Glutamate within the marmoset anterior hippocampus interacts with area 25 to regulate the behavioral and cardiovascular correlates of high-trait anxiety

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Glutamate within the marmoset anterior hippocampus interacts with area 25 to regulate the behavioral and cardiovascular correlates of high-trait anxiety

Abbreviated title: Hippocampus, medial PFC and trait anxiety

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Abstract

High-trait anxiety is a risk factor for the development of affective disorders and has been associated with decreased cardiovascular and behavioral responsivity to acute stressors in humans that may increase the risk of developing cardiovascular disease. Although human neuroimaging studies of high-trait anxiety reveals dysregulation in primate cingulate areas 25 and 32 and the anterior hippocampus (aHipp), and rodent studies reveal the importance of aHipp glutamatergic hypofunction, the causal involvement of aHipp glutamate and its interaction with these areas in the primate brain is unknown. Accordingly, we correlated marmoset trait anxiety scores to their post-mortem aHipp glutamate levels, and showed that low glutamate in the right aHipp is associated with high trait anxiety in marmosets. Moreover, pharmacologically increasing aHipp glutamate reduced anxiety levels in high anxious marmosets in two uncertainty based tests of anxiety – exposure to a human intruder with uncertain intent, and unpredictable loud noise. In the human intruder test, increasing aHipp glutamate decreased anxiety by increasing approach to the intruder. In the unpredictable threat test, animals showed blunted behavioral and cardiovascular responsivity after control infusions, which was normalized by increasing aHipp glutamate. However, this aHipp-mediated anxiolytic effect was blocked by simultaneous pharmacological inactivation of area 25, but not 32 – areas which when inactivated independently reduced, and had no effect on anxiety, respectively. These findings provide causal evidence in male and female primates that aHipp glutamatergic hypofunction, and its regulation by area 25, contributes to the behavioral and cardiovascular symptoms of endogenous high-trait anxiety.
Significance statement (120/120)

High-trait anxiety predisposes sufferers to the development of anxiety and depression. Although neuroimaging of these disorders and rodent modelling implicate dysregulation in hippocampal glutamate and the subgenual/perigenual cingulate cortices (areas 25/32), the causal involvement of these structures in endogenous high-trait anxiety, and their interaction, is unknown. Here we demonstrate that increased trait anxiety in marmoset monkeys correlates with reduced hippocampal glutamate, and that increasing hippocampal glutamate release in high-trait anxious monkeys normalizes the aberrant behavioral and cardiovascular responsivity to potential threats. This normalization was blocked by simultaneous inactivation of area 25, but not area 32. These findings provide casual evidence in primates that hippocampal glutamatergic hypofunction regulates endogenous high-trait anxiety, and the hippocampal-area 25 circuit is a potential therapeutic target.
Introduction

Trait anxiety refers to stable individual differences in how individuals respond to acutely stressful or threatening situations. High-trait anxious individuals are predisposed to develop anxiety and depression (Weger and Sandi, 2018), are more likely to interpret ambiguous stimuli as threatening (Mathews et al., 1989), and show reduced motivation (Ginty et al., 2015). Physiologically, increased anxiety is normally associated with increases in the autonomic indices of fear in response to fearful stimuli. However, a principle diagnosis in the domain of ‘anxiety’ covers a wide spectrum of anxiety-related and comorbid disorders, and while enhanced fear physiology is seen in specific phobia patients, there is increasing evidence that high chronic (trait) levels of anxiety and negative affect are associated with blunted cardiovascular responsivity, and a failure to show the adaptive cardiovascular changes that characterise a healthy ‘fight or flight’ response (Ginty and Conklin, 2011; Brindle et al., 2013; Lang et al., 2014, 2016; Souza et al., 2015). Moreover, the cardiovascular function of high-trait anxious and negative individuals does not discriminate between acutely stressful and non-stressful conditions (Carroll et al., 2017), an adaptive failure that may contribute to the increased incidence of cardiovascular disease in patients suffering from depression and anxiety (Stapelberg et al., 2011, 2012).

Numerous models of high-trait anxiety have been developed to investigate the neurobiology of these disorders. One such model of rodent prenatal stress, links a high-trait/depressive phenotype in the offspring with decreased depolarisation-evoked glutamate release in the ventral hippocampus (vHipp) (Marrocco et al., 2012, 2014). Although the relevance of such models to endogenous high-trait anxiety is uncertain, early life stress is a known risk factor for affective disorders and there is growing evidence that reduced hippocampal glutamatergic function contributes to affective disorder pathophysiology. Certainly hippocampal glutamatergic hypofunction correlates with depressive illness duration (Block et al., 2009; de Diego-Adelino et al., 2013), and disrupted glutamatergic
signalling pathways are associated with anxious/depressive behaviors in mice and humans (Tordera et al., 2007; Garcia-Garcia et al., 2009; Duric et al., 2013). However, these findings conflict with studies of non-anxious rodents in which reductions in vHipp glutamate function are anxiolytic in ethological tests of anxiety (see Barkus et al., 2010), and the anxiolytic effects of hippocampal lesions in non-anxious primates (Chudasama et al., 2008, 2009). Thus, there may be fundamental differences in how hippocampal glutamate contributes to healthy and high-trait anxious/affective disorder states. The hippocampus modulates such behavior, in part, via a network of medial PFC (mPFC) brain regions including Brodmann's area 25 and area 32, and changes in anterior hippocampal (aHipp, the primate equivalent of the vHipp) connectivity with the mPFC are implicated in affective disorders in humans (Dickie et al., 2011; Hamilton et al., 2011; Treadway et al., 2015). Rodent studies also highlight the importance of vHipp-mPFC connectivity in the control of anxiety-related behavior (Adhikari et al., 2010; Padilla-Coreano et al., 2016). However, the contributions of hippocampal glutamate, and hippocampal-mPFC circuitry in the behavioral and cardiovascular symptoms of endogenous high-trait anxiety remain unknown.

We have recently demonstrated the utility of a marmoset model for probing the behavioral and cardiovascular correlates of mPFC function and conditioned fear (Wallis et al., 2017), and described a subpopulation of marmosets with an endogenous high-trait anxiety phenotype that is associated with impaired discriminative Pavlovian conditioning, blunted amygdala serotonin function and reduced dorsal anterior cingulate cortex volume (Shiba et al., 2014; Mikheenko et al., 2015). However, the contribution of hippocampal glutamate to this phenotype is unknown. Consequently, the first aim of the present study (Experiment 1) was to assess whether reduced post-mortem hippocampal glutamate levels predicted heightened responsivity of a marmoset cohort to an unknown human. Having established a negative correlation between hippocampal glutamate and such responsivity, the second aim (Experiment 2) was to determine if hippocampal glutamate and high-trait anxiety were causally related. Thus, we used anatomically-specific intracerebral infusions
to increase presynaptic hippocampal glutamate release in high-trait anxious monkeys and assessed its effectiveness in ameliorating the behavioral and cardiovascular correlates of negative emotion in two anxiety tests – the human intruder paradigm which measures uncertainty-based behavior in the homecage, and an unpredictable threat test presented in an automated test apparatus which measures real time behavioral and cardiovascular responses. We then determined whether this amelioration utilised hippocampal-mPFC circuitry.

Methods

Animals

All the marmosets (Callithrix jacchus) used in these studies were bred on site at the University of Cambridge Marmoset Breeding Colony, and were housed in male/female pairs (males were vasectomised). They were kept in a 12 hour light-dark cycle (lights-on at 7am, lights-off at 7pm) in a controlled environment of 22 ± 1°C in temperature and 50 ± 1% humidity. Their cages contained a variety of environmental enrichment aids including boxes to play in, and suspended ladders, wooden branches and ropes to climb and swing on. Animals were fed a varied diet including fruit, rusk, malt loaf, peanuts, eggs, sandwiches and weekend treats and they had ad libitum access to water. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and the University of Cambridge Animal Welfare and Ethical Review Board.

Experiment 1

Hippocampal Neurochemical Quantification.

To assess the contribution of hippocampal neurochemistry to the high-trait anxious phenotype, stored hippocampal tissue from 12 marmosets (6 male, 6 female) with a range of anxiety scores on
the human intruder test (described below) was analyzed. Behavioral and cardiovascular data from these marmosets have previously been published elsewhere (Shiba et al., 2014). After euthanasia, the brain was removed and the anterior two-thirds of the left and right hippocampus was visualised, immediately dissected out and flash frozen into liquid nitrogen prior to storage at -80°C. For analysis, these tissues samples were thawed on ice and homogenised with 500 μl of Lysis Buffer NL from the Qiagen Qproteome Nuclear Protein Kit, before 250 μl of this sample was analyzed using reversed phase high performance liquid chromatography (HPLC) and electrochemical detection for both monoamines and glutamate as described previously (Tsai et al., 1997; Clarke et al., 2004). Briefly, monoamines were separated on a C18 silica-based analytical column (10 x 4.6 mm ODS3, Hypersil, Phenomoex, UK) using a mobile phase consisting of 13.6 g/l KH₂PO₄·H₂O, 185 mg/l octane sulphonic acid, 18% methanol (pH 2.75) delivered at 0.8 ml/min by a dual piston pump. Detection was achieved electrochemically using a dual electrode analytical cell and electrochemical detector with electrode 1 set at -150 mV and electrode 2 set at 180 mV with reference to a palladium reference electrode. For the measurement of glutamate, 6 μL of each sample was reacted with an equal volume of a derivatizing agent containing o-phthalaldehyde and β-mercaptoethanol for 2 min at room temperature. The derivatizing solution was prepared daily by diluting 1 mL of stock solution containing 27.5 mg o-phthalaldehyde, 1 mL methanol, 5 μL β-mercaptoethanol and 9 mL 0.4 M potassium tetraborate buffer (pH 10.4) in 3 mL of potassium tetraborate buffer. Glutamate was detected by HPLC and fluorescence detection (CMA 280) with excitation and emission wavelengths of 315–370 nm and 395–545 nm, respectively. Separation was achieved at 24°C using a Hypersil ODS 5 μm analytical column (80 × 4.6 mm; HPLC Technology, UK), and a mobile phase consisting of 100 mm Na₂HPO₄ and 30% methanol, adjusted to pH 6.38 with orthophosphoric acid, which was delivered at 1 mL/min. Before use, the buffer was filtered through a 0.2 μm filter under vacuum. The HPLC systems was calibrated using standards containing known amounts of monoamines and glutamate. Data were acquired on-line and the signals were integrated using Chromeleon software.
The resulting monoamine and glutamate levels of each animal were then compared to their anxiety scores.

Experiment 2

To determine whether increases in hippocampal glutamate activity could ameliorate the behavioral and cardiovascular responses associated with high-trait anxiety, we performed two behavioral experiments on marmosets that had been characterised as high anxious (described below). In Experiment 2a we investigated the effects of selectively increasing aHipp glutamate levels on behavioral performance in the human intruder test in six animals (3 males, 3 females). In Experiment 2b, in 6 animals (4 of which also contributed to Expt 2a; 2 females, 4 males), we investigated the behavioral and cardiovascular effects of selectively increasing aHipp glutamate levels in an unpredictable threat test, and also investigated the contribution of mPFC regions 25 and 32 to these effects (see Table 1).

To allow the selective anatomical manipulation of either the aHipp and/or areas 25 and 32, all ten animals in Experiment 2 underwent an aseptic surgical procedure to implant intracerebral cannulae targeting either the aHipp alone, or the aHipp and areas 25 and 32. Of these, the six animals who also undertook Experiment 2B, had another surgery to implant a telemetric blood pressure monitor into the descending aorta. Both surgeries were completed prior to the animals beginning any behavioral testing.

Cannulation Surgery

Marmosets were pre-medicated with ketamine hydrochloride (Vetalar; 0.05 ml of a 100 mg solution, i.m.; Amersham Biosciences and Upjohn, Crawley, UK) before being given a long lasting non-steroidal, anti-inflammatory analgesic (Carprieve; 0.03ml of 50mg/ml carprofen, s.c; Pfizer, Kent, UK). They were intubated and maintained on 2.0–2.5% isoflurane in 0.3 l/min O₂ and placed into a
stereotaxic frame modified for the marmoset (David Kopf, Tujunga, CA). Pulse-rate, O₂ saturation, breathing rate, and CO₂ saturation were all monitored by pulse-oximetry and capnography (Microcap Handheld Capnograph, Oridion Capnography Inc., MA, USA), and core body temperature was monitored by a rectal thermometer (TES-1319 K-type digital thermometer, TES Electrical Electronic Corp., Taipei, Taiwan). Cannulae (Plastics One, Roanoke, VA) were implanted into area 25 (double 7-mm-long cannulae, 1 mm apart, anteroposterior (AP) + 14, lateromedial (LM ± 0.5), area 32 (double 2mm long cannulae, 1mm apart; AP +17; LM ± 0.5 at a 30° AP angle) and the anterior hippocampus (double 15-mm-long cannula, 1 mm apart, AP + 6, LM ± 5.75/7.75, ventral + 5) with coordinates adjusted where necessary in situ according to cortical depth (Roberts et al., 2007). Postoperatively, and when fully recovered, all monkeys were returned to their home cage and then received the analgesic meloxicam (0.1 mL of a 1.5 mg/mL oral suspension; Boehringer Ingelheim) for 3 days after which they had at least a further 10 days recovery. Cannulae were cleaned every week (and caps and cannula blockers changed) to ensure the cannula site remained free from infection.

Telemetry Surgery

Animals were anaesthetised as before, the descending aorta was visualised within the abdominal cavity and the probe catheter of a telemetric blood pressure transmitter (Data Sciences International [DSI], St. Paul, MN, USA) implanted into the aorta as described previously (Braesicke et al., 2005). All monkeys received meloxicam as before in addition to prophylactic treatment with amoxicillin and clavulanic acid (Synulox; 50mg/ml solution, Pfizer, Kent, UK), for one day before and 6 days after telemetry surgery.

Drug Infusions

Approximately once a week prior to behavioral testing, animals received infusions of drug or vehicle down the cannulae. The use of chronically implanted cannulae and acute infusions allowed animals to act as their own controls and reduced experimental variation caused by inter-subject differences.

For all sterile central infusions the marmoset was held gently in a researcher’s hand, the caps and
cannula blockers were removed from the guide, and the site was cleaned with 70% alcohol. A sterile
injector (Plastics One) connected to a 2 μl gas tight syringe in a syringe pump was inserted into the
guide cannula for a 2 minute drug infusion. Following the infusion, the injector was left in place for a
further minute to allow the drug to diffuse before injector removal. Sterile cannula blockers and
caps were replaced and the marmoset was returned to its homecage for the relevant pre-treatment
time. To increase glutamate in the hippocampus 1μl of a mixture of the mGlu2/3 receptor antagonist
LY341495 (1ng/μl; Tocris Bioscience, UK), and the GABA\textsubscript{A} receptor antagonist, CGP52432 (1ng/μl;
Tocris Bioscience, UK) – the ‘LY/CGP cocktail’ – was bilaterally infused at a rate of 0.5 μl per minute,
with a 15 min pre-treatment time (Marrocco et al., 2012). These receptors both act to limit
presynaptic glutamate release, and their antagonism therefore acts to increase the amount of
glutamate released. To inactivate areas 25 or 32 we used the GABA\textsubscript{B} agonist muscimol and the
GABA\textsubscript{B} agonist baclofen (0.5 μl of 0.1 mM muscimol [Sigma, UK]/1.0 mM baclofen [Sigma, UK])
infusion at a rate of 0.25μl/min and a pre-treatment time of 25 mins (‘musbac’). Saline infusions
acted as vehicle controls.

Human intruder test

Defining a high anxiety trait

At approx. 2 years of age, high-trait anxious marmosets were identified according to their
performance on a human intruder test – a well-validated test of uncertainty-based anxiety in
primates (Carey et al., 1992; Oler et al., 2009; Fox et al., 2010; Mikheenko et al., 2015). In the
marmoset version of this test, the monkey is confined to the upper right hand quadrant of their
cage, away from their cage-mate, and a video camera focused on their quadrant to allow their
behavior to be quantified post-test. After an 8 min habituation period, an unfamiliar human
(wearing an unfamiliar latex human face mask) enters the room, stands 30 cm away from the
quadrant front and maintains eye contact with the monkey for two minutes (Agustín-Pavón et al.,
Because the intent of the unknown human is unclear, e.g. may provide treats or may try and hold/catch the monkey, the uncertainty tends to induce anxious behavior. There are many behaviors that can be analyzed in this paradigm, but one of the most relevant is the location of the animal within the cage. In contrast to normal animals, high-trait anxious marmosets characteristically retreat from the human intruder to the back (increased average depth from intruder) and/or the top (increased average height from intruder) of the cage, and therefore have a high score for time spent at the top/back of the cage and a very low score for time spent at the front (TSAF) of the cage. In particular, TSAF has been shown to be sensitive to anxiolytics (Carey et al., 1992; Santangelo et al., 2016) and marmosets with a TSAF score of less than 9% (based on the lower confidence interval of the median of 63 naïve marmosets tested on the human intruder) have been shown to display stable characteristic trait anxious behaviors and blunted serotonin signalling when compared to marmosets that scored over 25% TSAF (upper 95% confidence interval) (Mikheenko et al., 2015). Thus, those animals with a TSAF score of less than 9% were defined as high anxious in this study.

Drug treatments

To investigate whether increasing hippocampal glutamate release altered anxiety behaviors in the human intruder test, five high-trait anxious animals performed this test on an additional 3 occasions, a minimum of a week apart (4 animals had cannulae in areas 25, 32 and the aHipp, 2 animals had cannulae in the aHipp only). The first and third tests were presented after aHipp saline treatment while the middle test was presented after aHipp LY/CGP treatment. This treatment order allowed us to determine whether any drug effects were being confounded by habituation that might occur upon repeated exposure to a human intruder. To help minimize habituation and to ensure the intruder was novel in appearance on each occasion, the same intruder wore a different human rubber mask as a disguise for each test. These masks were counterbalanced between animals,
covered the whole head and hair of the experimenter and were close fitting so they did not compromise the ability to maintain eye contact.

Human Intruder Analysis

Behavior during the human intruder test was recorded and subsequently scored by an experimenter who was blind to the drug infusion used. During the 2 minute intruder period the time (seconds) that the animal spent in particular locations within the cage in terms of height (floor, low, medium, high, top of nestbox or average height) and depth (front, middle, back) was recorded as a percentage of the total time, alongside the number of jumps towards the intruder and the amount of time spent locomoting around the cage (number of seconds). Additional measures included species specific behaviors namely, the number of seconds spent head and body bobbing (a marmoset behavior indicative of anxiety (Carey et al., 1992; Agustín-Pavon et al., 2011; Santangelo et al., 2016)) and the number of various types of vocalizations based on differences in sound, length and frequency range. These include the tsik call, a proactive alarm call used either singly or repetitively in mobbing situations in response to a potential intruder, tse and egg calls which are both indicative of less proactive alarm/anxiety and are associated with vigilance behavior, and combinations of calls such as tsik-egg and tse-egg which may serve to coordinate mobbing, vigilance and anxiety between group members (Bezerra and Souto, 2008). All these behavioral variables have previously been shown to contribute to an animal’s overall response to a human intruder (Agustín-Pavón et al., 2012b), and some have been shown to load onto a single factor that was calculated from an exploratory factor analysis (EFA) that was carried out on human intruder test scores obtained as part of a screening procedure on 171 marmosets from the University of Cambridge Marmoset Breeding Colony. This EFA was used to predict the extent to which the different behaviors in the human intruder test are driven by any underlying latent variables. The EFA included: TSAF, TSAB, average height, proportion of time spent in locomotion, number of bobs, egg calls, tsik
call, tsik-egg calls, and tse-egg calls. In total, nine factors were identified but based on the point of inflection on a scree plot, a single factor was extracted accounting for 39.7% of the total variance in behavior. See table 2. The major behaviors that contributed to this factor were the percentage times spent at the front and back of the cage, the average height of the monkey within the cage, and the amount of head and body bobbing, tse-egg calls and locomotion. The minor contributory behaviors were the number of tsik-egg and egg vocalizations, and the influence of the tsik calls was negligible. Specifically, animals who scored highly on this composite score spent more time at the back of the cage and high in the cage, indicating a greater avoidance of the human intruder. They also spent more time head and body bobbing, less time locomoting and made fewer tsik vocalizations and more egg/tse-egg vocalizations, all signs of anxious behavior in marmosets (Carey et al., 1992; Bezerra and Souto, 2008; Agustín-Pavón et al., 2012a; Santangelo et al., 2016). Thus, the pattern in which the items cluster on this factor are all indicative of anxious behavior and suggest that these different behaviors are acting together to drive one underlying composite behavior that represents an animal’s overall anxiety towards the human intruder. This composite anxiety score was therefore calculated for each of the high anxious animals on each occasion they performed the human intruder test in this study using z scores derived from their performance as a group.

*Unexpected threat paradigm*

Behavioral testing using the unexpected threat paradigm took place within a custom made sound-attenuated testing chamber in a dark room. This apparatus enabled the measurement of real-time cardiovascular activity via a receiver situated directly under the animal. Animals were trained to enter a transparent Perspex carry box (l=21.5, w=19, h=25 cm), with air holes and a removable clear Perspex door in which they were transported to the behavioral test apparatus. The Perspex carry box was placed inside the test chamber, and the marmoset remained inside this box at all times during testing. The test chamber was lit by a 3W bulb (houselight), located in the middle of the ceiling of the chamber and contained a computer-controlled speaker and a siren generator (120dB;
Biotronix, Cambridge) through which 75dB auditory stimuli and a 117dB siren (mildly aversive loud noise) could be played. The apparatus was controlled by the Whisker control system (Cardinal and Aitken, 2010) and in-house software. Three video cameras were positioned in the test chamber so that the activity of the animal within the Perspex box could be recorded by video software (CyberLink, Power Director, CyberLink Corp), and directly observed by the experimenter during testing.

The aim of this test was to expose marmosets to a particular context with unpredictable threat (aversive loud noise) and then to determine their reactivity to a novel, unfamiliar cue presented within this unpredictable threat context. In order to investigate the effect of a variety of central brain manipulations on their reactivity to unfamiliar cues a repeated block design was used involving a 5-day cycle of sessions per block. In each session, the animal was positioned inside the Perspex carry box within the testing apparatus and the houselight was on. For the first 4 unpredictable threat days, animals were placed in the familiar environment of the testing box for 25 mins and presented intermittently with an aversive sound (unconditioned stimulus (US)) and an auditory cue. Each auditory cue was 20 sec long, 75dB and was presented 12 times with an intertrial interval (ITI) of 40-80 sec. Each US was a 0.4 sec, intrinsically aversive 117dB siren (Agustín-Pavón et al., 2012b) and was also presented 12 times per session with an ITI of 40-80 sec. The threat of the aversive US was unpredictable as there was no temporal relationship between the auditory cues and USs, thus the best predictor of the aversive noise was the context, namely the test apparatus. On the ambiguous cue probe (5th day), monkeys were presented with a novel, 20 sec, 75dB auditory cue presented 12 times with an ITI of 100-180 sec within this same threatening context. No USs were presented. All drug manipulations occurred on these ambiguous cue probe days. After the cue probe day, the next cycle then started with the novel auditory cue from the prior ambiguous cue probe day incorporated into the unpredictable threat training.
This paradigm therefore measures the response to an uncertain, novel stimulus in an environment associated with the unpredictable threat of a noise stressor (see Figure 3A). However, as the meaning of the novel cue on the probe session is ambiguous, it is potentially threatening – a potential that is amplified by it being presented within an environment associated with the risk of unpredictable threat. By including an unpaired auditory cue on the unpredictable threat sessions, it also becomes part of the context (although to a lesser extent than the testing apparatus), and prevents the marmosets learning that the every auditory cue presentation (the probe days) is associated with no US presentation. Performing the drug manipulations on the cue probe day therefore allows the investigation of the components of this acute stress response in the absence of the noise stressor itself.

Unexpected threat analysis

For cardiovascular analysis, blood pressure (BP) data was continuously transmitted by the implanted probe to a receiver (RPC-1, DSI), located beneath the testing apparatus. By comparison with an ambient pressure reference monitor (APR-1, DSI), a calibrated pressure output adapter (R11CPA, DSI) converted the absolute pressure into a gauge pressure in mmHg. The data was then collected, analyzed and stored offline using an analogue/digital converter (micro 1401, Cambridge Electronic Design (CED)) and data acquisition software (Spike2 Version7.01, CED). Any outliers and recording failures in the data were removed (blood pressure values above 200 mmHg or below 0 mmHg, or other abnormal spikes). Data collection was reliable overall, but data gaps of less than 0.4s were replaced by cubic spline interpolation and gaps of more than 0.4s were treated as missing values. Systolic and diastolic BP events were extracted as local maxima and minima for each cardiac cycle and used to calculate the mean arterial pressure (MAP; diastolic blood pressure + \frac{1}{3} (systolic blood pressure − diastolic blood pressure)), and interbeat intervals used to calculate HR and HRV (the root-mean-square standard deviation [RMSSD] measure of the time difference between
consecutive IBIs), for the 20 sec baseline and 20 sec aversive cue periods using custom-written code (RN Cardinal) and the HRV Toolkit (https://physionet.org/tutorials/hrv-toolkit/).

As HRV is determined by the interval between heartbeats it reflects how flexible the heart is in responding to internal or external stressors. Decreased HRV is associated with anxiety disorders and depression as well as cardiovascular disease and cardiovascular mortality. HRV reflects the balance between the parasympathetic (i.e. vagal) and sympathetic autonomic cardiac regulation and baroreflex activity and assesses the distinct contributions of vagal and sympathetic activity. Poincaré plots (plot of IBI$_{j+1}$ as a function of IBI$_j$) were created and the SD of the points perpendicular to the line of identity (SD1), and the SD of the points along the line of identity (SD2) (70) were then used to derive indices of autonomic activity. There were the cardiac vagal index (CVI), that represents the parasympathetic component of cardiac activity, and the cardiac sympathetic index (CSI), that represents sympathetic activity as well as some parasympathetic and baroreflex activity (Toichi et al., 1997; but see Rahman et al., 2011). For each trial, the mean parameter of interest was calculated for the 20 sec aversive cue presentation and for the immediately preceding 20 sec baseline (BL) period (the last 20 seconds of the preceding intertrial interval). Thus, during each cue presentation, the cue-directed HR was calculated as (HR during the cue) – (HR during BL). HRV was also assessed during each 20 sec baseline and cue period. This was possible because of the high resting heart rate of marmosets ensuring an adequate number of IBIs (approximately 100) were gathered across the two sampling periods (Toichi et al., 1997).

The normal healthy cardiovascular response to an ambiguous cue in a potentially threatening environment would be to show a heightened stress response to the potential threat, an increase in HR and CSI and a withdrawal of CVI leading to a transient reduction in HRV in preparation for fight or flight (de Geus et al., 1990; Boutcher and Stocker, 1996; Pagani et al., 1997). However, this cardiac responsivity is not seen in high trait anxious individuals in response to acute stress, who instead show no differences in cardiovascular function between the stress condition and the baseline. This
has been referred to as ‘cardiovascular blunting’ (Ginty and Conklin, 2011; Brindle et al., 2013; Levine et al., 2016; Carroll et al., 2017).

The behavior measured during the unexpected threat paradigm was vigilant scanning (VS), which is defined as a watchful scanning of surroundings accompanied by tense, vigilant body posture (Mikheenko et al., 2010; Agustín-Pavón et al., 2012b). The time the animal spent engaged in this behavior during the 20s cue period and 20s BL period was scored. Cue-directed VS was calculated as the difference between these two (synonymous to cue-directed HR). Studies of healthy individuals indicate that an acute mild stress response is normally associated with an increase in cue-directed VS as the animal engages with the potential threat (Agustín-Pavón et al., 2012b). However, high-trait anxious individuals show deficits in motivational behaviors and task engagement that could be considered a ‘behavioral blunting’ (Carroll et al., 2017).

A second person blind to the conditions of the experiment scored a subset of the discrimination sessions. Interscorer reliability was high ($R_{22} = 0.76, P < 0.001$).

**Experimental design and Statistical Analysis**

Glutamate levels from the 12 monkeys in Expt 1 were correlated to their anxiety-related behaviors using parametric bivariate correlation analyses in SPSS v22 (IBM, NY, USA) for both left and right hippocampi. Human intruder test data from Expt 2a were analyzed using repeated measures ANOVA and post-hoc paired (within-subjects) t-tests in SPSS v22 (IBM, NY, USA) using factors of test (saline 1, aHipp LY/CGP or saline 2). Cardiovascular and behavioral data from the unpredictable threat paradigm (Expt 2b) were analyzed in R version 3.2.2 (R Core Team, 2016) using repeated measures ANOVA and post-hoc paired (within-subjects) t-tests from the lmerTest package, and type III sums of squares with the Satterthwaite approximation for degrees of freedom. Factors included area (levels including aHipp, area 32, area 25 and combinations) and drug (including LY/CGP, saline and musbac). Although MAP was analyzed throughout, it was highly variable and was not statistically affected by
any of the manipulations used in the present study. MAP data is therefore not presented. In all cases
n=5 or above gives a high statistical power-to-subject ratio while minimizing the number of animals
used.

Post mortem assessment of cannulae placement

Animals were premedicated with ketamine hydrochloride (Vetalar; 0.05 ml of a 100 mg solution, i.m.; Amersham Biosciences and Upjohn, Crawley, UK) before being euthanized with pentobarbital sodium (Dolethal; 1ml of a 200mg/ml solution, i.v.; Merial Animal Health, Essex, U.K.). Animals were then perfused transcardially with 500ml 0.1M PBS, followed by 500ml of 4% paraformaldehyde fixative solution, over approximately 15 minutes. The brain was removed and left in the 4%
paraformaldehyde fixative solution overnight before being transferred to 30% sucrose solution for at least 48 hours. Brains were then sectioned on a freezing microtome (coronal sections; 60μm), mounted on slides, and stained with cressyl fast violet. The sections were viewed under a Leitz DMRD microscope (Leica Microsystems, Wetzlar, Germany). The cannula locations for each animal were schematized onto drawings of standard marmoset brain coronal sections and composite diagrams were then made to illustrate the extent of overlap between animals.

Results

Experiment 1:

Increased trait anxiety is associated with reduced glutamate levels within the primate right anterior hippocampus (aHipp).
To assess the contribution of aHipp neurochemistry to the trait anxious phenotype, glutamate and monoamine levels within the right and left aHipp of marmosets (from 17) were compared to the composite anxiety score (as determined by factor analysis) and the individual behaviors displayed in response to an unknown human. Hierarchical linear regression revealed that right aHipp glutamate levels were negatively correlated with the composite anxiety score of the animals, with a higher composite anxiety score correlating with reduced aHipp glutamate (EFA score: $R_{12} = -0.601$, $P = 0.039$).

Detailed inspection of correlations between right aHipp glutamate levels and individual performance variables revealed that reduced right aHipp glutamate was associated with the animals moving further away from the human intruder. Thus reduced glutamate was associated with an increased amount of time spent at the back of the cage and/or on the top of the nestbox – the furthest location possible from the intruder ($R_{12} = -0.649$, $P = 0.022$), and a trend towards an increase in average height (calculated from percentage time spent on the floor, low, middle and high locations within the cage; $R_{12} = 0.533$, $P = 0.075$). Reduced aHipp glutamate was also associated with increases in the amount of head and body bobbing – an anxiety related behavior ($R_{12} = -0.641$, $P = 0.025$), but no changes in the time spent at the front of the cage ($R_{12} = -0.430$, $P = 0.163$), the amount of locomotion ($R_{13} = 0.384$, $P = 0.217$) or in the types of vocalizations made in response to the intruder ($tse$, $R_{12} = 0.209$, $P = 0.514$; $tse$-egg, $R_{12} = 0.515$ $P = 0.087$; $tsik$, $R_{12} = 0.280$ $P = 0.3$; $tsik$-egg, $R_{12} = 0.309$ $P = 0.328$; egg, $R_{12} = -0.026$ $P = 0.925$).

These relationships were not seen with glutamate levels in the left aHipp, as adding left aHipp glutamate to the regression model predicting EFA did not improve the fit ($F_{1,9} < 1$, NS). The conclusions were also unchanged by analyzing according to the alternative sequence: left aHipp glutamate did not predict the EFA score ($F_{1,10} < 1$, NS) but adding right aHipp glutamate to this model significantly improved the fit ($F_{1,9} = 5.8319$, $p = 0.039$). Consistent with this, left aHipp glutamate did not correlate with average height ($R_{12} = -0.0.39$ $P = 0.904$), time spent at front ($R_{12} = -0.123$, $P = 0.703$), or back/top of nestbox ($R_{12} = -0.241$ $P = 0.450$) bobbing ($R_{12} = -0.007$ $P = 0.982$), any
Furthermore levels of DA, 5-HT and NA from both the left and right aHipp did not correlate with any of these individual behaviors or the composite anxiety score. However, both 5-HT and NA from the right aHipp, and DA from the left aHipp showed a positive correlation with the time spent at the back of the cage (5-HT, R₁₂ = 0.678 P = 0.015; NA, R₁₂ = 0.656 P = 0.021; DA, R₁₂ = 0.678 P = 0.015; see Table 2). These findings indicate that while overall levels of trait anxiety can be selectively linked to reduction in right aHipp glutamate, other neurochemicals may also contribute to specific avoidance behaviors within this test.

Experiment 2A

Pharmacologically increasing presynaptic glutamate release within the aHipp reversed the high anxiety phenotype on the human intruder test

Pharmacologically boosting aHipp glutamate levels in high-trait anxious monkeys significantly altered their anxiety-related behavior in response to the human intruder, but did not affect all behaviors equally. Bilateral infusion of aHipp LY/CGP did decrease the composite anxiety score (EFA) indicative of a decrease in anxiety. However there was also a tendency for EFA scores to show habituation between the first and second saline infusions (saline1, saline 2). Thus, despite EFA scores correlating with right aHipp glutamate levels in Expt 1, and showing a decrease after aHipp LY/CGP, its failure to return to saline 1 levels after saline 2 means that habituation effects cannot be ruled out (Table 3 and Figure 2). In contrast, the marked rise in the time spent at the front of the cage after aHipp LY/CGP was not accompanied by habituation between the first and second saline infusions indicating that the animals experienced less anxiety and spent more time in close proximity to the human intruder after aHipp LY/CGP only. Performance variables of height, movements (jumps, bobs and locomotion) and call types were unaffected.
Repeated measures ANOVA of the composite anxiety score revealed a main effect of test (saline 1, LY/CGP, saline 2) and a significant decrease in anxiety score between saline 1 and aHipp LY/CGP, but no difference between aHipp LY/CGP and saline 2 indicative of habituation (main effect of test3, F2,10 = 7.427, P = 0.011; first saline vs LY/CGP, t5 = 2.572, P = 0.042; second saline vs LY/CGP, t5 = 1.197 P = 0.285; see Table 3). Repeated measures analysis of all three depth measures (front, middle, back) also indicated that depth was sensitive to aHipp glutamate levels, but this was not due to alterations in time spent in the middle or back (no main effects of test3, Middle, F2,10 = 1.98, P = 0.189; Back, F2,10 = 2.239, P = 0.157), and was solely due to a main effect of TSA. Importantly this effect was not due to habituation as TSA was relatively low following both saline infusions, but was markedly increased after aHipp LY/CGP (effect of test3, F2,10 = 8.465, P = 0.007; first saline vs LY/CGP, t5 = 3.759, P = 0.013; second saline vs LY/CGP, t5 = 3.256, P = 0.023). No other performance variables of movement, height, or vocalizations (repeated measures analysis of all 6 height measures [tsik, tsik-egg, tse, egg and tse-egg, number of calls]) were affected (Locomotion, F2,10 = 1.17, P = 0.348; Jumps F2,10 = 1.027, P = 0.393; or Bobs F2,10 = 2.9, P = 0.101; height x test3, F10,50 = 1.69, P = 0.108; or vocalizations x test3, F10,50 = 1.776, P = 0.09).

Thus, enhancement of glutamate release in the aHipp specifically ameliorates the high-trait anxiety phenotype by increasing the approach to a human intruder.

**Experiment 2B**

The low cardiovascular responsivity shown by high-trait anxious monkeys to the presentation of an uncertain cue in a threatening environment was increased by aHipp presynaptic glutamatergic disinhibition.

To assess the contribution of aHipp-mPFC circuitry to anxiety we first assessed the response of high-trait anxious animals to a novel auditory cue in a context that has previously been associated with the threat of an unpredictable, aversive loud noise (Mikheenko et al., 2010). See Figure 3A.
High trait anxious marmosets display low cardiovascular responsivity to the presentation of an ambiguous cue in a threatening environment

Since the cardiovascular responsivity of high-trait anxious humans is blunted, we first determined whether the cardiovascular and VS response to the novel cue was changed when compared to the preceding baseline (cue minus baseline). High-trait anxious animals did show a slight increase in VS after bilateral aHipp saline infusion, but like high-trait anxious humans, they failed to show any cardiovascular alterations as cue-directed HR, HRV, CSI and CVI were all close to zero (Mikheenko et al., 2010) (one sample t-tests comparing to no change: VS, $t_5 = 3.744, P = 0.013$; HR, $t_5 = 0.888, P = 0.415$; HRV $t_5 = 1.078, p = 0.330$; CSI, $t_5 = 0.182, p = 0.863$; CVI, $t_5 = 1.043, p = 0.345$; see figure 4a).

Within-subjects correlation analysis of these changes in HR and VS revealed that those animals who showed the least HR responsivity, also showed the smallest changes in VS ($F_{1,65} = 19.001, P < 0.0001$), indicating a relationship between cardiovascular responsivity and behavioral engagement that we have previously shown to be independent of alterations in arousal or locomotion (Mikheenko et al., 2010; Wallis et al., 2017).

aHipp LY/CGP increased cardiovascular responsivity to the presentation of an ambiguous cue in a threatening environment

Compared to the non-responsiveness of the cardiovascular system to the novel auditory cue after a saline infusion, a marked increase in responsiveness was observed following aHipp LY/CGP infusions. A two-way ANOVA with factors of drug (saline or aHipp LY/CGP) and cue presentations (1-12) revealed that aHipp LY/CGP increased cue-directed HR (main effects of drug$_2$, $F_{1,107.67} = 11.25, P = 0.001$ and drug$_2 \times$ cue$_{12}$, $F_{11,103.22} = 2.65, P = 0.005$, figure 4). Similar analyses also revealed an increase in cue-directed CSI (main effects of drug$_2$, $F_{1,108} = 4.954, P = 0.0281$, and drug$_2 \times$ cue$_{12}$, $F_{11,101.35} = 2.63, P = 0.005$), a decrease in cue-directed HRV (main effect of drug$_2$ only, $F_{1,107.9} = 4.956, P = 0.028$) and no change in CVI (no main effect of drug $F_{1,107.9} = 1.446, P = 0.23$, drug$_2 \times$ cue$_{12}$, $F < 1$, NS).
In summary, high-trait anxious animals show a marked lack of cardiovascular responsivity in response to threatening cues, but increasing aHipp presynaptic glutamate release significantly increased both the cardiovascular and behavioral responsivity.

Inactivation of area 25 blocked the ability of aHipp LY/CGP to increase cardiovascular responsivity and VS

To determine whether the increases in cardiovascular responsivity induced by aHipp LY/CGP engage aHipp-area 25 circuitry, aHipp LY/CGP infusions were combined with simultaneous inactivation of area 25 with 0.5 μL of 0.1 mM muscimol/1.0 mM baclofen (‘musbac’).

This simultaneous inactivation was found to reduce the impact of aHipp LY/CGP on HR, VS, HRV and CSI. Thus repeated measures ANOVA with factors of manipulation (25 musbac, aHipp saline, aHipp LY/CGP, and aHipp LY/CGP + 25 musbac) and cue presentations (1-12) revealed changes in HR, VS, HRV and CSI but not CVI (HR, main effect of manipulation $F_{3,16.7} = 8.98$, $P = 1.491 \times 10^{-5}$; VS, main effect of manipulation $F_{3,177.46} = 4.82$, $P = 0.002$; HRV, main effect of manipulation $F_{3,180} = 3.4$, $P = 0.017$; CSI, a trending effect of manipulation $F_{3,148.22} = 2.269$, $P = 0.08$, and a manipulation x cue number interaction $F_{33,173} = 1.59$, $P = 0.02$; CVI, $F_{3,180} = 0.69$, $P = 0.55$).

Post hoc analysis revealed that simultaneous aHipp LY/CGP + area 25 musbac infusions reversed some of the cue-directed changes that were induced by aHipp LY/CGP alone. Specifically, the increases in HR that were seen after aHipp LY/CGP were abolished to below control levels, with the effects of aHipp LY/CGP + area 25 musbac no longer different from aHipp saline ($t_3 = 1.8$, $P = 0.157$). Similarly, the increases in VS and CSI seen after aHipp LY/CGP were returned to saline levels after aHipp LY/CGP + area 25 musbac (VS, aHipp saline vs aHipp LY/CGP + area 25 musbac $t_3 = 0.7$, $P = 0.49$, aHipp LY/CGP vs aHipp LY/CGP + area 25 musbac $t_3 = 1.45$, $P = 0.285$. CSI, aHipp saline vs aHipp LY/CGP + area 25 musbac $t_3 = 1.5$, $P = 0.21$, aHipp LY/CGP vs aHipp LY/CGP + area 25 musbac $t_3 =
6.43, P = 0.02). Although numerically the combined aHipp LY/CGP + area 25 musbac did reverse the
reductions in cue-directed HRV induced by aHipp LY/CGP, these did not reach significance due to
high variability (aHipp saline vs aHipp LY/CGP + area 25 musbac t2 = 1.5, P = 0.27, aHipp LY/CGP vs
aHipp LY/CGP + area 25 musbac t2 = 1.2, P = 0.27).

For control purposes we also investigated the effects of area 25 inactivation alone. However, in
contrast to the ability of area 25 inactivation to block the enhanced responsivity induced by aHipp
LY/CGP, area 25 inactivation on its own also enhanced responsivity in a similar manner to aHipp
LY/CGP. Thus, compared to saline, area 25 inactivation increased measures of both cue-directed HR
and VS (HR, t3 = 4.03, P = 0.027; VS, t3 = 4.03, P = 0.027; see Figure 4b). HRV, CSI and CVI were not
affected by area 25 inactivation (ts < 1, NS). As these HR and VS findings are very similar to those
seen after aHipp LY/CGP, the fact that both measures do not differ from saline after the combined
aHipp LY/CGP + area 25 musbac infusions indicates that the combined manipulation not only
abolishes the independent effects of aHipp LY/CGP manipulations alone, but also abolishes the
effects of 25 inactivation alone. This indicates reciprocal communication between these structures in
the behavioral and cardiovascular regulation of high-trait anxiety.

Area 25 activity is therefore an important determinant of aHipp LY/CGP’s ability to alter the
behavioral and cardiovascular indices of high-trait anxiety in response to an ambiguous cue, and
highlights the importance of reciprocal aHipp-area 25 connectivity in this phenotype.

Inactivation of area 32 did not alter the effects of aHipp LY/CGP.

In order to determine the contribution of aHipp-area 32 circuitry to the effects of aHipp LY/CGP, we
also combined aHipp LY/CGP infusions with simultaneous inactivation of area 32. However, unlike
area 25, area 32 inactivation did not modulate the effects of aHipp LY/CGP. Thus repeated measures
ANOVA of the area 32 manipulations with factors of manipulation (32 musbac, aHipp saline, aHipp
LY/CGP, and aHipp LY/CGP + 32 musbac) and cue number (1-12) revealed no main effects of
manipulation on cue-directed HRV or CVI (HRV, $F_{3,180} = 1.6$, $P = 0.18$; CVI, $F_{3,179.3} = 0.7$, $P = 0.55$).

Although similar analysis did reveal main effects of manipulation on both cue-directed HR and cue-directed VS, (HR, $F_{3,190.97} = 4.1$, $P = 0.007$; VS, $F_{3,189.4} = 4.36$, $P = 0.005$), and CSI approaching significance ($F_{3,179.7} = 1.6$, $P = 0.055$), these effects were not due to a modulatory role of area 32, but were due to the effects of aHipp LY/CGP alone, and not to the effects of simultaneous aHipp LY/CGP + area 32 inactivation.

Thus, although aHipp LY/CGP + area 32 inactivation did slightly reduce the increases in cue-directed HR seen with aHipp LY/CGP alone, this effect was not significant (aHipp LY/CGP vs aHipp LY/CGP + area 32 musbac, $t_3 = 1.4$, $P = 0.234$, see Figure 4c). Similarly, aHipp LY/CGP + area 32 musbac did not alter the effect of aHipp LY/CGP on cue-directed VS ($t < 1$, NS, aHipp saline vs area 32 musbac, $t < 1$, NS; aHipp LY/CGP vs aHipp LY/CGP + area 32 musbac, $t_3 = 1.53$, $P = 0.223$), CSI (aHipp saline vs area 32 musbac, $t_2 = 1.53$, $P = 0.223$) or CVI (aHipp saline vs area 32 musbac, $t < 1$, NS, aHipp LY/CGP vs aHipp LY/CGP + area 32 musbac, $t < 1$, NS).

Control inactivations of area 32 alone revealed no effects on cue-directed HR, VS, CSI (all $t < 1$, NS), HRV or CVI ($t > 1$, NS). Thus activity within area 32 does not impact on the ability of aHipp LY/CGP to increase the behavioral and cardiovascular indices of high-trait anxiety in response to an ambiguous cue.

Discussion

We have demonstrated that high-trait anxiety in marmosets is correlated with right, but not left aHipp glutamate levels, with reduced presynaptic glutamate in the right aHipp associated with increased anxiety. Pharmacologically elevating aHipp presynaptic glutamate release with aHipp LY/CGP in a cohort of high-trait anxious marmosets reduced their behavioral measures of anxiety in response to an unknown human. Glutamatergic elevations also increased the responsivity of the previously unresponsive cardiovascular systems of these high-trait anxious animals to unpredictable
threat cues, and simultaneously increased their vigilant scanning responses to the same cues.

Investigation into the specific pathways between the aHipp and mPFC that may underlie these cardiovascular and behavioral effects in high-trait anxious animals revealed that simultaneous inactivation of area 25, but not area 32, abolished the effects of elevating aHipp glutamate. Thus, we provide causal evidence in primates to support the hypothesis that aHipp glutamatergic hypofunction contributes to specific aspects of the behavioral and autonomic correlates of high-trait anxiety via an aHipp-area 25 pathway.

High-trait anxious marmosets in the current study showed a marked lack of cardiovascular responsivity to a threatening cue, and similarly low levels of VS. These findings contrast with the increases in HR and/or blood pressure and VS that normally signal increased fear in non-anxious animals during aversive fear conditioning, and in high-anxious marmosets responding in a similar, but less aversive, unpredictable threat paradigm (Mikheenko et al., 2010; Shiba et al., 2014; Wallis et al., 2017). Although one interpretation of these findings is that the paradigm was not aversive enough to cause a fear response, this is unlikely given the known aversive impact of the noise cue used (see Wallis et al., 2017). A more likely interpretation is based on the findings in high-trait anxious or depressed humans who show i) blunted cardiovascular responsivity to such stressors, and ii), reduced behavioral engagement that is most extreme in those individuals who show the greatest cardiovascular blunting (Ginty et al., 2015). Thus, the lack of cardiovascular responsivity to the threatening cues in the high-trait anxious marmosets may be equivalent to the cardiovascular blunting seen in high-trait anxious humans, although for confirmation a direct comparison with low anxious animals is required. Certainly, however we have reported previously a reduction in blood pressure, reminiscent of the blunted response seen here, in a subset of high anxious marmosets following repeated exposure to aversive Pavlovian conditioned stimuli (Shiba et al., 2014), an effect not seen in low anxious marmosets that displayed the expected elevation in cardiovascular activity to an aversive Pavlovian conditioned stimulus. Furthermore, like humans the high-trait anxious marmosets with the smallest HR changes also showed the smallest VS changes in Expt 2, thus the
low levels of VS seen in the present study may reflect decreased behavioral engagement with the
cue. These findings therefore most likely indicate that the high-trait anxious monkeys are displaying
a similar blunted phenotype to high-trait anxious humans and that this phenotype is linked to
reduced hippocampal glutamate.

Hippocampal glutamatergic hypofunction has previously been linked to affective illness severity (de
Diego-Adelino et al., 2013). However, despite strong evidence that a hippocampal network mediates
stressor-evoked behavioral and cardiovascular activity, few studies have investigated the role of the
hippocampus in cardiovascular responsivity (Neafsey et al., 1993; Bär et al., 2016; Kuntze et al.,
2016; Ajayi et al., 2018). Here we show that increasing aHipp glutamate with aHipp LY/CGP causes
corresponding increases in the HR and VS response to a threatening cue, along with increases in CSI
and decreased HRV. These are the components of the fight of flight response that are normally
shown in non-anxious animals responding to threat, and suggest that aHipp LY/CGP has had an
anxiolytic effect by normalizing the previously blunted responses. This is supported by the anxiolytic
effects of aHipp LY/CGP in the human intruder test. Here, aHipp LY/CGP increased the time animals
spent at the front of the cage and reduced the time spent deep in the cage, consistent with an
increased approach to, and engagement with, the potentially threatening intruder. Thus, both the
increased VS in the unexpected threat paradigm, and the increased approach behavior in the human
intruder test, may reflect increased engagement with the potential threat (be it aversive cue or
human intruder). Together, these findings indicate that elevation of aHipp glutamate can ameliorate
the high-trait anxiety phenotype, in part by returning cardiovascular and behavioral reactivity to the
healthy, non-blunted state (via a paradoxical increase in the fight or flight response). Glutamate
within the aHipp is clearly an important regulator of the cardiovascular and behavioral correlates of
high-trait anxiety.

Given that our data associated elevated trait anxiety with reductions of right aHipp
glutamate only, we could speculate that these alterations are due to the effect of increasing
 glutamate within the right and not the left aHipp despite our use of bilateral aHipp LY/CGP infusions.
Our manipulation data and human neuroimaging data are inconclusive regarding the laterality of the hippocampal abnormalities that are apparent in the affective disorders (see Malykhin and Coupland, 2015), and no hemispheric differences were seen in the hippocampal response to early life stress (maternal deprivation) in marmosets (Law et al., 2009). However, some neuroimaging studies do report a stronger relationship between right aHipp neurochemistry and the affective disorders than the left hippocampus (de Diego-Adeliño et al., 2013). Furthermore, brief, repeated exposure to a novel environment during the early life of rodents has been shown to preferentially alter the synaptic plasticity and volume of the right, not left, hippocampus (Verstynen et al., 2001; Tang et al., 2008). These rodent changes were small (and equivalent changes may have been masked by the lower group sizes in the marmoset study), but nevertheless suggest the right hippocampus may be particularly important for translating the effects of early-life experiences into adult temperament.

The hippocampus is connected to many other limbic brain regions through which it can enact these effects. These include the amygdala and the mPFC which have been extensively studied in rodent models of anxiety and fear behavior, but less so with respect to cardiovascular regulation. Certainly, rodent studies of threat regulation emphasise a tripartite mPFC-vHipp-amygdala circuit (Sierra-Mercado et al., 2010; Padilla-Coreano et al., 2016), and stronger amygdala-hippocampal network activity has been associated with higher levels of human trait anxiety (Kirkby et al., 2018). Although we have not investigated the contribution of the amygdala in this study, we have previously shown that levels of 5-HT are reduced in the amygdala of high anxious marmosets (Mikheenko et al., 2015), and the aHipp sends double-projecting neurons to both the mPFC and the basal amygdala, through which both regions can be modulated simultaneously (Kim and Cho, 2017). Communication specifically between the vHipp and the mPFC is vital for the behavioral correlates of threat conditioning in rodents, and studies have primarily emphasised the role of vHipp connectivity with the prelimbic (PL), rather than infralimbic (IL) subregion (LeDoux, 2000; Adhikari et al., 2010; Sierra-Mercado et al., 2010; Sotres-Bayon et al., 2012; Padilla-Coreano et al., 2016). As anatomical and connectivity studies suggest that rodent IL and PL are homologous to primate areas 25 and 32...
respectively, (Vertes, 2006; Vogt and Paxinos, 2014; Heilbronn et al., 2016), these rodent studies predict the importance of primate aHipp-area 32 connectivity for threat regulation. Despite this, the current data highlight the importance of aHipp-area 25 for the regulation of the behavioral and cardiovascular indices of cue-directed high-trait anxiety, and provide causal support for human neuroimaging studies highlighting the importance of aHipp-area 25 connectivity in the regulation of negative emotional behavior (Dickie et al., 2011; Hamilton et al., 2011; Treadway et al., 2015). One possible explanation for these differences is that hippocampal glutamate differentially regulates these mPFC subregions in healthy and high-trait anxious states. This would also explain why v/aHipp lesions and glutamate antagonism in non-anxious rodents and primates are anxiolytic (Chudasama et al., 2008, 2009; Barkus et al., 2010), yet increasing v/aHipp glutamate in high-trait anxious rats and marmosets is also anxiolytic (Marrocco et al., 2012; present study). However, confirmation of this hypothesis requires the testing of non-anxious animals on the current paradigm for comparison with the high-anxious cohort. Alternatively, the rodent IL and PL may have different functions to primate areas 25 and 32. Thus recent behavioral findings in marmosets showed that inactivation of areas 25 and 32 had opposite autonomic and behavioral effects on fear discrimination and extinction to these putative rodent homologs indicating uncertainty over the functional analogy of IL/25 and PL/32 (see Wallis et al., 2017). Inactivation of the IL also has no effect on cardiovascular control during baseline or stressful conditions, whereas area 25 inactivation causes a significant bradycardia (Müller-Ribeiro et al., 2012; Wallis et al., 2017). Nevertheless, vHipp-IL circuitry in the rat has been implicated in cardiovascular regulation, since electrical stimulation of the vHipp in anaesthetised rodents is only able to elicit a depression of cardiovascular activity (decreased HR), if the IL is intact (Ruit and Neafsey, 1990; Gianaros et al., 2004; Critchley et al., 2011). However, it should be noted that in both species, the hippocampus appears to connect primarily with the mPFC regions whose inactivation reduces fear.

Finally, inactivations of areas 25 and 32 alone had distinct effects. Inactivation of area 25 caused similar increases in HR and VS to aHipp LY/CGP which suggests that area 25 inactivation is also
anxiolytic in this paradigm. This replicates previous findings in which area 25 inactivation reduced the behavioral and cardiovascular indices of cued fear in healthy marmosets (Wallis et al., 2017), (but see Gianaros et al., 2004; Critchley et al., 2011). The findings that area 25 inactivation and aHipp LY/CGP have the same effects independently, but their simultaneous infusion blocks these effects, suggests functional dependence of these two structures in the regulation of high-trait anxiety. In contrast, area 32 inactivation had no effect on the cardiovascular and VS responses induced by the novel cue, and the combined aHipp LY/CGP + area 32 inactivation also failed to modulate the anxiolytic effects of aHipp LY/CGP alone. This diverges from the increase in threat responses seen after area 32 inactivation (with GABA agonists) during cued fear conditioning in non-anxious marmosets (Wallis et al., 2017), the positive relationship between perigenual GABA levels and human trait anxiety (Delli Pizzi et al., 2016). It is also different from the increase in HR and foreboding, or negative bias, that is seen after perigenual ACC stimulation in non-anxious epileptic patients and macaques respectively, that may have preferentially activated GABAergic interneurons (Amemori and Graybiel, 2012; Parvizi et al., 2013). However, it may not be possible to detect further increases in defensive behaviors in response to certain or uncertain threat in high-trait anxious animals, who may already be displaying maximal levels of this behavior. Clearly, the differing contributions of 25 and 32 to the modulation of threat responses has implications when considering treatment, particularly when considering GABAergic medications such as benzodiazepines (Bruijn et al., 2001).

To conclude, we have shown that aHipp glutamatergic hypofunction is a key predictor of a high-trait anxiety phenotype in marmoset monkeys and that by pharmacologically normalizing such hypofunction we can also normalize the abnormal behavioral and cardiovascular blunting associated with this phenotype. We provide evidence that an aHipp-area 25, but not aHipp-area 32 circuit underlies this specific anxiolytic effect and highlight the aHipp-area 25 pathway as a potential therapeutic target. Together these findings demonstrate the importance of investigating both the behavioral and autonomic aspects of emotional responding, and suggest that investigating
cardiovascular function in rodents may be a valuable adjunct when comparing rodent and primate mPFC function.

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**Table 1.**

| Cannulae targets | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |
|------------------|----|----|----|----|----|----|----|----|
| aHipp/25/32      | ✔  | ✔  | ✔  | ✔  | ✔  | ✔  | ✔  | ✔  |

**Human Intruder test (2a)**

|                  | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |
|------------------|----|----|----|----|----|----|----|----|
| Saline 1         | ✔  | ✔  | ✔  | ✔  | ✔  | ✔  | ✔  | ✔  |
| aHipp LY/CGP     | ✔  | ✔  | ✔  | ✔  | ✔  | ✔  | ✔  | ✔  |
| Saline 2         | ✔  | ✔  | ✔  | ✔  | ✔  | ✔  | ✔  | ✔  |

**Unpredictable Threat Test (2b)**
Table 1. Summary of subjects that took part in the behavioral studies. A tick indicates the subject took part in that phase of the study, and for Experiment 2a, the number in parentheses indicates the order in which the infusions were received. Subjects 3 and 6 only had cannulae targeting the aHipp as they underwent prefrontal microdialysis (not reported here). Post mortem histology for subject 5 revealed that their cannulae were not located in areas 25 or 32 and histology for subject 7 revealed that their cannulae were not located in area 25, therefore their data for these regions are excluded. However the overall number of infusions they received in these areas is included for completeness (including other drug combinations that are not reported here).

| Drug Combinations | Number of Infusions |
|------------------|---------------------|
| aHipp saline     | ✅ (2)   ✅ (4)  ✅ (1)  ✅ (2)  ✅ (1) |
| aHipp LY/CGP     | ✅ (4)   ✅ (5)  ✅ (2)  ✅ (1)  ✅ (3) |
| aHipp LY/CGP & musbac 25 | ✅ (1)   ✅ (1)  ✅ (3)  | ✅ (2) |
| aHipp LY/CGP & musbac 32 | ✅ (6)   ✅ (3)  ✅ (4)  | ✅ (4) |
| musbac 25        | ✅ (3)   ✅ (6)  ✅ (5)  | | ✅ (3) |
| musbac 32        | ✅ (5)   ✅ (2)  ✅ (6)  | ✅ (2)  ✅ (4) |

Number of infusions

| Drug Combinations | Number of Infusions |
|------------------|---------------------|
| aHipp            | 10 10 5 10 10 6 14 4 |
| Area 25          | 3 4  NA 3 3  NA 8 3  |
| Area 32          | 6 3  NA 2 4  NA 6 1  |

Table 2.
| Behavior                | Factor Loading |
|-------------------------|----------------|
| Average height          | 0.816          |
| Bobs                    | 0.769          |
| Time spent at back      | 0.688          |
| Tse egg calls           | 0.417          |
| Egg calls               | 0.332          |
| Tsik egg calls          | 0.323          |
| Tsik calls              | -0.091         |
| Locomotion              | -0.568         |
| Time spent at front     | -0.790         |

**Table 2. Factor loadings of individual behaviors within the EFA.** The pattern in which these individual behaviors load onto this EFA factor suggest that it represents the marmosets’ anxiety towards the intruder with a high factor score representing an animal high up and at the back of cage, far away from the intruder, remaining relatively still and performing a lot of head bobs, indicative of high anxiety.

| aHipp | Dopamine | Serotonin | Noradrenaline |
|-------|----------|-----------|---------------|
|       | EFA score |           |               |
|       | R_{12}=-0.332, P = 0.291 | R_{12}=-0.072, P = 0.825 | R_{12}=0.271, P = 0.394 |
|       | R_{12}=0.026, P = 0.935 | R_{12}=0.021, P = 0.949 | R_{12}=-0.127, P = 0.694 |
| % in depth zones | Front | | Middle |
|       | R_{12}=-0.362, P = 0.247 | R_{12}=0.227, P = 0.479 | R_{12}=-0.362, P = 0.247 |
|       | R_{12}=0.016, P = 0.960 | R_{12}=0.016, P = 0.960 | R_{12}=0.053, P = 0.869 |
|       | R_{12}=-0.362, P = 0.247 | R_{12}=0.016, P = 0.960 | R_{12}=-0.239, P = 0.454 |

**Table 3.**
| % in height zones          | Back  | Floor | Low  | Middle | High  | Top of nest box | Avg height | Movement | Calls |
|---------------------------|-------|-------|------|--------|-------|-----------------|-----------|----------|-------|
|                           | P = 0.913 | P = 0.661 | P = 0.540 | P = 0.015 | P = 0.503 | P = 0.325 |           |         |       |
| Back                      | R12=0.678, | R12=0.176, | R12=0.030, | R12=0.678, | R12=-0.057, | R12=0.656, | P = 0.015* | P = 0.015* | P = 0.021* |
|                           | P = 0.585 | P = 0.927 | P = 0.860 | P = 0.021* |         |         |           |         |       |
| Floor                     | R12=-0.364, | R12=-0.173, | R12=-0.160, | R12=-0.296, | R12=-0.068, | R12=0.223, |           |         |       |
|                           | P = 0.245 | P = 0.590 | P = 0.619 | P = 0.350 | P = 0.833 | P = 0.485 |           |         |       |
| Low                       | R12=-0.202, | R12=-0.354, | R12=-0.477, | R12=-0.246, | R12=-0.367, | R12=0.518, |           |         |       |
|                           | P = 0.530 | P = 0.259 | P = 0.117 | P = 0.441 | P = 0.240 | P = 0.085 |           |         |       |
| Middle                    | R12=-0.341, | R12=0.473, | R12=-0.073, | R12=-0.059, | R12=0.005, | R12=0.127, |           |         |       |
|                           | P = 0.278 | P = 0.120 | P = 0.822 | P = 0.845 | P = 0.989 | P = 0.693 |           |         |       |
| High                      | R12=0.545, | R12=0.076, | R12=0.377, | R12=0.369, | R12=0.345, | R12=-0.013, |           |         |       |
|                           | P = 0.067 | P = 0.814 | P = 0.227 | P = 0.237 | P = 0.272 | P = 0.968 |           |         |       |
| Top of nest box           | R12=-0.007, | R12=-0.137, | R12=-0.159, | R12=-0.044, | R12=-0.106, | R12=-0.216, |           |         |       |
|                           | P = 0.982 | P = 0.671 | P = 0.622 | P = 0.891 | P = 0.606 | P = 0.499 |           |         |       |
| Avg height                | R12=0.328, | R12=0.014, | R12=0.091, | R12=0.220, | R12=0.032, | R12=-0.281, |           |         |       |
|                           | P = 0.298 | P = 0.966 | P = 0.779 | P = 0.493 | P = 0.922 | P = 0.376 |           |         |       |
| Movement                  | Locomotion | R12=-0.364, | R12=0.041, | R12=0.049, | R12=-0.224, | R12=0.097, | R12=0.183, |           |       |
|                           |           | P = 0.245 | P = 0.900 | P = 0.880 | P = 0.484 | P = 0.765 | P = 0.569 |           |       |
|                           | Jumps    | R12=-0.0525, | R12=0.276, | R12=0.064, | R12=-0.241, | R12=0.019, | R12=-0.066, |           |       |
|                           |           | P = 0.080 | P = 0.385 | P = 0.842 | P = 0.450 | P = 0.952 | P = 0.838 |           |       |
|                           | Bobs     | R12=0.283, | R12=-0.06, | R12=0.106, | R12=0.269, | R12=0.067, | R12=-0.166, |           |       |
|                           |           | P = 0.373 | P = 0.854 | P = 0.742 | P = 0.397 | P = 0.837 | P = 0.605 |           |       |
| Calls                     | Tsik     | R12=-0.420, | R12=-0.053, | R12=-5.098, | R12=-0.054, | R12=-0.114, | R12=0.197, |           |       |
|                           |           | P = 0.174 | P = 0.870 | P = 0.857 | P = 0.867 | P = 0.725 | P = 0.540 |           |       |
|                           | Tsik-Egg | R12=0.376, | R12=-0.079, | R12=-0.098, | R12=0.525, | R12=-0.209, | R12=0.261, |           |       |
|                           |           | P = 0.228 | P = 0.807 | P = 0.762 | P = 0.08 | P = 0.515 | P = 0.413 |           |       |
Table 3. Hippocampal levels of 5-HT, Dopamine and Noradrenaline do not correlate with any of the behavioral measures sensitive to glutamate in the human intruder test. However, both right aHipp 5-HT and NA and left aHipp DA did correlate with the time spent at the back of the cage, suggesting that these neurochemical may contribute to specific behaviors within the human intruder test. * P < 0.05.

| Region  | Saline 1 | Saline 2 | LY/CGP  |
|---------|----------|----------|---------|
| EFA score* | 0.798 ± 0.179 | 0.161 ± 0.066 | 0.06 ± 0.121* |
| % in depth zones | | | |
| Front* | 0.957 ± 0.957 | 7.853 ± 4.572* | 20.55 ± 4.766* |
| Middle | 40.327 ± 16.098 | 60.089 ± 10.811 | 16.878 ± 7.839 |
| Back | 32.877 ± 9.965 | 18.990 ± 7.537 | 32.877 ± 9.965 |
| % in height zones | | | |
| Floor          | 0.00 ± 0.00 | 10.136 ± 8.180 | 10.106 ± 6.613 |
|---------------|-------------|----------------|----------------|
| Low           | 0.00 ± 0.00 | 3.7 ± 3.7      | 4.331 ± 2.842  |
| Middle        | 2.302 ± 2.302 | 16.935 ± 7.995 | 8.904 ± 3.967  |
| High          | 71.681 ± 8.089 | 66.738 ± 16.030 | 64.119 ± 10.841 |
| Top of nest box | 19.717 ± 7.8  | 1.738 ± 1.738  | 4.414 ± 2.328  |
| **Avg height** | 68.758 ± 3.697 | 58.79 ± 7.503 | 55.5 ± 5.593 |

**Movement**

| Locomotion    | 2.003 ± 0.832 | 3.208 ± 0.999 | 3.623 ± 1.119 |
| Jumps         | 1.667 ± 0.667 | 2.5 ± 1.057   | 3.5 ± 1.057   |
| Bobs          | 30.333 ± 8.313 | 19.333 ± 5.714 | 19.0 ± 7.546  |

**Calls**

| Tsik          | 3.833 ± 3.637 | 4.0 ± 2.696 | 1.0 ± 0.516   |
| Tsik-Egg      | 4.667 ± 3.721 | 4.0 ± 3.235 | 6.667 ± 5.690 |
| Tse           | 2.0 ± 1.095   | 0.667 ± 0.333 | 0.833 ± 0.477 |
| Tse-Egg       | 6.667 ± 2.951 | 2.833 ± 2.242 | 4.00 ± 1.949 |
| Egg           | 4.823 ± 2.926 | 1.667 ± 0.333 | 4.0 ± 1.770  |

Table 4. Summary of human intruder performances after saline and aHipp LY/CGP

Mean scores for all behaviors analyzed during the human intruder test after each presentation of the test (saline 1, LY/CGP and saline 2). Data presented as mean ± standard error of the mean. *P < 0.05. Asterisks in column 1 indicate a main overall effect of the measure, asterisks in columns 2-4 indicate significance compared to the preceding test.
Figure 1. Measures of high-trait anxiety correlate with glutamate levels within the right anterior hippocampus.

A. Schematic of the human intruder test apparatus detailing the depth (front, middle, back) and height (floor, low, middle, high, top of nest box) locations that the monkey could occupy.

B. Schematic showing the dissection of the hippocampus from AP 4 to AP 6.5 prior to glutamate analysis.

Decreased levels of hippocampal glutamate levels correlated with increased exploratory factor analysis score (EFA score; C), increased head and body bobbing (D), but not time spent at the front of the cage (E).

Figure 2. aHipp LY/CGP treatment reversibly increases the time spent at the front of the cage and reduces the EFA score in the human intruder test

A. Schematic showing the position of the cannulae tips and cannulae locations for each animal. All aHipp cannulae were located within the ranges of AP, 4.8-5.6, respectively, and are plotted here on a single coronal section. Cytoarchitectonic parcellation was based on (Burman and Rosa, 2009). The circles represent the estimated maximal spread of the muscimol/baclofen or saline infusions (West et al., 2011).
B. The time spent at the front of the cage is reversibly increased by aHipp LY/CGP infusion, but this is not shown in the composite anxiety score (C) due to habituation between the 2 saline infusions. *P < 0.05.

Figure 3. Unpredictable threat test and cannulae schematics

A. In the unpredictable threat test (n = 6), animals were placed in a familiar environment for 25 mins and played two types of auditory stimuli, a novel auditory cue and an unconditioned stimulus (US) for the 4 unpredictable threat days. Each cue was 20 sec long, 75dB and was presented 12 times with an intertrial interval (ITI) of 40-80 sec. Each mildly aversive US was 0.4 sec long, 117dB and presented 12 times with an ITI of 40-80 sec. The threat of the aversive US was unpredictable as there was no relationship between the order of the cues and USs. On the ambiguous cue probe (5th day), monkeys were presented with a novel, ambiguous 20 sec, 75dB cue presented 12 times with an ITI of 100-180 sec in the same context. No USs were presented. All drug manipulations occur on ambiguous cue probes. The cycle then repeated with the novel cue incorporated into the unpredictable threat training.

B. Glass brain illustrating the rostro-caudal locations of the aHipp, area 25 and area 32, and the double intracerebral cannulae targeting each area.

C. Representative histological sections with arrows marking the position of the cannulae tips and cannulae locations for each animal. All aHipp, area 25 and area 32 cannulae were located within the ranges of AP 4.8-5.6, 12.5-14, and 15.8–16.6 respectively, and are plotted here on a single coronal section for each target area. Cytoarchitectonic parcellation was based on (Burman and Rosa, 2009). The circles represent the estimated maximal spread of the muscimol/baclofen or saline infusions (West et al., 2011).
Figure 4. aHipp LY/CGP ameliorates the behavioral and cardiovascular correlates of cue-induced anxiety, but these effects can be blocked by simultaneous area 25 inactivation.

Graphs show the changes in heart rate (HR, beats per min), vigilant scanning (VS), HRV, CSI, and CVI under drug and saline conditions, during the cue relative to the baseline (the last 20 secs of the immediately preceding ITI). Positive numbers indicate an increase from baseline, while negative numbers indicate a decrease compared to baseline. Data = mean ± SEM, * P < 0.05.

a. Compared to saline, aHipp LY/CGP infusion altered responding in a cue-dependent manner, as assessed by cue-induced increases in HR, VS, and CSI, and decreases in HRV.

b. Simultaneous aHipp LY/CGP + area 25 inactivation abolished the increases in HR, VS, and CSI that were seen with aHipp LY/CGP alone. Area 25 inactivation also increased HR and VS in its own right.

c. Simultaneous aHipp LY/CGP + area 32 inactivation did not alter the changes in VS and cardiovascular activity seen with aHipp LY/CGP alone. Area 32 inactivation also had no effect on its own.

Figure 5. Those animals who show the smallest cue-induced changes in HR, also show the smallest changes in VS.

Within-subjects correlation analysis of the cue-induced changes in HR and VS after infusion of saline into the aHipp revealed that those animals who showed the least HR responsivity, also showed the smallest changes in VS. *** P < 0.0001.
A. Human Intruder Test

B. aHipp dissection

C. Glutamate vs EFA score

D. Glutamate vs head and body bobbing

E. Glutamate vs time spent at front cage
Figure 2

A. Locations of hippocampal cannulae

B. Relative time monkeys spent at the front of the cage

C. Composite anxiety score

Figure 3

A. Unpredictable Threat Test

Unpredictable threat: Unpaired presentations of 12 x cues and 12 x USs. Creates a context of unpredictable threat.

B. Cannula placements

C. Schematics and representative histology of cannula placements

Anterior Hippocampus (aHipp)

Area 25

Area 25

AP = 14

AP = 5.8

Area 32

AP ~17

n=4

n=5

Figure 4
Figure 5

A scatter plot showing the relationship between change in heart rate (HR) and change in visual stimuli (VS) from baseline. The data points are color-coded by subject, with each subject having a distinct symbol and color. The plot includes trend lines for each subject, indicating a positive correlation between the variables. The significance level is denoted by stars (***) for the overall trend.