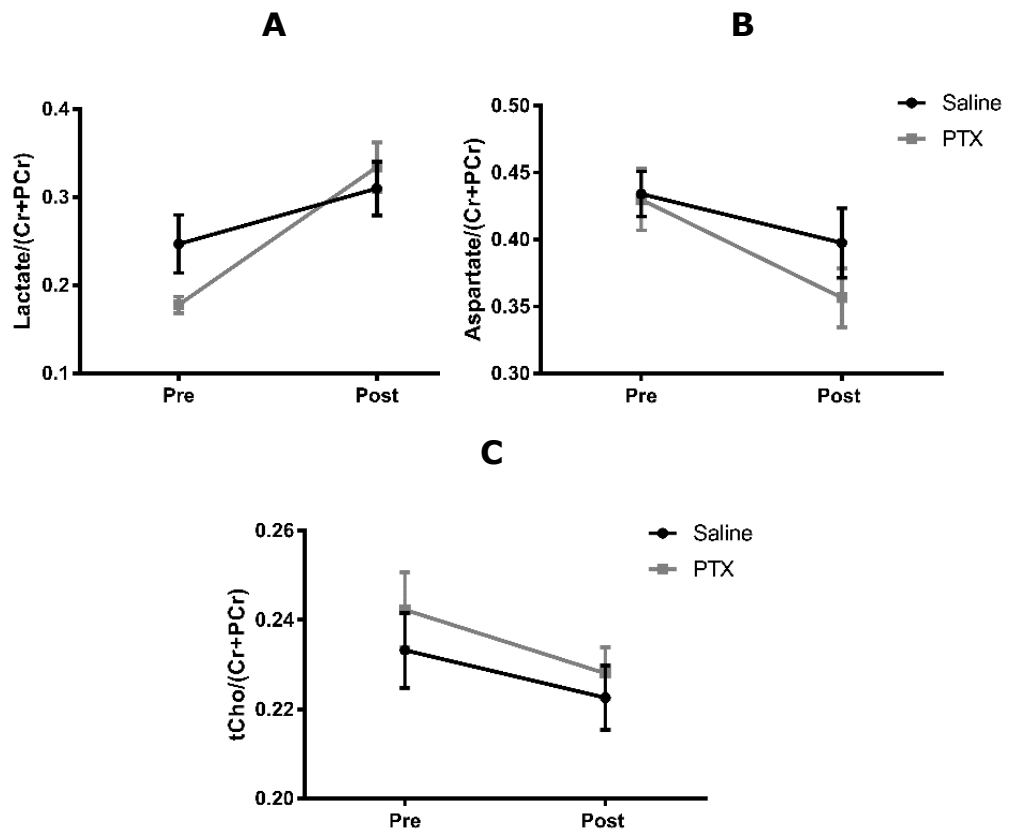


Figure 12. Concentrations (mean \pm SEM) of lactate **(A)**, aspartate **(B)** and total choline **(C)** as a ratio to total creatine (Cr+PCr) for both drug infusion conditions, saline (black) and picrotoxin (grey), measured before and after hippocampal drug infusion. The overall concentration of lactate significantly increased between pre and post measurements with no difference present between infusion conditions. Concentrations of total choline and aspartate in both infusion conditions (saline and picrotoxin) reduced significantly between pre and post infusion measurements.

The concentrations of both aspartate (Fig. 12B) and tCho (Fig. 12C) were significantly lower post infusion compared to pre infusion (tCho: $F_{(1, 13)} = 8.69, p = 0.012$; Asp: $F_{(1, 12)} = 9.24, p = 0.01$) with no effect of drug group or significant interaction of these factors (tCho: Drug x Time interaction: $F_{(1, 13)} = 0.15, p = 0.71$, drug effect: $F_{(1, 13)} = 0.66, p = 0.43$; Asp (Drug x Time interaction: $F_{(1, 12)} = 0.58, p = 0.48$, drug effect: $F_{(1, 12)} = 1.2, p = 0.30$). These changes therefore reflect baseline drift from the pre-infusion to the post-infusion measurement.

In addition, glucose was not fitted from the acquired spectra prior to intra-hippocampal infusion, according to our exclusion criteria, in two animals in the saline group and two animals in the picrotoxin group. Interestingly, after the hippocampal infusion, however, glucose was not fitted for seven animals in the picrotoxin condition, whilst in only 3 animals that received saline it was not possible to accurately fit glucose. This indicates that glucose was fitted with less precision after picrotoxin infusion compared to saline infusion, suggesting a possible decrease in glucose concentrations by hippocampal picrotoxin. However, the lack of sensitivity to gain accurate measurements of glucose after infusion makes drawing precise conclusions challenging.

Discussion

This study investigated the effect of picrotoxin induced ventral hippocampal disinhibition on the concentrations of neuro-metabolites within the mPFC, using short-TE ^1H MRS in anaesthetised rats. Using this method, we were able to compare the concentration of seventeen metabolites before and after hippocampal picrotoxin infusion. The results of this study revealed a significant decrease in mPFC GABA and significant increase in mPFC *myo*-inositol concentrations caused by hippocampal picrotoxin, compared to saline infusion. Hippocampal disinhibition did not clearly affect the concentrations of any of the other measurable metabolites in this study, including glutamate and glutamine, and also did not affect the glutamine/glutamate ratio. Although direct comparison with other studies is difficult due to methodological differences, the concentrations as a ratio to total creatine, and CRLB values of neurometabolites including glutamate, glutamine, *myo*-Inositol, taurine and NAA are consistent with those of several studies using MRS in rodent models at similar magnetic field strengths (Napolitano et al., 2014, Jupp et al., 2020, Vernon et al., 2015, Zhou et al., 2012). For example, metabolites including glutamate and NAA show comparatively high concentrations compared to glutamine, glutathione and GABA. Therefore, using MRS in combination with intra-cerebral pharmacological manipulations it was possible to reliably quantify alterations in concentration of a number of neurotransmitters and metabolites in the PFC.

Hippocampal disinhibition increased prefrontal myo-inositol

Ventral hippocampal disinhibition caused a significant increase in *myo*-inositol concentration in the mPFC. *Myo*-inositol is often seen as a marker of glial cells (Brand et al., 1993) and is considered as a proxy for neuro-inflammation (Chang et al., 2013), although it does have a diverse role in several processes including autophagy (Sarkar and Rubinsztein, 2006), osmotic regulation (Fisher et al., 2002) and intracellular signalling transduction via the phosphatidyl-inositol (PI) pathway (Berridge, 2009, Kim et al., 2005). However, the distribution of *myo*-inositol in the brain is not well understood and while it appears to be present in greater quantities in glial cells (Brand et al., 1993), *myo*-inositol is also present in neuronal cells (Fisher et al., 2002, Novak et al., 1999). Brain *myo*-inositol can be regulated by several processes including by *de novo* synthesis from glucose-6-phosphate (G-6-P), mediated by *myo*-inositol-3-phosphate synthase, recycling via the PI pathway, and uptake from cerebral blood flow via plasma membrane inositol transporters (Rae, 2014). Therefore, increased mPFC *myo*-inositol seen in this study could be due to several mechanisms including, increased uptake from cerebral blood supply, changes in osmotic regulation, and increased recycling via the PI cycle. However, only around 3% of brain *myo*-inositol is taken up from the blood stream (Spector, 1988). and it can take several hours for significant uptake of *myo*-inositol from the bloodstream to the brain (Patishi et al., 1996). Therefore, it is unlikely that uptake from cerebral blood flow accounts for the increased *myo*-inositol concentrations caused by hippocampal disinhibition in this study. It is

also unlikely that increased mPFC *myo*-inositol concentration caused by hippocampal disinhibition reflect changes in osmotic regulation as, in the present study, there were no corresponding changes in NAA concentration, which also acts as an intracellular osmolyte (Taylor et al., 1995). Importantly, the majority of brain *myo*-inositol is derived from recycling inositol containing compounds as part of the PI cycle and *de novo* synthesis from G-6-P (Colodny and Hoffman, 1998, Fisher et al., 1992). Considering the major source of *myo*-inositol in the brain is produced by the PI cycle and many neurotransmitter receptors, including glutaminergic, serotonergic, adrenergic, dopaminergic and cholinergic, are coupled to the PI secondary messenger system (Fisher et al., 1992) it is possible the increase in *myo*-inositol is caused by increased neurotransmission. It has been shown previously that NMDA can potentiate muscarinic receptor stimulated PI turnover (Vadnal and Bazan, 1987) and electroconvulsive stimulation induces IP3 accumulation (Challis et al., 1994), which suggests increased neural activity drives increases in *myo*-inositol concentrations. Therefore, the increase in *myo*-inositol caused by hippocampal disinhibition likely reflects increased PI turnover which is driven by increased mPFC neurotransmission. However, using MRS does not allow for the resolution of metabolites at a cell type specific or metabolic pathway level and, therefore, it is not possible to probe specific mechanisms or pathways, which control the regulation of neuro-metabolites.

Whilst it is unclear which mechanism drives increased *myo*-inositol concentration in the mPFC, this finding is interesting considering changes in *myo*-inositol have been reported in schizophrenia. Increased *myo*-inositol concentrations have been reported in the striatum of first episode psychosis patients (de la Fuente-Sandoval et al., 2013, Plitman et al., 2016), although a recent meta-analysis of mPFC *myo*-inositol levels in schizophrenia found a small overall reduction (Das et al., 2018). However, reduced PFC *myo*-inositol is correlated with depressive symptoms in patients with schizophrenia and major depressive disorder (Chiappelli et al., 2015, Coupland et al., 2005). Therefore, the finding of reduced *myo*-inositol in the PFC may not be exclusive to schizophrenia. Overall, it seems that changes in *myo*-inositol metabolism might have implications in the onset and treatment of psychiatric disorders. Recently, Jupp et al. (2020) identified a functional relationship between ventromedial PFC *myo*-inositol concentration and the control of behavioural impulsivity, whereby rats with higher impulsivity have reduced *myo*-inositol concentrations compared to low impulsive rats. Therefore, the increase in mPFC *myo*-inositol caused by ventral hippocampal disinhibition could have functional consequences related to psychiatric disorders, although the functional effects of this alteration in metabolism are yet to be examined.

Hippocampal disinhibition decreased prefrontal GABA

We also found a significant change in mPFC GABA concentration following hippocampal picrotoxin, compared to saline, infusion. The

difference between infusion conditions was mostly mediated by an increase in GABA concentration from pre-infusion baseline to post-infusion spectra in the saline condition, which was absent in the picrotoxin condition. The increase in GABA from baseline in the control condition is likely due to the effect of isoflurane anaesthesia on the GABAergic pathway. Basal GABA release is increased in cortical nerve terminals by isoflurane anaesthesia (Westphalen and Hemmings, 2006), and the overall concentration of GABA in the brain is increased by isoflurane as measured using *in vivo* MRS (Boretius et al., 2013). However, in the picrotoxin condition, there was no increase in GABA concentration from the pre-infusion to the post-infusion period, if at all, the picrotoxin infusion caused a small numerical (albeit non-significant) reduction in GABA concentration. This suggests that hippocampal disinhibition negatively modulates prefrontal GABA concentrations. Consistent with this finding Iltis et al. (2009) and Napolitano et al. (2014) also reported decreased PFC GABA concentrations caused by systemic administration of NMDA antagonists. NMDA receptor antagonism reduces GABAergic interneuron activity, subsequently leading to increased pyramidal cell activity (Homayoun and Moghaddam, 2007). Therefore, the negative modulation of GABA concentrations by hippocampal disinhibition in the present study may also reflect changes in the excitatory/inhibitory balance within the mPFC.

GABA is synthesised from glutamate by glutamate decarboxylase (GAD 65/67) and is taken up by both GABA neurons and astrocytes

after its release during neurotransmission (Waagepetersen et al., 2007). In neurons, GABA then either re-enters vesicles for neurotransmission or is recycled via the TCA cycle into glutamate and subsequently converted to GABA. Astrocytic GABA is also recycled via the TCA cycle into glutamate, which is converted to glutamine and released into the extracellular space and then taken up by either glutamatergic or GABAergic neurons (Bak et al., 2006, Rae et al., 2003). Two pools of GABA exist in neurons, which are cytoplasmic or vesicular, and most GABA under basal conditions is thought to be in the cytoplasmic pool and is synthesised by GAD67 which is widely localised across the neuron (Dericioglu et al., 2008, Maddock and Buonocore, 2012). However, GAD65, which is localised to nerve terminals, can be upregulated to increase production of the vesicular GABA pool (Patel et al., 2006).

How much of the vesicular pool can be detected using MRS is unclear, and it is likely that GABA measured using MRS mainly corresponds to the large cytoplasmic pool (Maddock and Buonocore, 2012). Therefore, reductions in GABA concentrations as measured using MRS likely reflect a reduction in the overall pool of cytoplasmic GABA. The functional significance of this pool is not clear; it could contribute to replenishing vesicular GABA stores, although some evidence suggests the cytoplasmic GABA pool could be an important source for extrasynaptic GABA release, which plays a role in mediating tonic and phasic GABA activity (Dericioglu et al., 2008, Farrant and Nusser, 2005, Wu et al., 2007). However, the reduction in GABA caused by

hippocampal disinhibition could reflect a reduction in cytoplasmic GABA pools caused by an increase in prefrontal metabolism, whereby GABA recycled via the TCA cycle is used in cellular metabolic processes.

Hippocampal disinhibition did not affect glutamate and glutamine

In contrast to our hypothesis, we observed no changes in glutamatergic metabolism, reflected by stable levels of glutamine, glutamate and the ratio of these metabolite after picrotoxin infusion. Previous studies in rats reported changes in glutamate metabolism after neural manipulations, including increased mPFC glutamine concentrations caused by systemic administration of either PCP or ketamine (Iltis et al., 2009, Napolitano et al., 2014). However, the use of high doses of systemic PCP and ketamine used by Iltis et al. (2009) and Napolitano et al. (2014) make direct comparison to this study challenging. Systemic administration of NMDA antagonists can cause increased activation of pyramidal cells within the PFC, but may also affect distal brain regions and thus metabolite changes caused by systemic NMDA antagonists may be related to activation changes in several regions.

We also might have expected to identify reductions in glucose concentration similar to that reported in human MRS studies, where visual stimulation leads to reductions in glucose concentrations (Mangia et al., 2009). This decrease in glucose concentration is linked to increased utilisation of glucose (Mangia et al., 2009), and

glutamate release is strongly correlated with glucose oxidation (Hyder and Rothman, 2012). In addition, Just and Faber (2019) reported decreased glucose concentrations within the somatosensory cortex upon forepaw stimulation in rats, suggesting increased cortical activity causes transient reductions in overall glucose concentrations. Furthermore, increased lactate concentrations have been reported under trigeminal nerve stimulation within the barrel cortex of rats (Just et al., 2013). In contrast, Iltis et al. (2009) reported transient increases in mPFC glucose concentrations with corresponding reductions in lactate after systemic PCP administration. However, systemic PCP administration can disrupt glucose utilisation (Weissman et al., 1987), which likely accounts for these conflicting findings. In line with a negative impact of hippocampal picrotoxin infusion on mPFC glucose, we were unable to quantify glucose in the majority of rats after picrotoxin infusion. This finding suggests an overall decrease in glucose concentration caused by hippocampal disinhibition as the levels of glucose were below the detection threshold. However, as it was not possible to reliably quantify glucose from all spectra this limits the conclusions concerning the effect of hippocampal disinhibition on mPFC glucose concentrations.

One major difference between this study and that of Iltis et al. (2009) is their use of higher magnetic field strengths (9.4T), which may have enabled more accurate quantification of glucose and lactate as reflected by lower CRLB values (<20%). Although Just et al. (2013) achieved CRLB values more comparable to those achieved in this

study, between 30-40%, for the quantification of glucose and lactate at 9.4T. This was, however, achieved with much shorter acquisition times (10mins) compared with the acquisition time used in this study (34mins). Therefore, to achieve reliable measurements of glucose and lactate concentrations use of spectral editing techniques (de Graaf et al., 2000, Lange et al., 2006) and higher magnetic field strengths are necessary. This notwithstanding, those spectra which enabled the quantification of lactate exhibited a significant increase in concentration over time, with no difference between drug infusion groups. This finding is consistent with the known effects of isoflurane anaesthesia, which has been shown to increase baseline lactate concentrations (Boretius et al., 2013, Valette et al., 2007).

Technical limitations

Whilst using ^1H MRS is a powerful tool which allows for the quantification of a complex neurochemical profile and possible direct translation between pre-clinical and clinical studies, the use of anaesthesia is a confounding factor which has to be considered. The use of anaesthesia is necessary when using rodent models for MRS acquisition to remove movement artefacts and reduce the stress of restraint and significant noise generated by MR scanners (Haensel et al., 2015). However, inhalant anaesthetic regimens such as isoflurane can modulate basal neuronal activity, modify neurovascular coupling and reduce cerebral metabolic consumption of oxygen and glucose (Alkire et al., 1997, Masamoto et al., 2007, Masamoto and Kanno, 2012, Toyama et al., 2004). In addition, isoflurane has the potential

to impact the neurochemical profile measured by MRS (Boretius et al., 2013). Therefore, general anaesthesia used during MRS acquisition could mask infusion related effects on neuronal activity and subsequent changes in metabolism confounding the analysis of spectroscopy data. Nevertheless, previous studies in rodents have identified changes in neuro-metabolites using acute pharmacological manipulations (albeit systemic injections) under anaesthesia (Iltis et al., 2009, Napolitano et al., 2014), and we see some moderate changes in the neurochemical profile acquired under isoflurane in this study. In addition to anaesthesia related effects on neural activity and metabolism, the acquisition of data from the entire PFC may have impacted the findings from this study. Our previous brain-wide SPECT imaging experiments (chapter 3) revealed significantly increased neural activation only in small sub-regions of the mPFC, including the anterior cingulate cortex and the most ventral mPFC. However, the voxel positioning in the mPFC used for MRS acquisition covers a heterogeneous region containing ventral and dorsal prefrontal regions. The duration of 1^{H} MRS acquisition required (~ 34 mins) to achieve sufficient signal to noise for the accurate quantification of neuro-metabolites prevented the analysis of multiple VOIs within the mPFC. Therefore, specific localised changes in metabolism in individual sub regions might not be revealed across the overall voxel and, therefore, any changes in the neurochemical profile may become diluted.

Conclusion

In summary, using ^1H MRS allowed for the non-invasive exploration of the effect of ventral hippocampal disinhibition in the mPFC. In this study we were able to reliably measure a number of neuro-metabolites in combination with intra-cerebral drug infusion. Picrotoxin infusions into the ventral hippocampus increased *myo*-inositol and decreased GABA concentration in the mPFC, whilst having little effect on the rest of the measured neurochemical profile. The cellular mechanisms which cause these changes are unclear, however the findings of this study suggests aberrant hippocampal function may contribute to changes in GABA and *myo*-inositol which have been reported in clinical imaging studies of schizophrenia.

General discussion

The work in this thesis addressed the effect of hippocampal disinhibition (i.e. reduced inhibitory GABA transmission) on cognitive deficits related to the positive symptoms of schizophrenia and on neural activity within and beyond the hippocampus. Clinical imaging studies have shown that hippocampal hyperactivity is a key feature of psychiatric disorders, including schizophrenia, and is thought to be caused by dysfunctional inhibitory neurotransmission (Heckers and Konradi, 2015). In addition, hippocampal hyperactivity is correlated with the severity of the positive symptoms of schizophrenia and the progression to psychosis (Schobel et al., 2013). Therefore, disrupted hippocampal GABA transmission could cause hippocampal hyperactivity and disrupt neural circuits leading to the onset of psychosis. To determine the neural and behavioural effects of hippocampal disinhibition we combined pharmacological manipulation of hippocampal GABA transmission, behavioural testing and neuroimaging approaches. Overall, the key findings of this project demonstrate hippocampal disinhibition had no effect on latent inhibition, but disrupted aversive conditioning; caused prominent metabolic hyperactivity in the hippocampus around the site of drug infusion, plus marked activation changes in projection sites as measured by SPECT; and caused moderate reductions in prefrontal GABA and increases in *myo*-inositol levels as revealed by MRS.

To explore the relationship between hippocampal disinhibition and psychosis symptoms we performed two experiments using a conditioned emotional response paradigm to test the effects of hippocampal disinhibition on aversive conditioning and its latent inhibition (chapter 2). Aberrant allocation of salience to irrelevant stimuli is thought to be the psychological process which causes delusions and hallucinations (Kapur, 2003), and latent inhibition is commonly used in preclinical research to model such deficits (Weiner and Arad, 2009). In the first latent inhibition experiment we performed ventral hippocampal infusions prior to the pre-exposure and conditioning phases of the CER paradigm. The paradigm generated robust LI in controls, whilst ventral hippocampal picrotoxin infusions disrupted conditioning to the light cue as reflected by reduced fear conditioning in the non-pre-exposed animals, which abolished LI in these rats. Ventral hippocampal disinhibition also disrupted contextual fear conditioning compared to controls. However, we found no evidence for disrupted LI due to a ceiling effect of conditioning. These findings are in agreement with previous studies which have shown ventral hippocampal hyperactivity disrupts cue and contextual conditioning (Zhang et al., 2001) and suggest that hippocampal disinhibition may contribute to the impaired aversive conditioning that has been reported in patients with schizophrenia (Jensen et al., 2008).

Following on from this we carried out a second LI experiment, in which we performed hippocampal infusions prior to pre-exposure

only, which would remove any detrimental effect of disrupted conditioning caused by ventral hippocampal disinhibition at conditioning. Ventral hippocampal infusion of picrotoxin prior to pre-exposure resulted in a similar level of conditioning to that in saline infused animals and thus latent inhibition was unaffected. These data suggest that ventral hippocampal disinhibition disrupts associative processing, reflected by reduced cue and contextual conditioning, but does not cause deficits in LI, at least not by disrupting attention or salience processing during pre-exposure. Whilst we found no evidence for the disruption of LI by hippocampal disinhibition, future studies are necessary to further understand the effects of hippocampal hyperactivity on salience processing. One possible future experiment would be to use an appetitive LI paradigm which would remove the confounding effects of hippocampal disinhibition at conditioning and allow for the evaluation of disrupted hippocampal activity on the processing of irrelevant stimuli.

To investigate the relationship between dysfunctional GABA transmission and neural activation patterns within and beyond the hippocampus we used brain-wide SPECT imaging. This technique allows the measurement of spatial patterns of cerebral blood flow, which reflects regional neural activity (Oelschlegel and Goldschmidt, 2020). The findings from our whole-brain neuroimaging analysis (chapter 3) demonstrate that ventral hippocampal disinhibition causes neural activation changes within local hippocampal circuits and in a range of distal sites to the drug infusion site. The metabolic

hyperactivity seen in the ventral hippocampus after picrotoxin infusion resembles the anterior metabolic hyperactivity seen in schizophrenia, and thus provides evidence for a causative relationship between disrupted GABAergic function and hippocampal neural hyperactivity (Heckers and Konradi, 2015). In addition, ventral hippocampal disinhibition caused a significant reduction in neural activity within the dorsal hippocampus, which could have significance when considering the impact of ventral hippocampal hyperactivity on contextual fear conditioning as shown in chapter 2. This deactivation also resembles deactivation of the posterior hippocampus accompanied by anterior hippocampal hyperactivity seen in patients with schizophrenia (Ragland et al., 2017). Furthermore neural activation changes were seen across cortical and subcortical sites which might have relevance to the disruption of cognitive functions in psychiatric disorders. In particular, hippocampal disinhibition caused significant increases in neural activity within regions of the prefrontal cortex consistent with anatomical connectivity between the two regions. Significantly, aberrant hippocampal-prefrontal connectivity may have functional significance as hippocampal disinhibition disrupts prefrontal-dependent attentional processes (McGarrity et al., 2017).

We also observed strong increases in neural activity within the medial and lateral septum, which are again consistent with strong functional and anatomical connectivity (Muller and Remy, 2018, Risold and Swanson, 1997). In addition, hippocampal infusion of picrotoxin

induced neural activation changes within olfactory regions including the piriform cortex and anterior olfactory nucleus. Olfactory deficits are present in early stage schizophrenia and preclinical evidence has demonstrated a link between olfactory processing and ventral hippocampal circuits (Aqrabawi et al., 2016, Moberg et al., 2014). In addition, hippocampal disinhibition also caused significant hyperactivity of the lateral hypothalamus, which could have particular importance considering hippocampal-hypothalamic circuits are implicated in the modulation of anxiety (Jimenez et al., 2018).

Following on from the SPECT studies, we investigated the neuro-metabolic effects that are associated with mPFC neural activation caused by ventral hippocampal disinhibition. To this end, we used ^1H MRS in combination with hippocampal picrotoxin infusions (chapter 4). To the best of our knowledge, there are currently no reported studies which have performed site-specific neuronal manipulations at a distal region to the area of MRS measurements. This technique allows for the measurement of a neurochemical profile including neurotransmitters and energy metabolites. Overall, we were able to robustly acquire a neurochemical profile comparable to previous rodent studies. This study revealed hippocampal disinhibition causes increased *myo*-inositol within the mPFC. As discussed in Chapter 4, this finding may reflect increased recycling of intracellular messenger, which is consistent with increased cellular activity. We also found some evidence for a reduction in mPFC GABA. This change was in part mediated by an increase in GABA concentration in the control group,

probably due to changes in baseline GABA caused by isoflurane anaesthesia (Boretius et al., 2013). However, this increase was not present in the picrotoxin infusion group, suggesting hippocampal disinhibition negatively modulates mPFC GABA concentrations possibly by depletion of the overall metabolic pool of GABA. However, hippocampal disinhibition had little effect on the remaining neuro-metabolites measured in this study including glutamate containing compounds involved in excitatory neurotransmission.

Conclusions

Ventral hippocampal neural disinhibition causes associative processing deficits, reflected by reduced cue and contextual conditioning, and wide-spread neural activation changes both within and beyond the hippocampus. The findings from this study provide evidence of a causative relationship between disrupted GABA transmission and hippocampal hyperactivity which is a key feature of schizophrenia pathophysiology (Heckers and Konradi, 2015). In addition neural activation changes at hippocampal projection sites could help to explain some of the behavioural and cognitive deficits caused by hippocampal disinhibition, including attentional impairments, locomotor hyperactivity and fear conditioning deficits (Bast et al., 2001a, McGarrity et al., 2017). Future experiments would be important to investigate the functional consequences of changes in neural activation caused by aberrant drive of hippocampal projections. Whilst we reported disrupted cue and contextual conditioning mediated by hippocampal disinhibition we found no evidence for an effect on LI. Further studies are necessary to understand the effects of hippocampal disinhibition on salience and attentional processing, considering the hypothesised link between dysfunctional hippocampal activity and psychosis (Katzel et al., 2020). Overall this work adds to the idea that hippocampal neural disinhibition can disrupt behaviours relevant to psychiatric disorders by disrupting neural activity both locally within the hippocampus and at key projection sites.

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Appendices

Appendix 1: Published conference abstracts

Williams S, Hock R, Oelschlegel AM, Goldschmidt J, Bast T (2020)
Brain-wide activation changes caused by hippocampal neural disinhibition: A translational imaging study using SPECT and MRS in a rat model. FENS Abstr. 2959.

Williams S, Gwilt M, Hock R, Stevenson C, Cassaday H, Bast T (2019)
Impact of hippocampal neural disinhibition on latent inhibition in a conditioned emotional response procedure. British Neurosc. Assoc. Abstr. 25: PM004.

Williams S, Hock R, Oelschlegel AM, Goldschmidt J, Bast T (2019)
Brain-wide activation changes caused by hippocampal neural disinhibition. British Neurosc. Assoc. Abstr. 25: PS127.

Appendix 2: Additional work

FENS 2018 abstracts

Poster number: F106

Theme: Animal studies: Pharmacology of learning and memory - part I

A role for the nucleus accumbens in the hippocampal learning-behaviour translation

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The hippocampus is required for important types of rapid everyday learning, including place learning. However, the pathways via which hippocampal place learning is translated into behaviour remain to be determined. The intermediate hippocampus is critical for the hippocampal learning-behaviour translation and combines neural substrates of accurate place encoding with links to prefrontal and subcortical behavioural control sites, which may contribute to this translation (Bast et al., 2009, PLoSBiol; Bast, 2011, Curr Opin Neurobiol). The nucleus accumbens (NAc) is a main candidate due to strong hippocampo-NAc projections that have been implicated in behaviour based on place memory (Humphries & Prescott, 2010, Prog Neurobiol). To examine the role of the NAc, we combined functional

inhibition (via microinfusion of the GABA agonist muscimol) with measurements of behavioural performance based on hippocampus-dependent rapid place learning using the watermaze delayed-matching-to-place (DMP) test (Bast et al., 2009). Electrophysiological and sensorimotor experiments (locomotor activity, startle response/prepulse inhibition) showed that muscimol (125-250ng/0.5 µl/side) reduced NAc neuron firing around the infusion site by about 50% and caused, if at all, only moderate sensorimotor effects. Watermaze Experiment 1, testing the impact of NAc muscimol within-subjects, supported that the NAc is required for DMP performance; however, there appeared to be a carry-over effect, with NAc muscimol eroding the typical DMP search strategy on subsequent test days, possibly reflecting that NAc is required to reinforce the strategy. Watermaze Experiment 2, using a between-subjects design, confirmed that NAc muscimol impairs expression of hippocampus-dependent rapid place learning on the DMP test. Overall, these findings support that the NAc contributes to the hippocampal learning-behaviour translation.

Poster number: P105

Theme: Animal studies: Pharmacology of learning and memory - part I

Too little and too much: impact of functional inhibition and disinhibition of the medial prefrontal cortex on cognitive flexibility assessed using an operant strategy-shifting task

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Attentional performance requires balanced medial prefrontal cortex (mPFC) activity, with functional inhibition or disinhibition (by micro-infusion of GABA agonist muscimol or antagonist picrotoxin, respectively) causing impairments (Pezze et al., 2014, J Neurosci). Cognitive flexibility has also been suggested to require balanced mPFC activity (Brady & Floresco, 2016, JoVE). To confirm this, we examined how mPFC muscimol and picrotoxin affect cognitive flexibility in Lister-hooded rats, using an operant strategy-shifting task involving shifts from egocentric-spatial to cue light-based responses or vice versa (Brady&Floresco,2016). Remarkably, Lister-hooded rats (irrespective of infusion) required three times as many trials compared to previous studies in other strains to shift from egocentric to cue light-based responses (Brady & Floresco, 2016). These slow shifts were unaffected by the prefrontal manipulations.

However, disinhibition, but not inhibition, impaired egocentric-response expression and increased trial omissions, showing that regional neural disinhibition can disrupt functions not normally requiring the disinhibited region (Bast et al., 2017, BrJPharmacol). Additional behavioural studies showed that although Lister-hooded rats struggled to shift from egocentric to cue-based responses, they readily acquired cue-based responses when they had not first been trained the egocentric strategy. Moreover, they then readily shifted from cue-based to egocentric strategy, and could perform egocentric-response reversals. However, there was always a higher shift-cost for egocentric-to-cue than for cue-to-egocentric shifting. Overall, although Lister hooded rats readily acquired both strategies, they overcame the egocentric strategy very slowly, and this slow strategy change was not mPFC dependent. Contrastingly, rats readily performed cue-to-egocentric-strategy shifts; we are currently examining the impact of mPFC functional inhibition and disinhibition on such flexible shifts.

BNA 2019 abstracts

Poster number: PM028

Theme: Learning and memory

Too little and too much: impact of functional inhibition and disinhibition of the medial prefrontal cortex on behavioural flexibility assessed using an operant strategy-shifting task

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Attentional performance requires balanced medial prefrontal cortex (mPFC) activity, with functional inhibition or disinhibition by micro-infusion of the GABA agonist muscimol or antagonist picrotoxin, respectively, causing impairments (Pezze et al., 2014, JNeurosci). Aspects of behavioural flexibility, linked to the mPFC and local GABA transmission (Brady & Floresco, 2016, JoVE), may also require balanced mPFC activity. We examined how mPFC muscimol (62.5ng/side) and picrotoxin (300ng/side), as compared to saline control infusions, affect behavioural flexibility in male Lister Hooded rats, using an operant strategy-shifting task. The task involved 'shifts' (across three sessions) from a spatial-response condition, where food reward was associated with left or right lever responses, to a cue light-based

response condition, where food reward was associated with panel light illumination, or vice versa (Brady&Floresco,2016)(see Figure). Trials to criterion and percentage of correct responses, omissions and errors (perseverative or never reinforced) were analysed by ANOVA using infusion group as between-subjects factor and, if appropriate, shift session as within-subjects factor. Fisher's LSD test was used for post-hoc comparisons. Remarkably, irrespective of infusion, to shift from spatial-response to cue light-based responses, Lister hooded rats required three times as many trials as reported in previous studies in other strains (e.g. Brady&Floresco,2016). These slow shifts were not impaired by mPFC inhibition or disinhibition. However, in addition to increasing trial omissions, disinhibition impaired spatial-response expression and, during shifts, reduced perseveration of the spatial-response condition. Although Lister-Hooded rats struggled with spatial-response-to-cue-response shifts, they readily acquired cue-based responses when they had not first been trained in the spatial-response condition. Moreover, they then readily performed cue-to-spatial-response-strategy shifts. Interestingly, there was a strong trend for mPFC disinhibition to slow down the cue-to-spatial-response-shift, with picrotoxin infused rats showing a lower percentage of correct responses than saline-infused rats during shift session 2. In conclusion, although Lister hooded rats readily acquired both spatial-response and cue strategies they were slow to shift from the spatial-response strategy. This was unaffected by mPFC inhibition or disinhibition. In contrast, they readily performed a cue-to-spatial-

response-strategy shift, and prefrontal functional disinhibition, but not inhibition, tended to disrupt this shift.

Poster number: PS045

Theme: Learning and memory

A role for the nucleus accumbens in the hippocampal learning-behaviour translation

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The hippocampus is required for rapid place learning, but the pathways via which hippocampal place learning is translated into behaviour remain to be determined. The intermediate hippocampus is critical for the hippocampal learning-behaviour translation and combines neural substrates of accurate place encoding with links to prefrontal and subcortical behavioural control sites, which may contribute to this translation (Bast et al.,2009,PLoSBIol; Bast,2011,CurrOpinNeurobiol). The nucleus accumbens (NAc) is a main candidate due to strong hippocampo-NAc projections that have been implicated in behaviour based on place memory (Humphries&Prescott,2010,ProgNeurobiol). We combined NAc functional inhibition in Lister Hooded rats by microinfusion of the GABA agonist muscimol with in vivo electrophysiological measurements around the infusion site to verify neural effects and with locomotor and startle/prepulse-inhibition (PPI) assays to rule out gross sensorimotor impairments (adapting methods from Pezze et al.,2014,JNeurosc). We then tested the impact of NAc muscimol on

behavioural performance based on hippocampus-dependent 1-trial place learning using the watermaze delayed-matching-to-place (DMP) test (Bast et al.,2009), with muscimol infused between trial 1 (learning) and 2 (expression of memory). ANOVA was used whenever the independent variable had more than two levels or to analyse the impact of multiple independent variables. Muscimol (125-250ng/0.5 µl/side) dose-dependently reduced NAc neuronal firing around the infusion site by 30-50% and caused moderate sensorimotor effects. Interestingly, NAc muscimol impaired between-session locomotor habituation. With habituation memory linked to the hippocampus (Fanselow,2000,BehavBrainRes), this is consistent with the idea that NAc is important for hippocampal memory expression. Our first watermaze experiment, testing the impact of NAc muscimol within-subjects, indicated that the NAc is required for DMP performance. However, there appeared to be a carry-over effect: after repeated testing with muscimol, rats failed to show the typical DMP search strategy (persistent searching in correct location) even on drug-free test days, possibly reflecting that NAc is required to reinforce the strategy. A second watermaze DMP experiment, using a between-subjects design to avoid carry-over effects, confirmed that NAc muscimol impairs expression of hippocampus-dependent rapid place learning. Overall, these findings support that the NAc contributes to translation of rapidly-acquired place memory into behaviour.

FENS 2020 abstracts

Poster number: 2960

Theme: Animal cognition and behaviour: Neural circuit mechanisms

Can levetiracetam ameliorate neurocognitive effects of hippocampal disinhibition? In vivo electrophysiological and behavioural studies

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Hippocampal neural disinhibition (decreased GABA function) has been implicated in cognitive disorders, including schizophrenia and age-related cognitive decline (Bast et al, 2017, BrJPharmacology). We previously showed that hippocampal disinhibition in rats by local microinfusion of the GABA-A antagonist picrotoxin disrupts memory and attention, increases locomotor activity, and enhances hippocampal multi-unit burst firing under anaesthesia (McGarrity et al, 2017, CerebCortex). Low doses of levetiracetam, the second-generation antiepileptic, have been shown to decrease both age-related hippocampal neural hyperactivity and cognitive deficits in humans and rodent models (Haberman et al, 2017, Neurotherapeutics). Here, we tested if systemic levetiracetam would antagonize 1) the enhanced hippocampal burst firing and 2) the open-field locomotor hyperactivity caused by hippocampal disinhibition by

picROTOXIN. 1) Using multi-unit recordings under anaesthesia, we replicated that hippocampal picROTOXIN (150ng) enhances hippocampal burst firing. Levetiracetam selectively attenuated the increased burst duration, with this effect being significant at 10mg/kg (i.p.), but not 50mg/kg; 50mg/kg slightly reduced the peak frequency in bursts under baseline conditions. Based on these findings, we used 10mg/kg for the locomotor studies. 2) Levetiracetam (10mg/kg) attenuated locomotor hyperactivity caused by hippocampal disinhibition; this was supported by a strong trend towards an interaction of systemic injection (levetiracetam vs. saline) and hippocampal infusion (picROTOXIN vs. saline) in a 2X2 within-subjects study ($p=0.0523$). Low-dose levetiracetam attenuated some neural and behavioural effects of hippocampal disinhibition. Levetiracetam's impact on clinically relevant cognitive impairments caused by hippocampal disinhibition (McGarrity et al, 2017, CerebCortex) remains to be examined.

Poster number: 1461

Theme: Animal learning and memory

Too little and too much: impact of functional inhibition and disinhibition of the medial prefrontal cortex on an operant strategy-shifting task of behavioural flexibility

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Attentional performance requires balanced medial prefrontal cortex (mPFC) activity, with both functional inhibition or disinhibition (by micro-infusion of GABA agonist muscimol or antagonist picrotoxin) causing impairments (Pezze *et al.*, 2014, *JNeurosci*). Does this finding extend to behavioural flexibility? We examined effects of mPFC muscimol (62.5ng/side) and picrotoxin (300ng/side), as compared to saline-control infusions, on behavioural flexibility in male Lister-hooded rats, using a food-reinforced operant (lever-press) strategy-shifting task (Brady&Floresco, 2016, *JoVE*). The task involved 'shifts' (across three sessions) from a spatial (left/right) response to a cue-light-based response, or vice versa. Irrespective of infusion, shifting from spatial to cue responses required three times as many trials as reported in studies using other rat strains (e.g., Brady&Floresco, 2016). These slow shifts were unaffected by mPFC-

manipulations. However, mPFC-disinhibition increased trial omissions and impaired spatial-response expression.

However, Lister-hooded rats readily acquired cue responses when they had not first been trained to perform spatial responses; then, they readily shifted from the cue to the spatial response. mPFC disinhibition tended to slow this shift, reducing the percentage of correct responses during shift session 2. If mPFC was disinhibited both during initial-rule acquisition and shift-sessions, this also increased perseveration during shifting. In Lister-hooded rats, prefrontal disinhibition impaired aspects of behavioural flexibility, slowing shifting from cue to spatial responses. Rats were slow to shift from spatial to cue responses, which was unaffected by mPFC inhibition or disinhibition. A Bayesian, trial-by-trial analysis of response strategies is ongoing, which may reveal additional, more subtle, effects of prefrontal manipulations on behavioural flexibility.

Appendix 3: PIPS reflective statement

Note to examiners:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

PIPS Reflective Statement

For my PIPS placement I spent 3 months in the experimental psychology department at the University of Oxford where I worked in the group of Professor David Bannerman and Dr Mark Walton. During my time in the department I was working on a project assessing the role of cholinergic interneurons in learning and behaviour. This project was a new venture for the lab which would complement the previous work they had done investigating the role of midbrain dopamine in learning and reward. The aims of the project were to understand how activity of cholinergic interneurons within the striatum effects reward related behaviours and prediction errors. This project was based on previous findings from Dr Stephanie Cragg's lab which highlighted the role that cholinergic interneurons play in mesolimbic dopamine neuron activity and dopamine release. During

my time in the department I worked closely with Dr Lauren Burgeno, a research fellow based in both the Walton and Cragg labs. The project was carried out using *in vivo* techniques in a rodent model. Initially this required training water restricted mice on a two-step probability-based task which had been adapted from a human based task by the lab.

This training period took several weeks due to the complex nature of the task used and necessity for robust behaviour to ascertain useful data. After completion of this training period we injected viruses and implanted fibre optics into the dorsal and ventral striatum to allow for specific recording of cholinergic interneurons during task completion using fibre photometry. Running of the task and data analysis required an understanding of coding using python, and therefore this project allowed me to gain experience in using python to complete data collection. Unfortunately, due to the length of the experiment and time taken to analyse the vast amount of photometry and behavioural data the results of the study were finalised after completion of my placement. However, the skills I gained during this period will be very useful for me going forward in my career.

In addition to the project I was working on, I was also able to observe and learn about the other projects going on in the department which included techniques such as voltammetry and electrophysiological recordings in awake animals. During my time in Oxford I had the opportunity to go to several seminars and talks which helped broaden

my knowledge base and drive my scientific curiosity. I was also able to attend a seminar on 'open science' where a number of speakers, including one of the co-founders of bioRxiv and the CEO of eLife aired their views on various aspects of open access journals, pre-print publications and the publication process in general.

Overall, my time at the University of Oxford was very useful and the placement gave me lots of opportunities to learn new skills such as, surgical techniques, photometry and coding. The placement also gave me the opportunity to interact and learn from a diverse group of people which has given me new insights into a range of scientific avenues. Thanks to my time in the department for experimental psychology, this has helped me to determine that I would like to continue within academia upon completion of my PhD. This is due to my enjoyment of working within a creative environment whereby there is more freedom to explore interesting scientific questions.