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Title:

Opposing roles of primate areas 25 and 32 and their putative rodent homologs in the regulation of negative emotion

Authors and affiliations

Chloe U. Wallis. [1,2], Rudolf N. Cardinal [2,3,4], Laith Alexander [1,2], Angela C. Roberts [1,2], Hannah F. Clarke[1,2,*]

[1]Department of Physiology, Development and Neuroscience, Downing Street, Cambridge, CB2 3DY, UK

[2]Behavioural and Clinical Neuroscience Institute, University of Cambridge, Downing Site, Cambridge, CB2 3EB, UK

[3]Department of Psychiatry, University of Cambridge, Box 189, Level E4, Cambridge Biomedical Campus, Cambridge CB2 OQQ, UK

[4]Liaison Psychiatry Service, Cambridge and Peterborough NHS Foundation Trust, Box 190, Cambridge Biomedical Campus, Cambridge, CB2 OQQ, UK

*__Corresponding author__, email: hfc23@cam.ac.uk

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Disorders of dysregulated negative emotion such as depression and anxiety also feature increased cardiovascular mortality and decreased heart-rate variability (HRV).These disorders are correlated with dysfunction within areas 25 and 32 of the ventromedial prefrontal cortex (vmPFC), but a causal relationship between dysregulation of these areas and such symptoms has not been demonstrated. Furthermore, cross-species translation is limited by inconsistent findings between rodent fear extinction and human neuroimaging studies of negative emotion. To reconcile these literatures we applied a novel investigative approach to the brain-body interactions at the core of negative emotional dysregulation. We show that in marmoset monkeys (a non-human primate which has far greater vmPFC homology to humans than rodents), areas 25 and 32 have causal yet opposing roles in regulating the cardiovascular and behavioural correlates of negative emotion. In novel Pavlovian fear conditioning and extinction paradigms, pharmacological inactivation of area 25 decreased the autonomic and behavioural correlates of negative emotion expectation, while inactivation of area 32 increased them via generalisation. Area 25 inactivation also increased resting HRV. These findings are inconsistent with current theories of rodent/primate prefrontal functional similarity, and provide new insight into the role of these brain regions in affective disorders. They demonstrate for the first time that area 32 hypoactivity causes

behavioural generalisation relevant to anxiety, and that area 25 is a causal node governing both the emotional and cardiovascular symptomatology relevant to anxiety and depression.

Significance Statement

Affective disorders are associated with increased cardiovascular mortality and enhanced negative emotion. While neuroimaging studies of such disorders reveal dysregulation in numerous frontal brain regions including the subgenual/perigenual cingulate cortices (areas 25 and 32), the causal involvement of this dysregulation is unknown, and translation from rodent studies is limited. Here we demonstrate that in the marmoset monkey, inactivation of area 25 increases parasympathetic modulation of resting cardiovascular function, and decreases the cardiovascular and behavioural correlates of negative emotion. In contrast area 32 inactivation increases these correlates. These findings provide the first direct evidence that these primate areas differentially regulate negative emotion, and link the cardiovascular symptomatology of affective disorders to central neural, rather than peripheral cardiac, dysfunction.

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Introduction

The ventromedial prefrontal cortex (vmPFC) including the anterior cingulate cortex (ACC), has emerged as a key node within the network of brain regions implicated in the regulation of negative emotion. Correlational imaging studies have consistently identified morphological and functional abnormalities in human vmPFC in a range of affective disorders associated with negative emotion dysregulation (1–5). Most prominently, depression has been linked to hyperactivity in caudal subgenual (sg)ACC (centred on area 25) and hypoactivity in perigenual (pg)ACC (centred on area 32 but also including to varying extents areas 10, 24 and 14), which are reversed following successful treatment with pharmacological and psychological therapies, as well as deep brain stimulation (DBS) targeting area 25 (6, 7), Alterations in both pgACC and sgACC activity and sgACC grey matter volume are also seen, however, in generalised anxiety disorder (GAD), post-traumatic stress disorder, phobia, and in response to successful psychological therapy for GAD (8–11), indicating a more generalised role for these regions in the regulation of negative emotion. A neuroimaging meta-analysis of the vmPFC has proposed that activity in caudal sgACC is associated primarily with negative affect whilst activity in more anterior regions that include areas 32, 24 and 10 are associated primarily with positive affect (12). Notably, the vmPFC also responds to stimulation of the vagus nerve, the primary parasympathetic regulator of the cardiac cycle. and vmPFC blood flow correlates with heart rate variability (HRV), a parasympathetic-controlled index of cardiac autonomic function that is chronically low in affective disorders such as depression and anxiety (13–15). This anatomical overlap may explain the strong bi-directional association between affective disorders and cardiovascular dysfunction, such as the increased risk of ischaemic heart disease in depression (14, 16, 17).

Despite this wealth of correlative evidence, the causal contribution of the vmPFC to dysregulation of negative emotion, and in particular the independent roles of the component brain regions that together comprise vmPFC, are poorly understood. Indeed, these changes in area 25 and 32 activity may not necessarily be a primary cause of negative emotion dysregulation. Instead, they could reflect compensatory changes caused by dysfunction in other areas of the PFC that are causing affective symptoms, or represent a correlate of decisions made elsewhere in the vmPFC about the individual's

affective state (18). Addressing this issue of causality is vital to refine our understanding of affective disorders, and for the development of novel, more targeted treatments. While stimulation throughout the ACC has been shown to induce autonomic changes in humans, such cardiovascular or emotional effects cannot be specifically attributed to a particular area (19) without targeted interventions.

Such targeted interventions have primarily been made in rodents studying the role of the medial PFC in fear conditioning and extinction. These studies have largely ignored the cardiovascular components that are a characteristic feature of depression and anxiety (but see , 20, 21) and have focused primarily on the behavioural correlates of negative emotion. Importantly, these behavioural findings related to rodent fear cannot currently be reconciled with the human neuroimaging data discussed above. The rodent mPFC consists of infralimbic (IL) and prelimbic (PL) subregions, whose anatomical connectivity is very similar to that of primate areas 25 and 32 respectively (22–24). Given this similarity in connectivity, comparable functions would also be predicted. However, although these regions are differentially involved in the regulation of a conditioned negative emotional response, the similarities end there. IL neurons are active during recall of extinction, and both lesions and temporary inactivation of the IL enhance conditioned fear and inhibit extinction recall, leading to the hypothesis that the IL is associated with the recall (and storage; , 25) of extinction memory (12, 26–29). In contrast, activity in PL is associated with the expression of conditioned fear (12, 26–28, 30). Thus activity in the IL and PL have opposite effects to the activity of areas 25 and 32 in humans, with IL activity reducing negative emotional responding (compared to the increase seen with area 25 activity) and PL activity increasing negative emotional responding in the rodent (compared to the increased positive affect associated with area 32) (26, 31). Furthermore, the rodent data are also inconsistent with the neuroimaging of fear extinction in humans where the focus of activity related to extinction recall is far more rostral than area 25 (32). In light of the failure to develop major new psychopharmacological treatments for the affective disorders over the last twenty years (33), these cross-species behavioural inconsistencies raise serious questions about the translational relevance of rodent medial PFC conditioned fear studies to our understanding of these regions in the primate brain.

To resolve these discrepancies a new experimental approach is required which takes into account the brain-body interactions at the core of emotion and uses a non-human primate in which there is far greater prefrontal homology to humans than rodents. This allows the investigation of primate areas 25 and 32, on classic rodent tasks of conditioned fear that implicate the IL and PL in order to bridge the translational gap in our current understanding. Here we describe the effects of temporarily inactivating areas 25 and 32 selectively in marmoset monkeys on both behavioural and cardiovascular indices of negative emotion. Marmoset monkeys were tested in three paradigms: i) an emotionally neutral condition, ii) a novel version of a mildly aversive Pavlovian discrimination task (34) in which the probability of receiving an aversive loud noise was predicted differentially by three distinct auditory cues, and iii) a novel adaptation of the classic rodent fear conditioning and extinction paradigm in which the sight of a rubber snake replaces footshock (26). We used real-time telemetric cardiovascular monitoring (35) and behavioural assessment, combined with anatomically specific intracerebral infusions of γ-aminobutyric acid (GABA) agonists that temporarily inactivate cell bodies, nerve terminals and glia, without affecting fibres of passage (36) (or saline control), and anxiolytic drug treatment with diazepam (Figure 1).

Results

Area 25 regulates baseline cardiovascular activity via the parasympathetic system

To assess the impact of area 25 and area 32 inactivation on cardiovascular regulation, animals were assessed first in a familiar, emotionally neutral environment and their cardiovascular function measured

in the absence of any cues (*n* = 13; Figure 1A). Bilateral inactivation of areas 25 and 32 with 0.5 µL of 0.1 mM muscimol/1.0 mM baclofen had different autonomic effects (Figure 2). Inactivation of area 25 decreased heart rate (HR) and mean arterial blood pressure (MAP) and increased HRV, while inactivation of area 32 only increased MAP. HRV reflects the balance between the parasympathetic (vagal) and sympathetic autonomic regulation of the heart. Consequently, we fractionated our HRV measure into the cardiac vagal (CVI) and sympathetic (CSI) indices (37). Area 25 inactivation selectively increased CVI without changing the CSI (Figure 2D), indicating that alterations in HRV and cardiac vagal control can be specifically linked to area 25 dysfunction.

Area 25 inactivation reduces, and area 32 inactivation enhances, aversive Pavlovian conditioned responding

To assess the impact of area 25 and 32 inactivation on the regulation of negative emotion, a subset of monkeys then learned the novel aversive Pavlovian conditioned discrimination (Figure 1B/C) in which three auditory CSs predicted an aversive loud noise with 100%, 50% or 0% probability. Loud noise is frequently used as an aversive cue in humans (38), and has been used in non-human primates as an alternative to the footshock used in non-primate species (39). In marmosets, loud noise causes punishment-induced suppression of responding (40), and response generalisation in high trait anxious individuals (41), similar to that seen in humans and rats. In addition, there is no evidence of habituation to the loud noise, with conditioned responses being maintained across repeated sessions (Figure 3C), and, most critically, loud noise is avoided in instrumental choice tasks (42; Figure S1). Multiple lines of evidence therefore indicate that the loud noise has aversive value, rather than just being salient. As animals learned this auditory discrimination, both their CS-directed HR and CS-directed behavioural orienting (vigilant scanning, VS) increased in proportion to the extent that the CS predicted the aversive loud noise (Figure 3). The cue periods were too short to allow the measurement of HRV (see Methods), while MAP was unaffected by the CSs.

Bilateral inactivation of area 25 or area 32 disrupted this Pavlovian discrimination, and revealed that these areas have opposing roles in the evaluation of, or the expression of the response to, emotionally significant stimuli (Figure 4). Area 25 inactivation reduced anticipatory negative emotional responding: the cardiovascular and behavioral responses were attenuated in a CS-dependent manner, with the response to the CS fully predictive of aversive noise decreasing the most (Figure 4A/D). This pattern was mirrored by systemic diazepam, an anxiolytic drug (Figure 4C/F). These findings were not due to a change in overall locomotor activity impacting upon cardiovascular activity (see Figure S2/ Methods). Neither were they due to a generalised blunting of cardiovascular reactivity, or to changes in arousal, as the magnitude of the unconditioned arousal response to the loud noise was unaffected by drug treatment (Figure 5). Muscimol/baclofen did not affect US-induced HR changes in either area 25 or 32, and nor did diazepam.

In contrast to the reduction in conditioned negative responding following area 25 inactivation, area 32 inactivation increased negative emotional responding. The HR and behavioral responses were increased in a non-CS-specific manner, with a generalisation of the negative emotional responses (previously only seen to the most aversive CS) to the mid and least aversive CSs.

Area 25 inactivation enhances, and area 32 inactivation impairs, fear extinction

The findings from the aversive discrimination are consistent with data from human neuroimaging suggesting that activity within area 25 is associated with increased negative affect, and activity within area 32 is associated with increased positive affect. However they are inconsistent with data derived from the rodent fear conditioning literature in which the suggested function of the rodent PL is to enhance negative

emotion, while the IL acts to reduce it. It is possible that the observed inconsistencies could be due to differences between our mildly aversive discriminative fear paradigm – which uses multiple auditory cues, requires substantial training and does not investigate extinction – and rodent paradigms, which use a single cue to predict footshock and which are learnt in one session. We therefore adapted the classic rodent paradigm for use with marmosets by replacing the rodent US (footshock) with the sight of a plastic snake (see figure 6). Snakes are natural predators of marmosets and are ethologically, highly aversive stimuli (43) that are commonly used in laboratory tests of negative emotion (44). Snake stimuli also resist habituation (45), making them suitable for within-subjects investigation of the behavioural and cardiovascular effects of saline or muscimol/baclofen infusions into areas 25 and 32 as before.

As in the rodent paradigms, animals acquired a general (cue + context) conditioned behavioural response (vigilant scanning, VS) in one session that slowly extinguished under saline the next day and was followed by subsequent reinstatement and re-extinction of the conditioned response. They also showed a general conditioned cardiovascular response, with MAP showing a similar pattern (HR also increased but it was too variable to use as a measure of conditioning). These responses were stable across the repeated blocks of conditioning, which were distinguished from one another by having distinct contextual backgrounds, and were readily extinguished under saline conditions (see Figure 7).

As with the discrimination findings, bilateral inactivation of area 25 or area 32 during extinction revealed that these areas have opposing roles in the regulation of fear extinction responding (Figure 7). Compared to saline, inactivation of area 25 enhanced fear extinction of VS and MAP fear responses, indicating a reduction in fear (Figure 7A). In contrast, inactivation of area 32 did not affect the gradient of extinction for MAP but appeared to delay the extinction of VS such that VS increased across the early stage of extinction before it began to decline (Figure 7B). This VS increase also persisted throughout the next session when marmosets were tested for recall of extinction while drug-free. Recall was unaffected by area 25 inactivation. These effects on extinction were not due to an alteration in the ability of marmosets to express conditioned fear, as there was no difference in VS or MAP during the first CS pair of the extinction session after either manipulation. Thus area 25 inactivation reduces the autonomic and behavioural components of the conditioned fear response to enhance extinction, while area 32 inactivation causes an overgeneralised increase in the behavioural component of the fear response and blocks extinction.

Discussion

Using a novel experimental approach that takes into account brain-body interactions, the present study uses two different fear conditioning paradigms to show that area 25 inactivation reduces the behavioural and cardiovascular correlates of conditioned fear, and area 32 inactivation increases them via overgeneralisation. They provide the first causal evidence in primates to support the hypothesis that area 25 activity promotes negative affect and area 32 activity reduces negative affect, in both the behavioural and autonomic domains of emotion. These findings show remarkable similarity to correlative human imaging studies in which negative affect (both induced and pathological) is associated with activity in a subgenual, posterior ventromedial region centred on area 25, while positive affect is associated with activity in a distinct, more anterior region that includes areas 32, 24 and 10 (12). They also provide the first causal evidence that activity in area 25 regulates cardiovagal control in both the resting state (neutral condition) (compare , 46), and during alterations in emotional states in healthy subjects (aversive Pavlovian discrimination) (compare , 47).

The finding that area 32 inactivation in a primate caused overgeneralisation of emotional responses (in both the conditioned fear discrimination and single cue fear extinction paradigms) suggests that area 32 normally suppresses such generalised fear responding. Behavioural overgeneralisation of fear is a characteristic symptom of negative emotion and is seen in anxious humans (48), anxious marmosets (41) and anxious rats (49, 50), and has been associated with perigenual vmPFC (areas 10/24/32) hypoactivity in humans (51). We now causally link area 32 hypoactivity to such overgeneralisation in primates. When compared to the more limited autonomic effects of area 32 inactivation in emotionally neutral conditions, the discrimination findings provide support for human neuroimaging data that implicate area 32 in cardiovascular modulation during emotion regulation (see also , 52), and less in the tonic regulation of cardiovascular activity in non-emotional situations (13). In contrast to area 32, area 25 inactivation not only regulated the emotional responding induced by external stimuli, but also had profound effects on cardiovascular activity in an emotionally neutral condition, decreasing HR and MAP but also increasing CVI, the component of HRV that is reduced in both anxiety and depression (eg. , 15, 53). Furthermore, the demonstration that area 25 inactivation reduces anticipatory negative emotional responding and increases resting CVI suggests that area 25 overactivity directly contributes to the maladaptive and emotional inflexibility seen in such disorders. This may explain aspects of the strong bidirectional relationship between depression and cardiovascular disease (14, 54).

It is notable that HR (and not MAP) was more tightly linked to conditioning in the fear discrimination paradigm, but MAP (and not HR) was more tightly linked to conditioning in the fear extinction paradigm. Although it is aversive, the noise US used in the fear discrimination is not an ethologically relevant stimulus, and is therefore less likely to trigger a fight or flight response than the highly ethologically-relevant snake US used in the fear extinction paradigm. In contrast to HR, which is primarily controlled by the parasympathetic nervous system, MAP and fight or flight responses are predominantly sympathetically mediated, which may explain the dominance of the MAP response to the snake. The increase in MAP and HR seen after area 32 inactivation during negative emotional provocation is consistent with the autonomic activation and area 32 hypoactivity seen in some forms of anxiety (51).

As such, the convergence between the current findings in marmosets and human data is a marked improvement on the discrepancies between the rodent and human data. As judged by the dominant theory of rodent/primate vmPFC homology (55), our behavioural results differ from the large body of rodent fear conditioning studies in which inactivation of the putative rodent homologs of area 25 (the infralimbic cortex, IL) and area 32 (the prelimbic cortex, PL) have the opposite effects to that seen in marmosets, increasing and reducing negative emotional responding respectively (26, 31). It is possible that the anatomical similarity between IL/25 and PL/32 (24) may not translate into equivalent functionality, or that the functional role of IL and PL in fear conditioning and extinction is more complex than initially proposed (56). Certainly, the focus on negative emotion ignores similar roles for these regions in the recall and expression of positive emotion (57), in the control of goal directed instrumental responding (58, 59) and in social cognition (60). It has been suggested that the function of the PL region transcends fear expression and appetitive goal-directed instrumental responding, and is important in the ability to attend selectively to the elements of the environment that best predict an outcome, be it discriminating between a cue and its associated context in a simple Pavlovian paradigm using a single cue (46) or between two cues of a compound stimulus in a Pavlovian overshadowing procedure (61). Such a hypothesis could explain the generalised increases in conditioned fear responses seen here after inactivation of area 32 in fear extinction. However it remains to be determined whether it can explain the situation seen in the discriminative fear conditioning paradigm, where animals with inactivation of area 32 did not generalise conditioned fear responses to the context but instead showed generalisation from a CS+ to a CS-. IL, in contrast, has been implicated not only in inhibiting Pavlovian conditioned fear and conditioned appetitive responses during extinction in rodents (26), but also in attenuating the influence

of goal directed behavior when this conflicts with habitual stimulus-response associations (62). Of note, ablations of area 25 in a rhesus monkey alter the autonomic coding of appetitive stimuli (63). Recent reviews have attempted to reconcile these two aspects of IL function, particularly given that IL is central to visceromotor/autonomic circuits, and proposed that IL may 'allow' state-based habits to dominate behavior (64, 65). If so, the inconsistency of our results with current findings in rodents suggests that at the very least, more work is need to determine how IL/area 25 inactivation can induce opposing effects on fear extinction across rodent and marmoset studies. Alternatively we need to rethink the current views on cross species functional similarities and its translational implications.

Our understanding of the contribution of these areas to cardiovascular modulation is more limited, but both IL activation or PL inactivation have been shown to suppress the cardiovascular responses to acute restraint stress (21, 66) – again suggesting that these regions have opposing effects in rats, but in the opposite direction to that in marmosets and humans. Furthermore, in direct contrast to the cardiovascular alterations seen in the neutral condition in the present study, manipulations of the rodent medial PFC are consistently reported to have no effect on HR or BP in emotionally neutral resting conditions (21, 66).

In summary, we have isolated the specific contributions of areas 25 and 32 of the primate anterior cingulate cortex to the regulation of the cardiovascular and behavioural components of negative emotion. These results provide mechanistic insight into the correlative human imaging studies that implicate these regions in the regulation of negative emotion. This insight will be vital for understanding how dysfunction within areas 25 and 32 contributes to the behavioural and physiological symptomatology of emotion dysregulation in depression and anxiety, and how current and novel treatments may be better targeted. We demonstrate the utility of a novel primate model to study complex brain-body interactions, and highlight its importance for our understanding of the neural basis of affective processing.

Materials and Methods

Subjects and Housing

Seventeen experimentally naïve marmosets (*Callithrix jacchus*, 8 female, 9 male), bred on-site at the University of Cambridge Marmoset Breeding Colony, were housed in male/female pairs (males were vasectomised). Of these, 13 were used in the present studies, and 4 are referred to in the Supplement (see table S1). 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 suspended ladders, wooden branches and ropes to climb and swing on, and boxes to play in. 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 local Animal Welfare and Ethical Review Board.

Behavioural Testing Apparatus

Behavioural testing took place within a sound-attenuated box in a dark room. Animals were trained to enter a transparent Perspex carry box in which they were transported to the behavioural 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 auditory stimuli and a siren could be played. The apparatus was controlled by the Whisker control system (67) and in-house software (R. Cardinal). Three video cameras were positioned in the test chamber so that the movement of the animal within the Perspex box could be recorded by video software (CyberLink, Power Director, CyberLink Corp.). The video display was also shown on a monitor outside of the test apparatus, so the animal could be observed by the experimenter during testing.

Surgical procedures

All animals underwent two aseptic surgical procedures, one to implant intracerebral cannulae targeting either the area 25, area 32, or both, and one to implant a telemetric blood pressure monitor into the descending aorta. Both surgeries were completed prior to the animal beginning any behavioural 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 O_2 and placed into a stereotaxic frame modified for the marmoset (David Kopf, Tujanga, CA). Pulse-rate, O_2 saturation, breathing rate, and CO_2 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 the sgACC (26 gauge double cannulae, 7mm long, 1mm apart, Anteroposterior[AP] +14, Lateromedial [LM] +/- 0.5) and the pgACC (double cannulae, 2mm long, 1mm apart, AP +17, LM +/- 0.5 at a 30^O AP angle) adjusted where necessary in situ according to cortical depth (68). 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 Probe Surgery

Animals were anaesthetised as before, the descending aorta 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 just about the aortic bifurcation as described previously (35). 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 Treatments

For all sterile drug treatments the marmoset was held gently in a researcher's hand. For central infusions, 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 and 0.5µl of saline or 0.5µl of 0.1 mM muscimol/1.0 mM baclofen was bilaterally infused at a rate of 0.25 µl per minute. 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 25 minutes. For peripheral injections, the injection site was cleaned with alcohol and injected with either diazepam (0.25 mg/kg, i.m.; Wockhardt Ltd.) or an equal

volume of saline vehicle 30 min before testing. All cardiovascular and behavioural effects of drug treatments had returned to normal by the subsequent testing session.

Behavioural Testing Paradigms

1. Neutral Condition

After full recovery from both telemetry surgery and cannulation surgery, monkeys were habituated to the test apparatus for 4-8 fifteen-minute sessions during which time they were positioned inside the Perspex carry box in the test apparatus with the light on. No sounds were played. The number of habituation sessions was dependent on how quickly they acclimatised to the test apparatus. Each monkey was considered to be habituated when their heart rate did not change from one habituation session to the next, and they were observed to be completely calm for the whole session. They then received additional identical 15 minute sessions, prior to which they received infusion of saline or muscimol/baclofen into either area 25 or 32. Infusion days were interspersed with days when the animal was given a test session without an infusion to ensure any alterations in cardiovascular activity had returned to normal. All marmosets took part in the neutral condition.

2. Discrimination Condition

In contrast to most rodent fear conditioning paradigms which use a simple, single cue to predict electric shock presentation (26), here we use a milder, discriminative fear conditioning paradigm with multiple cues associated with different probabilities of aversive loud noise.

During each session, the animal was positioned inside the Perspex carry box within the testing apparatus and the houselight was on. Before the discrimination started, each animal received a probe session in which they were given with 4 presentations each of three distinct auditory conditioned stimuli (CSs; clicks, 10 clicks per second at 75 dB; intermittent tone, 440 Hz, 1 sec on, 0.1sec off at 75 dB; white noise at 75 dB). The innate, unlearned responses to each CS were assessed according to response magnitude (behavioural and cardiovascular). The CS that resulted in the largest response became CS1 and was paired with the least aversive US. The CS with the smallest response became CS3 and was paired with the most aversive US. The intermediate CS became CS2. This was to ensure that the discriminative responses shown by the animal were conditioned responses and not innate. Upon commencing the discrimination, each session contained twelve trials, with each trial consisting of the presentation of one of the three CSs (20s, 70-75dB), and the presentation of an unconditioned stimulus (US) immediately afterwards. The US was either a mildly aversive loud siren (117-120dB, 0.4-0.7s) or a non-aversive brief period of darkness (0.4s). These were combined to make 3 CS-US pairs that varied in aversivity (valence) and were each presented 4 times per session: CS1 was always followed by the mildly aversive siren, CS3 was always followed by the non-aversive darkness, and CS2 was followed on 50% of trials by the siren, and by darkness on the other 50%. The trials were presented in a pseudorandom order with a variable intertrial interval (ITI = 100-160s) and the three different CS-US pairings were counterbalanced across animals. Of the 9 animals who went on to learn the aversive discrimination, one animal failed to learn it and was dropped from the study.

For each trial, the mean heart rate (HR) was calculated for the 20 sec CS presentation and for the immediately preceding 20 sec baseline (BL) period (the last 20 seconds of the preceding intertrial interval). For each CS presentation, the CS-specific HR was calculated as (HR during the CS) – (HR during BL). Significant discrimination performance was defined as a difference in CS-directed HR between the most aversive and the least aversive CS that was statistically significant (within data from that subject) across two consecutive sessions. Upon reaching discrimination criterion, the marmoset received a drug treatment before testing on the next two test sessions. This could be central infusions or peripheral injections of diazepam. Following this, animals were required to regain discrimination criterion before

another infusion was performed. Two animals failed to regain significant discrimination performance after peripheral diazepam treatment which meant that the effect of peripheral saline could not be assessed. For this reason the diazepam results are compared to the effects of saline infusions into area 32, as it was the most recent, and therefore comparable, saline treatment in those animals.

3) Fear conditioning and extinction

Animals were given four cycles of fear conditioning, extinction and recall. Each cycle consisted of five sessions, spread over 5 consecutive days; two sessions of habituation to the context, one session of fear conditioning, one session of fear extinction, and one session of extinction recall. During each session, animals were positioned inside the Perspex carry box within the testing apparatus as before, but each time the cycle was repeated there was a different context. This was achieved by covering the inside of the testing apparatus with distinctive black and white patterned screens. For the two habituation sessions animals were given 12 unconditioned stimulus (US) presentations whereby a sliding door was opened to reveal a hidden chamber, for 5s. The animal was then given one session of fear conditioning; 12 trials consisting of the presentations of an auditory conditioned stimulus (CS, 25s, 70-75dB), paired with a US presentation which started 20s after CS onset, so that the last 5s of the CS was concurrent with the duration of the US. There was a variable inter-trial interval of 110-130s. The first five CS presentations were paired with a US^{--} where the sliding door was opened to reveal a hidden chamber; however, the subsequent seven CS presentations were paired with a US+ where the sliding door opened to reveal a hidden chamber containing a life-like plastic cobra snake. The following day, the animal had a session of fear extinction: 20 trials consisting of the presentation of the CS-US- pairing, on a variable inter-trial interval of 60-80s. Finally, the animals were given a session to test for recall of fear extinction: 15 trials consisting of CS-US- pairing, on a variable inter-trial interval of 60-80s.

Data Analysis

Telemetry data collection and analysis

Blood pressure (BP) data was continuously transmitted by the implanted probe to a receiver for offline analysis using Spike2 (Version 7.01, CED), as described previously (35). 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. For the neutral condition, mean arterial blood pressure (MAP) was calculated from the systolic and diastolic blood pressure for each cycle using MAP = diastolic BP + (1/3 (systolic BP – diastolic BP)). To obtain HR and heart rate variability (HRV) measures for the 15 minute duration of the neutral condition, the interbeat intervals (IBIs, calculated as the time interval between systolic BP events) were imported to Kubios HRV version 2.1 (69) and were corrected for artefacts, using the low artefact correction setting. In contrast to the low setting, artefact correction with the strong setting increases the risk of accidental elimination of valid data. Analysis with the strong setting, however, did not change the results (see Supplement). Average HR and HRV (root mean squared standard deviation (RMSSD) of the time difference between consecutive IBIs, a time-domain measure of HRV) were calculated for each session. In order to assess indices of vagal and sympathetic activity, Poincaré plots (plot of IBI_{j+1} as a function of IBI_j) were created in Kubios. The standard deviation of the points perpendicular to the line of identity (SD1), and the standard deviation of the points along the line of identity (SD2) (70) were used to derive indices of autonomic activity; the cardiac vagal index (CVI) and the cardiac sympathetic index (CSI) (37). HRV analysis requires a certain number of IBI pairs to be valid (approximately 100). While the 15 minute session of the neutral condition is ample (an average of 14000 IBIs), it was therefore not possible to calculate HRV measures in response to the 20 s cue

presentations in the fear discrimination or fear extinction paradigms. There was no evidence that animals deviated from sinus rhythm (see Supplement).

Behavioral analysis

Behavior during the discrimination was recorded and subsequently scored by an experimenter. The specific behavior scored was vigilant scanning (VS), defined as CS-related anxious-like behaviours including watchful scanning of surroundings accompanied by tense, vigilant body posture (71, 72). The time the animal spent engaged in this behavior during the 20s CS period and 20s BL period was scored. CS-specific VS was calculated as the difference between these two (exactly as for CS-specific HR). A second person blind to the conditions of the experiment scored a subset (one in five) of the discrimination sessions. Interscorer reliability was high (r_{480} = 0.817, P < 0.005). For control purposes, overall locomotor activity (defined as the duration of movement including translational movement with all four paws moving and upper body movement causing a change in direction of the animal of 90 degrees or more) was also scored (see Supplement).

Statistical Analysis

For the neutral condition, the average HR and MAP were calculated for the 15 min session, and this data used to calculate the HRV as before. It was not possible to quantify VS during the neutral condition. This is because VS is by definition, CS-related, and because the neutral condition did not include any CSs, there was no VS.

For the discrimination, each monkey's CS-directed HR or VS during the experimental manipulation days (saline, muscimol/baclofen or diazepam) was standardized to the same monkey's CS-induced heart rate during the preceding 2 day block of baseline behaviour, using y' = (y - y_mean) / y_SD, where y = individual CS-induced change in HR or VS during experimental days, y' = standardized y, y_mean = subject's own mean CS-induced change in HR or VS during baseline days and y_SD = subject's own standard deviation of CS-induced change in HR or VS during baseline days. This was done because of substantial inter-monkey variability in baseline HR/VS and the magnitude of HR/VS changes. Within-subject standardization therefore increases the power to detect drug-induced changes. A small number of CS datapoints were removed from the dataset due to animals vocalising during the BL or the CS, which materially changed the HR. In all cases the VS/HR during a 20 sec CS was also compared to the immediately preceding 20 sec baseline period. This was so that any changes seen in the neutral condition could be eliminated from the discrimination data analysis: e.g. where area 25 inactivation reduced HR in the absence of cues, it would do so across the whole session and affect both baseline and CS periods equally. Thus, any measured changes in CS responses were due to the altered interpretation of the CSs and not due to an overall reduction in HR. For the US analysis the period around the onset of the US was compared to the last 5 seconds of the baseline and averaged across all 8 of the most aversive CSs. The peak US-induced HR increase was then compared to the HR immediately preceding the US onset.

ANOVA was performed with R version 3.2.2 (73) using the lme4 package for linear mixed-effects modelling, statistical tests from the lmerTest package and type III sums of squares with the Satterthwaite approximation for degrees of freedom (here reported to the nearest integer). For the discrimination, area (sgACC or pgACC) and manipulation (saline vs drug) were both factors, and CS valence was a linear/continuous predictor (-1 least, 0 mid, 1 most). The assumption of a normal distribution of residuals was verified with the Shapiro-Wilk test. In 2 analyses (the impact of drug infusions on CSI on the neutral condition, and the impact of saline infusion on visual scanning at baseline) there were marginal violations of the normality assumption ($p < .05$), which were minor as judged by a Q-Q plot,

and to which ANOVA is robust (74). Alpha was controlled per ANOVA and for all sub-analysis thereafter, as is the norm (74).

For the fear extinction the overall mean ΔMAP during CS 2-6 of the conditioning sessions (when the animal is expecting the US-) was subtracted from the remaining data points for each subject, to normalise each subject's snake conditioning scores to their own individual MAP baseline. This was due to high inter-individual variability in MAP. CS1 was not used as it was the first time the animal had heard that CS and could therefore be confounded by novelty-induced arousal. ΔVS was calculated the same way.

For analysis of both ΔMAP and ΔVS data, trials were averaged in trial blocks of two (26). ANOVA was performed to analyse the effects of area 25 and area 32 infusions on the CS ΔMAP/VS responses with R as above. Area (25 or 32), drug treatment (saline or muscimol/baclofen) were discrete factors, and subject a random factor. For analysis of conditioning and recall sessions CS-trial block was analysed as a discrete factor. However, for the purpose of analysing the *gradient* of extinction, CS-trial group was treated as a linear predictor. For analysis of CS-directed effects, the scores for the preceding 20 sec baseline (the last 20s of the ITI) were subtracted from the CS scores without normalisation to CS2-6 (the comparison to baseline providing the normalisation).

Since all animals had at least four repeats of conditioning, extinction and recall, an ANOVA was performed to assess whether this repetition affected the acquisition of fear conditioning. As conditioning took place without any infusions, each animal's first four conditioning sessions were included in this analysis, even if the extinction and recall data was not analysed further.

***Post mortem* assessment of cannulae placement**

Animals were premedicated with ketamine hydrochloride (Vetelar; 0.05 ml of a 100 mg solution, i.m.; Amersham Biosciences and Upjohn, Crawley, UK) before being euthanized with pentobarbitol 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.

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Author Contributions

- CUW, Study design, Acquisition of data, Analysis and interpretation of data, Drafting the article
- RNC, Analysis and interpretation of data, Drafting the article
- LA, Acquisition of data, Drafting the article
- ACR, Conception and study design, Drafting the article
- HFC, Conception and study design, Analysis and interpretation of data, Drafting the article

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Figure legends

Figure 1: Behavioral conditions and prefrontal cortex cannulae. **A**. In the neutral condition (n=13), animals were placed in a familiar environment for 10 min in the absence of any cues. **B**. In the discrimination condition (n=7), animals were played three distinct conditioned stimuli (CSs) that were followed probabilistically by two distinct outcomes (unconditioned stimuli; USs) that varied in aversiveness (**C**). **D**. Experimental sequence. Monkeys experienced the neutral condition before learning the aversive discrimination **E**. Each 20 second CS was presented four times in a pseudorandom order, with an intertrial interval (ITI) of 100-160 secs. **F**. Glass brain illustrating the rostro-caudal locations of area 25 and area 32 and the double intracerebral cannulae targeting each area. **G.** Representative histological sections with arrows marking the position of the cannulae tips and cannulae locations for each animal. All area 32 cannulae were located within the range of AP 15.8–16.6 and all area 25 cannulae were located within the range of 12.5-14, plotted here on a single coronal section for each target area. Black circles indicate animals within the neutral condition only (area 25, n=6; area 32, n=7) and gray circles indicate the animals within both the neutral condition and the discrimination (area 25, n=4; area 32, n=4). Cytoarchitectonic parcellation was based on (73). The circles represent the estimated maximal spread of the muscimol/baclofen or saline infusions (74).

Figure 2. Inactivation of area 25 and area 32 had differential effects on cardiovascular activity in an emotionally neutral environment. Compared to saline, anatomically selective inactivation of area 25 and area 32 with muscimol/baclofen (musbac) had different effects (area x treatment) on each of HR (beats per minute; $F_{1,17.7}$ = 5.966, P= 0.025, η^2 =0.052), MAP (mmHg; $F_{1,18}$ = 17.9, P= 0.0004, η^2 = 0.09) and HRV ($F_{1,17.8}$ = 7.514, P= 0.013, η^2 =0.052). Area 25 inactivation decreased HR ($F_{1,5}$ = 19.151, P= 0.007, d=2.01) and MAP ($F_{1,5}$ = 6.32, P= 0.05, d=1.61) and increased HRV ($F_{1,5}$ = 10.392, P = 0.023, d=1.17). The HRV change was due to an increase in the cardiac vagal index (CVI; $F_{1,5}$ = 8.1436, P= 0.035, d=0.61) without

change in cardiac sympathetic index (CSI; $F_{1,19.1}$ = 1.2, not significant [NS]). In contrast, area 32 inactivation increased MAP only ($F_{1,9.99}$ = 12.4, P= 0.0052, d=0.36; HR and HRV: F < 1, NS). Bars show mean ± standard error of the mean (SEM); SED: standard error of the difference for the interaction term; *P < 0.05.

Figure 3: Animals learned to discriminate the three conditioned stimuli (CSs) and this discrimination was not disrupted by central infusions of saline. A. Example learning traces from one animal show how the traces separate out as learning progresses, with CS-directed heart rate increases (in beats per minute; bpm) that mirror the aversiveness of each CS. In each case the heart rate during the CS is compared to the last 20 seconds of the preceding ITI (the 'baseline'). Criterion was defined as displaying a significant difference in HR between the most and least aversive CSs across two consecutive days of testing. Central infusions commenced once the criterion had been reached. Saline infusion into either area 25 or area 32 did not disrupt the significant heart rate (**B**) or behavioral (**C**) discriminations. Baseline values were averaged across both areas, and before both drug and saline manipulations (therefore 4 baselines per animal; baseline discrimination: heart rate, $F_{1, 13}$ = 7.4, P= 1.6 x $10^{-6,}$ d = 2.6 ; vigilant scanning, $F_{1, 13}$ = 104.03, P= 1.43 x 10^{-7}, d =2.7). Saline infusions were averaged across each area (therefore 2 saline treatments per animal; saline discrimination, heart rate, $F_{1, 13}$ = 34.94, P= 5x $10^{-5,}$ d = 1.8; vigilant scanning, $F_{1, 13}$ = 24.47, P= 0.00026, d = 1.8). Saline infusions did not disrupt discrimination performance (all terms including treatment (saline or baseline); P> 0.05, NS). The number of dots on each column indicate the per-subject mean for each baseline/saline treatment; the discriminative response remained stable during the experiment. * P<0.05. Least, mid, most refer to the CS aversiveness; n=4 for each area.

Figure 4: Area 25 inactivation and diazepam treatment abolished the increases in heart rate (HR) and vigilant scanning (VS) induced by conditioned stimuli (CSs) associated with aversive outcomes, with effects that were more pronounced as the CSs became more predictive. Area 32 inactivation caused overgeneralisation in a way that did not depend on the CS. Main figures show the CS-induced change in HR or VS, under drug and saline conditions, relative to the last 20 seconds of the immediately preceding ITI (the 'baseline'), standardized to that subject's own variability (measured independently; see Methods). Positive numbers indicated an increase in HR or VS from baseline, while negative numbers indicate a decrease compared to baseline. * P< .05, $^{\#}$ P< .05 manipulation x CS interaction, † P< .05 main effect of manipulation. SED shown is for the drug × CS interaction; n = 4. Insets show the drug-induced changes relative to saline (error bars: SEM; L/Mi/Mo, least/middle/most aversive CS). **A, D:** Area 25 inactivation with muscimol/baclofen abolished discrimination, altering responding in a CS-dependent manner, as assessed by CS-induced changes in HR (drug × CS, $F_{1,17}$ = 5.11, P= .037, η^2 = 0.117; effect of CS during inactivation, $F_{1,7}$ = 1.87, NS) and VS (drug × CS, $F_{1,17}$ = 7.84, P= .012, η^2 =0.113; effect of CS during inactivation, $F_{1,7}$ = 1.57, NS). Discrimination was retained under saline infusion (effect of CS: P = .009, η^2 = 0.557 for HR and .0003 for VS, η^2 = 0.685). These effects differed from those of area 32 inactivation (HR: drug × area, $F_{1,34.7}$ = 14.7, P= .0005, η^2 = 0.165; VS: drug × area, $F_{1,33.4}$ = 24.2, P= .00002, η^2 =0.198). **B, E:** Area 32 inactivation similarly impaired discrimination, but here the effects of inactivation were not specific to a particular CS (HR: drug, $F_{1,20}$ = 16.3, P= .00065, η^2 = 0.384; drug × CS, $F_{1,20}$ = 2.50, P= .129, η^2 =0.059; effect of CS during inactivation, F < 1, NS; VS: drug, $F_{1,17}$ = 5.27, P= .035, η^2 =0.107; drug × CS, $F_{1,17}$ = 2.45, P= .136, η^2 =0.050; effect of CS during inactivation, $F_{1,10}$ = 3.3, P= .099, η^2 =0.248). Discrimination was similarly retained under saline (P= .0047 for HR, η^2 =0.566 and .0125, η^2 = 0.473 for VS). **C, F:** Systemic diazepam (0.25 mg/kg) impaired discrimination by altering responding in a CS-dependent manner (HR:

drug × CS, $F_{1,20}$ = 3.77, P= .066, η^2 = 0.085; drug, $F_{1,20}$ = 7.97, P= 0.011, η^2 =0.180; VS: drug × CS, $F_{1,17}$ = 8.16, P= .011, η^2 =0.159), similar to the effects of area 25 inactivation.

Figure 5. The US response to the most aversive CS is unaffected by inactivation of area 25, area 32, or diazepam treatment, indicating that alterations specifically in conditioned, but not unconditioned, negative arousal are responsible for the alterations in heart rate seen during CS presentation. US-induced change in heart rate (HR, beats per minute; bpm) under drug (muscimol/baclofen and diazepam) and saline conditions, relative to the last 5 seconds of the baseline. Data is shown for the last 4 seconds of the CS and the first 4 seconds after the US onset at the 40 second time point (see Supplementary) and is the mean of the 8 presentations of the CS fully predictive of the aversive US for each condition. Inset: the absolute change in HR response (the difference between HR at US onset and the peak HR response). Repeated measures ANOVA revealed a significant difference in HR between US onset and US peak ($F_{1,3}$ =57.843, P = 0.005, η^2 = 0.951) but no interaction with drug treatment ($F_{4,12}$ =0.663, P = 0.630). There were also no significant differences in US-induced HR increases between area 25 saline and area 25 muscimol/baclofen (t_3 =-0.452, P= 0.682), area 32 saline and area 32 muscimol/baclofen (t_3 =-0.762, P = 0.501), or area 25 muscimol/baclofen and diazepam (t_3 =0.812, P = 0.476).

Figure 6. Within-subject fear conditioning and extinction paradigm. A. Animals were given four cycles of fear conditioning, extinction and recall. Each cycle consisted of five sessions, spread over 5 consecutive days: two sessions of habituation to the context, one session of fear conditioning, one session of fear extinction, and one session of extinction recall. On days 1 and 2 **(i)**, animals were given two days of habituation where they received 12 presentations of the US- (5 s duration, ITI = 110-130 s; door opens to reveal an empty chamber). On day 3 **(ii)** marmosets received one session of fear conditioning which comprised 5 presentations of an auditory CS (25 s, 70dB) paired with the US-, followed by 7 presentations of the same CS paired with the US+ (5 s, ITI=60-80 s; the door opening to reveal the chamber containing the plastic snake for the last 5 s of the CS). On days 4 and 5 **(iii)** the marmosets were tested for extinction and recall where the CS was presented in extinction (extinction, 20 CS-US- presentations; recall, 15 CS-US- presentations; ITI = 60-80 s). **B.** Each time the cycle was repeated the inside of the test apparatus was covered with distinctive black and white patterned screens to create a different context and a different CS cue was used. Contexts, cues and context/cue combinations were counterbalanced across animals.

Figure 7. Area 25 inactivation enhanced the behavioural and cardiovascular correlates of fear extinction. Area 32 inactivation impeded behavioural correlates of fear extinction in a way that did not depend on the CS. Graphs show the change in VS or MAP under drug and saline conditions, Figures **ii** and **iii** in each block represent the CS responses irrespective of baseline. Positive numbers indicate an increase in HR or VS from baseline, while negative numbers indicate a decrease compared to baseline. * P< .05, $^{\#}$ P< .05 manipulation x CS interaction, † P< .05 main effect of manipulation, error bars = ± SEM. Area 25, n = 3. Area 32 VS, n = 5. Area 32 MAP, n = 4. **Ai/Bi**. Representative cannulae locations for each animal, plotted here on a single coronal section for each target area. A separate cohort of 8 monkeys learned this new paradigm. **A/B ii and iii.** Subjects across both groups acquired conditioned behavioural and cardiovascular responses in one session. There was a robust effect of the CS (analysing raw pre-US data for the first three CS pairs as a four-level factor, across area 25/32 subjects with area as an

additional predictor; VS: $F_{1,53.9} = 46.521$, $p = 8.14 \times 10^{-9}$, $\eta^2 = 0.333$; MAP: $F_{1,47.1} = 5.00$, $p = 0.030$, $\eta^2 = 0.018$), with no differences in conditioning between subjects by subsequent infusion area (terms involving area: $F < 1$, NS, for both MAP and VS). This response was not altered across repeated conditioning cycles (analysing data as before, across all cycle repeats, with repeat and area as additional predictors; terms involving repeat, $F < 1.64$, $p > 0.2$, NS, both for VS and MAP). These responses were readily extinguished under saline treatment in both areas (VS: CS pair, $F_{9, 54} = 6.169$, $P < 0.001$, MAP: $F_{9, 45} = 5.513$, $P < 0.001$). **Aii-iii**. Area 25 inactivation with muscimol/baclofen reduced negative emotion by enhancing the extinction of both the VS and MAP responses. There was a greater reduction in the expression of VS over the course of the extinction sessions (VS: drug x CS-pair, $F_{1, 54} = 8.297$, $P = 0.0057$, $\eta^2 = 0.030$, **Aii**) and a trend towards a greater decrease in MAP (drug x CS-pair, $F_{1, 54} = 3.046$, $P = 0.08661$, $\eta^2 = 0.012$, **Aiii**). These effects were not due to an effect on marmosets' ability to express conditioned fear, as there was no difference in VS/MAP during the first CS-pair of the extinction session (VS: $t_2 = 0.48$; $P = 0.678$, MAP: $t_2 = -0.32843$, $P = 0.7738$), and were different from those of area 32 inactivation (VS: area x drug x CS-pair: $F_{1, 146} = 6.233$, $P = 0.0136$, η^2=0.011, MAP: drug x CS-pair, $F_{1, 127} = 4.253$, $P = 0.0412$, $\eta^2 = 0.009$; area x drug x CS-pair, $F_{1, 127} = 1.205$, $P = 0.2743$). **Bii-iii.** Area 32 inactivation also altered extinction, but here the effects of inactivation were not specific to the CS-pairs and the gradient of extinction was not altered for VS or MAP (VS: drug x CS-pair, $F_{1, 92} < 1$; drug, $F_{1, 92} = 1.8140$, $P = 0.1813$; MAP, drug x CS-pair $F_{1, 73} < 1$). However, given that VS behaviour was much higher after inactivation of area 32, a *post hoc* analysis asked whether inactivation affected VS independently of the extinction process. This revealed an extinction-independent increase in general VS (drug, $F_{1, 76} = 128.171$, $P < 2.2 \times 10^{-16}$, $\eta^2 = 0.502$; drug x CS-pair, $F_{9, 76} < 1$, **Bii**), and indicates a significant generalised increase in fear throughout the session, despite no difference in VS during the first CS-pair of the extinction session ($t_4 = 1.13$; $P = 0.32$). This generalised increase in VS (not MAP, **Biii**) after area 32 inactivation persisted throughout the next session when marmosets were tested on extinction recall while drug free, but recall after area 25 inactivation was unaffected (area x drug: VS, $F_{1, 36} < 1$; MAP, $F_{1, 64} < 1$. Area 32: VS; drug, $F_{1, 52} = 40.69$, $P = 4.82 \times 10^{8}$, $\eta^2 = 0.233$, drug x CS-pair, $F_{6,52} < 1$; MAP: drug, $F_{1, 39} < 1$. Area 25: drug; VS, $F_{1, 26} < 1$, MAP, $F_{1, 26} < 1$).