Manipulating immune pathways differentially impact emotionality: dissociable effects of complement C3 and C3aR on learned fear and innate anxiety

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Abstract

Background Recent findings implicate complement, a key component of the immune system, in healthy brain functioning and risk for psychiatric disorders. Altered emotional function, in particular maladaptive fear and anxiety, is a pervasive and clinically important symptom across several neuropsychiatric disorders. In this work we examined the extent to which different complement pathways impact on dissociable components of anxiety and learned fear.

Methods: *C3*$^{-/-}$ and *C3aR*$^{-/-}$ mice were assessed in a battery of tests assaying innate anxiety and learned fear. The effects of systemic diazepam (2mg/kg) and the C3aR antagonist SB290157 (10mg/kg) on behaviour was assessed. Expression of genes linked to anxiety, stress responses and risk for psychiatric disorder were assessed in brain regions implicated in fear and anxiety.

Results: The data indicated a robust and highly reproducible double-dissociation of C3 and C3aR on learned fear and innate anxiety, respectively. We probed downstream complement pathways responsible for the C3-specific effects on learned fear and excluded contributions from the terminal pathway, C6 to C9. The selective anxiety phenotype in *C3aR*$^{-/-}$ mice was resistant to the anxiolytic properties of diazepam and acute SB290157 administration in wildtypes did not phenocopy constitutive *C3aR* knockout, suggesting *C3aR*$^{-/-}$ anxiety phenotypes resulted from long-term *C3aR* deficiency. Expression of the calcium voltage-gated channel subunit Alpha1c (*Cacna1c*) mRNA, an established psychiatric risk gene, was selectively increased in *C3aR*$^{-/-}$ mice.

Conclusions: These findings reveal novel functions and unexpected dissociations between the C3 and C3aR pathways impacting on specific emotional behaviours relevant to psychiatric disorder.

Introduction

The complement system is a key component of the immune system that plays a pivotal role in inflammation and host-defence. Complement activation occurs via several pathways, all of which lead to cleavage of the central protein C3 (see Supplementary Figure S1)(1). Activation of C3 generates the fragments C3a and C3b. C3a is an anaphylatoxin which upon binding to its canonical G-protein coupled receptor C3aR, induces both pro- and anti-inflammatory effects(2). The C3b fragment plays a role in opsonization by macrophages and microglia via complement receptor 3 (CR3) and propagates further complement activation by contributing to the cleavage of complement component 5 (C5). Akin to C3, C5 cleavage yields C5a, a ligand for the C5a receptor (C5aR), and C5b, which triggers the terminal complement pathway by binding proteins C6, C7, C8 and C9 which form the membrane attack complex (MAC), leading to lytic cell death or activation(3). Neurons, astrocytes and microglia express complement receptors and are capable of synthesising complement proteins(4; 5), the expression patterns of which vary over the course of brain development 6).

It is increasingly recognised that complement has versatility beyond its canonical immune functions(7). In the CNS, evidence has implicated complement in neurodevelopmental processes including neurogenesis(8), migration(9) and synaptic pruning(10), and in the pathogenesis of neurodevelopmental, neurodegenerative and psychiatric conditions. In Alzheimer's disease, genetic variants in complement related loci have been associated with increased disease risk(11; 12), and complement knockout mice exhibit reduced age-related synapse loss(13) and neuropathology(14). Alterations in complement proteins and activation have also been reported in serum from individuals with autism-spectrum disorder(15)

schizophrenia(16), major depressive disorder(17), bipolar disorder(18) and post-traumatic stress disorder(19). In the case of schizophrenia, an important finding comes from elegant genetic work demonstrating that structural variation in the *C4* locus is associated with risk of developing the disease(20). C4 cleavage generates fragments that contribute to the activation of C3, yielding C3a and C3b. Given the known roles for C3b/CR3 in synaptic pruning(10; 21), it has been suggested that *C4* variants may impact on psychiatric risk via this mechanism, leading to abnormal connectivity and disruption of neural networks(20). Variants in *C3* and putative complement-control genes *CSMD1* and *CSMD2* have also been implicated in genetic susceptibility for schizophrenia(22; 23).

Altered emotional function, in particular excessive fear and anxiety, is a pervasive and clinically important symptom that is comorbid across several of the DSM-5 and ICD-11 defined disorders in which complement has been implicated. Anxiety is characterised by sustained arousal, negative affect, apprehension and hypervigilance surrounding anticipated or potential threats, while fear is an acute response to an experienced, imminent danger(24). It is important to note that while these processes share some underlying neurobiology, they also have contributions from distinct neuronal circuitries(25). Although links between anxiety and inflammation have been documented(26), there has been little investigation of the specific role of complement in anxiety and fear. In animal models, prenatal manipulations increasing C3 deposition in the fetal brain are associated with adult anxiety phenotypes(27). Additionally, complement inhibitors reduced anxiety in a model of Alzheimer's disease(28) and aged *C3* deficient mice exhibit reduced anxiety alongside enhanced contextual fear responses(13). These studies suggest that complement influences fear and anxiety; however, the precise relationship

between signalling within discrete pathways of the complement system (e.g., C3a/C3aR or C3b/CR3 axes) and their relationship to dissociable aspects of anxiety and fear and underlying brain function is unknown.

In this work we used knockout mouse models to manipulate C3 and C3aR signalling in order to functionally parse anxiety and fear related phenotypes. In homozygous *C3* knockout mice(29) complement cannot be activated beyond C3, and therefore these animals lack C3 activation fragments (C3a, C3b) and downstream activation products (C5a, C5b) thus cannot activate the terminal complement pathway. Phenotypic effects in this model could therefore be the result of disturbance to any downstream effector molecule(s). We compared the *C3*$^{-/-}$ model with homozygous *C3aR* knockout mice(30). In these mice, complement is intact apart from the capacity for C3a to bind to its canonical receptor C3aR and here we tested the extent to which phenotypic effects were the result specifically of C3a/C3aR signalling. *A priori*, because C3a is an obligate cleavage fragment of C3 we hypothesised that any phenotypes dependent on the binding of C3a to C3aR would be apparent in both *C3*$^{-/-}$ and *C3aR*$^{-/-}$ models.

In contrast to this initial expectation we found completely different effects of the two models on emotional behaviours. *C3aR*$^{-/-}$ mice displayed a profound innate anxiety phenotype across a range of measures that was lacking in the *C3*$^{-/-}$ model. When we examined learned fear (where a previously neutral cue generates a fear response as a result of predicting an aversive outcome) we found that the dissociation was reversed, with the *C3*$^{-/-}$ mice exhibiting an enhanced fear response to a conditioned cue, but no differences between wildtype and *C3aR*$^{-/-}$mice. We probed downstream complement pathways responsible for the C3-specific effects on learned fear and excluded contributions from the terminal pathway by using *C6*$^{-/-}$ mice. These findings

point to a hitherto unrecognized complexity of complement effects on brain function and behaviour of potential relevance to emotional dysfunction in psychopathology.

Materials and Methods

For additional materials and methods, see Supplementary Information.

Animals. Wildtype and *C3*$^{-/-}$ strains were sourced from The Jackson Laboratory (strain B6.PL-Thy1^{a}/CyJ stock#000406 and B6;129S4-C3tm1Crr/J stock#003641 respectively), whereas *C3aR*$^{-/-}$ mice were provided by Craig Gerard of Boston Children's Hospital, USA and *C6*$^{-/-}$ mice were an in-house strain. *C3*$^{-/-}$, *C3aR*$^{-/-}$ and *C6*$^{-/-}$ strains were maintained via homozygous breeding on a C57Bl/6J background. Male mice (3-8 months old) in littermate groups (2-5 mice/cage) were kept in a temperature and humidity-controlled vivarium (21±2°C and 50±10%, respectively) with a 12-hour light-dark cycle (lights on at 07:00hrs/lights off at 19:00hrs). Home cages were environmentally enriched with cardboard tubes, soft wood blocks and nesting materials. Standard laboratory chow and water were available *ad libitum*. All procedures were performed in accordance with the requirements of the UK Animals (Scientific Procedures) Act (1986). Sample sizes for each experiment are provided in the corresponding Figure.

General behavioural methods. Testing took place between the hours of 09:00 and 17:00, with random distribution of testing for subjects of different genotypes throughout the day. Mice were habituated to the test rooms for 30 min prior to testing. All assays involved individual testing of mice and apparatus was cleaned thoroughly with a 70% ethanol solution between subjects. Data for the EPM, EZM

and Open Field were collected using EthoVision XT software (Noldus Information Technology, Netherlands).

The elevated plus maze (EPM). Subjects were placed at the enclosed end of a closed arm and allowed to freely explore for 5 min. Data from each pair of arms were combined to generate single open and closed arm values. In addition, the following parameters were manually scored: number of stretch-attend postures (SAPs; defined as the animal slowly and carefully reaching towards the open arms in a low, elongated body posture(31; 32)), number of head dips (looking over the edge of an open arm) and grooming frequency.

The elevated zero maze (EZM). Subjects were placed at the border of one of the open and closed regions and allowed to freely explore for 5 min. Data from each pair of regions were combined to generate single open and closed region values. In addition, the number of head dips (as above) were measured.

Assessment of fear potentiated startle (FPS). FPS was assessed using a pair of ventilated and soundproofed SR-LAB startle chambers (San Diego Instruments, CA, USA). FPS consisted of three separate sessions presented over a two-day period (see Figure 7A). FPS was determined as the fold change between pulse-alone trials and pulse+CS trials within the post-conditioning session.

Pharmacological manipulations

Diazepam A three-day dosing regimen of diazepam (2 mg/kg, i.p., Hameln Pharmaceuticals, UK) or an equivalent volume of vehicle (0.1 M phosphate buffered saline, pH 7.4) was used. Following 2 days of pre-treatment, diazepam or vehicle was administered 30 min prior to testing on the EPM on the 3rd day.

C3aR antagonist Two dosing regimens were used to assess effects of SB290157(33) (N2-[2-(2,2-Diphenylethoxy)acetyl]-L-arginine trifluoroacetate salt; 10 mg/kg, i.p, Sigma Aldrich, UK) or equivalent volume of vehicle (0.1 M phosphate buffered saline, pH 7.4, with 1% DMSO) on behaviour.

Acute SB290157 or vehicle was administered 1 hour prior to testing on the EPM.

Semi-chronic SB290157 or vehicle was injected twice daily for five days and subjects were tested on the EPM on the 6th day.

Corticosterone measurements in mice subjected to the EPM. Testing took place between the hours of 10:00 and 14:00 to account for the diurnal pattern of corticosterone release(34). Subjects were culled 20 minutes post-EPM by cervical dislocation. Control mice were removed from their home cage and immediately culled. Plasma was collected from trunk blood and frozen at -80°c until required. Corticosterone ELISA was performed according to manufacturer's instructions (ADI-900-097, Enzo Life Sciences, UK).

Quantitative Polymerase Chain Reaction (qPCR) Tissue from behaviourally naïve mice of each genotype was used to assess gene expression using standardised quantitative qPCR methods (see Supplementary Information), with quantification using the $2^{-\Delta\Delta Ct}$ method (35). All samples were run in triplicate and samples differing by >0.3 Cts were excluded. The change in expression of genes of interest, after normalisation to two house-keeping genes (ΔCt) was transformed to yield $2^{-\Delta\Delta Ct}$ values. Relative changes from wildtype expression were calculated for each gene of interest.

Statistical analysis. All statistical analyses were carried out using GraphPad Prism 8.0 (GraphPad Software, CA, USA). Data was assessed for equality of variances using the Brown-Forsythe test and then appropriate parametric (one-way or two-way ANOVA) or non-parametric (Kruskal-Wallis) tests used. *Post hoc* pairwise comparisons were performed using the Tukey or Dunn's tests for parametric or non-parametric analyses, respectively. Exact p values were reported unless $p<0.0001$. All p values were multiplicity adjusted(36). Data are expressed as mean + standard error of the mean.

Results

Increased innate anxiety in *C3aR*$^{-/-}$ but not *C3* $^{-/-}$ mice

We assessed emotional reactivity in the elevated plus maze (EPM), a well-established test of innate anxiety in rodents(37; 38). As demonstrated in Figure 1A, heatmaps indexing overall maze exploration indicated significant differences in open arm exploration between genotypes (see Supplementary Video 1 for representative examples). In comparison to wildtype and *C3*$^{-/-}$ mice, *C3aR*$^{-/-}$ mice took longer to first enter the open arms (Figure 1B) and spent less time there per entry (Figure S2A), leading to a reduced overall duration spent in the open arms (Figure 1C). The ethological parameters of head dips and SAPs also differed (Figure 1D,E), with *C3aR*$^{-/-}$ mice exhibiting a significant reduction in the former and significant increase in the latter, consistent with increased anxiety(39). We noted a significantly increased frequency of head dipping in *C3*$^{-/-}$ mice (Figure 1D) which may indicate reduced anxiety(40). These initial data were consistent with an anxiogenic phenotype present in *C3aR*$^{-/-}$ mice but absent in *C3*$^{-/-}$ mice. As might be predicted

from a heighted state of anxiety, we observed reduced locomotion around the maze in *C3aR*$^{-/-}$ mice (Figure S2B). Given that several measures were dependent on movement, it was important to eliminate potential locomotor confounds. To address this issue, we measured activity independently in a non-anxiety provoking environment over three days and found no group differences (Figure S2C) indicating that EPM measures were not influenced by movement confounds.

In a further cohort of animals, we assessed behaviour in the elevated zero maze (EZM), another established assay of anxiety related-behaviour(41). These data recapitulated the pattern of findings seen in the EPM with the *C3aR*$^{-/-}$ mice avoiding the open arms of the maze to a much greater extent than the wildtype and *C3*$^{-/-}$ animals (Figure 1F-J). Additional data from the open field test were consistent with the maze data in demonstrating a *C3aR*$^{-/-}$ specific anxiety phenotype (Figure S3).

Excessive grooming in *C3aR*$^{-/-}$ mice

During behavioural testing we noticed an increase in grooming in the *C3aR*$^{-/-}$ mice that was present in all *C3aR*$^{-/-}$ cohorts tested. We quantified this behaviour in one of the EPM sessions, as shown in Figure 2A&B. In the context of the EPM, grooming may be a displacement behaviour(42) and is positively correlated with anxiogenic phenotypes(43). Furthermore, excessive grooming in the *C3aR*$^{-/-}$ mice was chronic and present in the home-cage environment, resulting in substantial hair loss and occasionally skin lesions (see Figure 2C for representative examples). Again, this phenotype was not observed in *C3*$^{-/-}$ or wildtype mice.

Altered sensitivity of *C3aR*$^{-/-}$ and *C3*$^{-/-}$ mice to diazepam in the elevated plus maze

In a separate cohort of animals, we repeated the EPM experiment and in addition tested sensitivity of anxiety-related behaviour to 2mg/kg diazepam, an established clinically effective anxiolytic drug(37; 44). Vehicle-treated animals replicated the initial findings across all behavioural indices of anxiety, again showing a dissociation between the *C3aR*$^{-/-}$ and *C3*$^{-/-}$ mice (Figure 3A). In wildtype mice diazepam led to a trend for increased time on the open arms (Figure 3B), an effect often held to index reduced anxiety(37; 44; 45). While these effects did not reach statistical significance there was a highly significant reduction in SAPs consistent with anxiolysis(40; 45) Figure 3C). In contrast to wildtypes, diazepam was without effects in *C3aR*$^{-/-}$ mice (Figure 2A,B,C) and produced an anxiogenic pattern of effects in *C3*$^{-/-}$ mice (Figure 2A,B and Figure S4B,C). Locomotor activity monitored across all areas of the maze (Figure S4D) indicated that wildtype and *C3aR*$^{-/-}$ mice were unlikely to have been influenced by sedation. In *C3*$^{-/-}$ mice however, activity was significantly suppressed under drug conditions indicating a possible sedative effect.

Enhanced corticosterone response to the EPM in *C3aR*$^{-/-}$ mice

In a sub-cohort of wildtype, *C3*$^{-/-}$ and *C3aR*$^{-/-}$ mice treated with vehicle in the diazepam study we assayed plasma corticosterone 30 minutes after exposure to the EPM and compared levels to those of a group of animals who remained in their home-cages (baseline condition). There were no genotype differences in basal levels of corticosterone (Figure 4); however, being placed on the EPM increased plasma corticosterone 6-15-fold in all genotypes, suggesting that the EPM was a

potent stressor. *Post hoc* analyses indicated a significantly greater EPM-evoked corticosterone response in the *C3aR*$^{-/-}$ animals, consistent with their anxiety-like behaviour observed on the maze.

Changes in expression of stress and anxiety related genes in *C3*$^{-/-}$ and *C3aR*$^{-/-}$ mice

Using qPCR we measured expression of genes linked to anxiety and the stress response in brain regions implicated in fear and anxiety(25). Both knockouts demonstrated significantly greater cerebellar expression of *Nr3c1* encoding the glucocorticoid receptor (Figure 5A) and there was a trend in *C3aR*$^{-/-}$ mice for increased expression in the ventral hippocampus (vHPC) and medial prefrontal cortex (mPFC). Expression of *Crhr1* (encoding the corticotrophin-releasing hormone receptor 1) and *Gabra2*, which encodes the $GABA_A$ receptor $\alpha2$ subunit responsible for mediating benzodiazepine anxiolysis (46) was unaltered (Figure 5C,D). Since activation of C3aR is known to stimulate calcium influx(47; 48), we also investigated the expression of *Cacna1c,* a gene which encodes the $Ca_v1.2$ subunit of the L-type calcium channels and has established links to psychiatric disorder and anxiety(49; 50). We found a significant increase in expression (Figure 5B) that was genotype and brain region specific; with selective increases in expression in *C3aR*$^{-/-}$ mice in the vHPC and cerebellum, but not the mPFC.

Administration of the C3aR antagonist SB290157 did not phenocopy *C3aR*$^{-/-}$ mice

We next examined the extent to which systemic administration of the C3aR antagonist SB290157(33) to wildtype mice, at doses which have been shown to influence brain phenotypes(8; 48), would phenocopy *C3aR*$^{-/-}$ mice. We tested the effects of both acute and semi-chronic SB290157 treatment in the EPM. While the previous finding of enhanced anxiety was replicated in vehicle-treated *C3aR*$^{-/-}$ mice in both acute (Figure S5) and semi-chronic dosing regimens (Figure 6) neither SB290157 treatment had any effects on behaviour in wildtype mice.

Enhanced learned fear in *C3*$^{-/-}$ but not *C3aR*$^{-/-}$ mice

In a separate group of animals, we next assessed fear potentiated startle (FPS; see Figure 7A)(51; 52), an established means of assaying learned (or conditioned) fear(53; 54). In the pre-conditioning session, pulse-alone trials indicated increased basal startle reactivity in both *C3aR*$^{-/-}$ and *C3*$^{-/-}$ animals relative to wildtype (Figure 7B). Increased reactivity to the unconditioned foot shock stimulus (in the absence of any startle stimulus) during the conditioning session was also observed in both knockouts (Figure 7C). The potentiation of the startle response by the CS in the post-conditioning session is illustrated in Figure 7D. Here, in contrast to the previous assays of innate anxiety an opposite pattern of effects was observed, with *C3*$^{-/-}$ animals showing a selective enhancement of conditioned fear relative to the *C3aR*$^{-/-}$ and wildtype animals.

No effects on learned fear in *C6*$^{-/-}$ mice

The demonstration of enhanced fear learning in *C3*$^{-/-}$, but not *C3aR*$^{-/-}$ mice suggested that effects in *C3*$^{-/-}$ mice were mediated by molecules downstream of C3, independent from the C3a/C3aR axis. As there is no activity beyond the C3 convertase in *C3*$^{-/-}$ mice, C3 cannot be cleaved and therefore *C3*$^{-/-}$ mice lack C3a/C3b and C5a/C5b in addition to the terminal pathway (C6-C9). Therefore, it is possible that indirect loss of function of these molecules may contribute to the observed phenotype in *C3*$^{-/-}$ mice, although it is unclear which one. We therefore compared *C3*$^{-/-}$ with *C6*$^{-/-}$ mice (which have intact C3b/CR3, C5b and C5a/C5aR function but lack the terminal pathway) and hypothesised that should *C6*$^{-/-}$ mice show comparable behaviour to *C3*$^{-/-}$ mice then the phenotype would likely be due to a loss of terminal pathway activity. On the other hand, if *C6*$^{-/-}$ mice demonstrated no fear learning phenotype, this would confine the likely mediating molecules to either C3b or the downstream protein C5 and its breakdown products. Using a separate cohort, we replicated the previous *C3*$^{-/-}$ finding of enhanced learned fear (Figure 8). However, across all measures *C6*$^{-/-}$ mice were comparable to wildtype mice, suggesting that loss of C3b/CR3 or C5a/C5aR signaling, but not the terminal pathway, was involved in the *C3*$^{-/-}$ fear learning phenotype. Additionally, we did not observe innate anxiety-like phenotypes in *C6*$^{-/-}$ mice (Figure S6).

Discussion

We have used knockout models manipulating specific complement proteins to reveal dissociable effects on emotional behaviours. A main finding was a profound innate anxiety phenotype in the *C3aR*$^{-/-}$ mice that was absent in *C3*$^{-/-}$ mice. The specificity of

the anxiety phenotype exhibited by *C3aR*$^{-/-}$ mice at the behavioural level was paralleled by EPM-evoked corticosterone levels, confirming the validity of the EPM as an index of anxiety-like behaviour. *C3aR*$^{-/-}$ mice also displayed a pathological grooming phenotype, which has previously been linked to the immune system and microglial dysfunction (55). A second main finding was a double dissociation in terms of learned fear. Here we observed the opposite pattern of effects whereby *C3*$^{-/-}$ and not *C3aR*$^{-/-}$ mice displayed enhanced fear reactivity. Although the data presented here was in males, we can report that the same highly specific double dissociation was also seen in females (Figures S7,S8 & S9) extending the broader applicability of our findings. Together, these data are suggestive of distinct effects mediated by closely related elements of the complement system that impact differentially on the neural mechanisms underlying innate anxiety and learned fear.

Since C3a is solely produced via C3 cleavage, and C3aR is the canonical receptor for C3a, we expected that any phenotypes dependent on the binding of C3a to C3aR would be apparent in both knockout models. Given the divergence in phenotypes observed, one explanation is that the *C3aR*$^{-/-}$ phenotypes are C3a independent and instead mediated by an alternative ligand. TLQP-21, a cleavage fragment of the neuropeptide precursor protein VGF (non-acronymic; 56; 57) was recently reported to bind the C3aR. This peptide has pleiotropic roles including the stress response(58) and its precursor VGF is widely expressed throughout the CNS with high levels found in the hypothalamus, a region associated with stress reactivity and abundant C3aR expression(59; 60). Determining whether TLQP-21/C3aR interactions mediate the phenotypes observed will be a priority for future work.

We probed mechanisms underpinning the *C3aR*$^{-/-}$ innate anxiety phenotype by assessing the effects of the anxiolytic drug diazepam. We found that a dose of

diazepam that was anxiolytic (i.e. reduced SAPs) in wildtype mice had no effect in *C3aR*$^{-/-}$ mice. SAPs are highly sensitive to pharmacological manipulation(40; 61) and in agreement with our own findings, diazepam has been shown to specifically decrease SAPs in the absence of effects on open arm exploration (62). Importantly, *C3aR*$^{-/-}$ mice consistently performed more SAPs than other genotypes, and therefore floor effects cannot account for the pattern of results observed. Benzodiazepines act on $GABA_A$ receptors(46) however we found no significant changes in expression of *Gabra2*, a $GABA_A$ receptor subunit responsible for anxiolytic actions of benzodiazepines in tests of innate anxiety(46). We did however see changes in glucocorticoid receptor gene expression and a highly specific increase in expression of *Cacna1c* in *C3aR*$^{-/-}$ brain tissue. The latter finding is of particular interest given the strong evidence implicating *CACNA1C* variants in susceptibility for psychiatric disorders including schizophrenia and bipolar disorder(49). Alterations in anxiety have also been reported in human risk variant carriers(63) and in *Cacna1c* animal models(50; 64). Hence, it may be speculated that since C3aR activation is known to trigger Ca^{2+} influx(47), alterations in calcium channels may be a compensatory mechanism resulting from *C3aR* deletion.

Another question relating to potential mechanisms was whether anxiety phenotypes seen in the *C3aR*$^{-/-}$ mice were a result of acute, ongoing effects of *C3aR* deletion in the adult brain or instead the consequence of neurodevelopmental impacts of *C3aR* deficiency. At the outset, both were possibilities on the basis of previous findings implicating C3aR in developmental neurogenesis(65) and acute changes in C3aR expression in the adult brain after behavioural manipulations(66). The C3aR antagonist SB290157 had no effects on behaviour in wildtype animals and hence adult anxiety phenotypes in C3aR$^{-/-}$ mice were likely a consequence of long-term

constitutive knockout of C3aR, suggestive of developmental mechanisms. It should be noted that SB290157 has attracted criticism due to evidence of agonist activity(67). However, the negative results obtained in this work were not consistent with agonist effects which would be hypothesized to reduce anxiety in the EPM.

In contrast to innate anxiety, we observed a specific effect of *C3* knockout on conditioned fear. Lack of comparable phenotypes in *C3aR*$^{-/-}$ and *C6*$^{-/-}$ mice suggested that the effects were unlikely to be dependent on either C3a/C3aR or the terminal pathway. These observations lead to several possibilities regarding the mediating pathways. Firstly, effects could be mediated via loss of C3b/CR3 dependent synaptic elimination(10; 21), suggesting a developmental mechanism. In support, complement mediated synaptic pruning has now been demonstrated in brain regions relevant to fear learning such as the amygdala(68). The C3b/CR3 pathway could also be involved acutely in learning, as *C3* mRNA is upregulated during discrete stages of fear learning(66) and microglial CR3 is implicated in long term depression(69). Another possibility is that lack of the C5a/C5aR axis, which has a range of developmental roles(65), mediates enhanced fear-learning phenotypes in *C3*$^{-/-}$ mice, although there is less evidence for a role of C5a/C5aR in this context at present.

While our experiments have focused on anxiety and learned fear these endophenotypes are commonly comorbid with depression, and recent preclinical work has suggested a protective role for C3a/C3aR in chronic-stress induced depressive behaviour(70). Given the common co-occurrence of anxiety and depression, our findings of enhanced anxiety in *C3aR*$^{-/-}$ mice might seem at odds with the reported resilience of *C3aR*$^{-/-}$ to depressive phenotypes(70). However, the chronic unpredictable-stress paradigm used by Crider et al (2018) is likely to evoke

significant inflammation, and therefore the extent to which our data in acutely stressed animals can be compared is questionable. In summary, our findings add significantly to the evidence that perturbations of the complement system, whether reduced complement activation as in the present work or excessive activation as is predicted by *C4* variants(20; 71), have major and dissociable effects on brain and behavioral phenotypes of relevance to core clinical symptoms of psychiatric disease.

Author's contributions

LJW, TH, WPG and LSW designed the study. LJW and TH performed behavioural experiments with assistance from NH, SAB, EB and MT. ALM, SAB, EB, MT and LJW performed molecular analyses. BPM and TRH provided animals. JH, MJO, JR, WPG, NH, TRH, BPM, LSW and TH assisted in data interpretation and conceptualisation of experiments. LJW, TH, WPG and LSW drafted the manuscript. All authors approved the final manuscript.

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Competing financial interests

The authors declare no competing financial interests.

Materials and correspondence

All data from this study are available from the corresponding authors upon reasonable request.

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Figures and legends

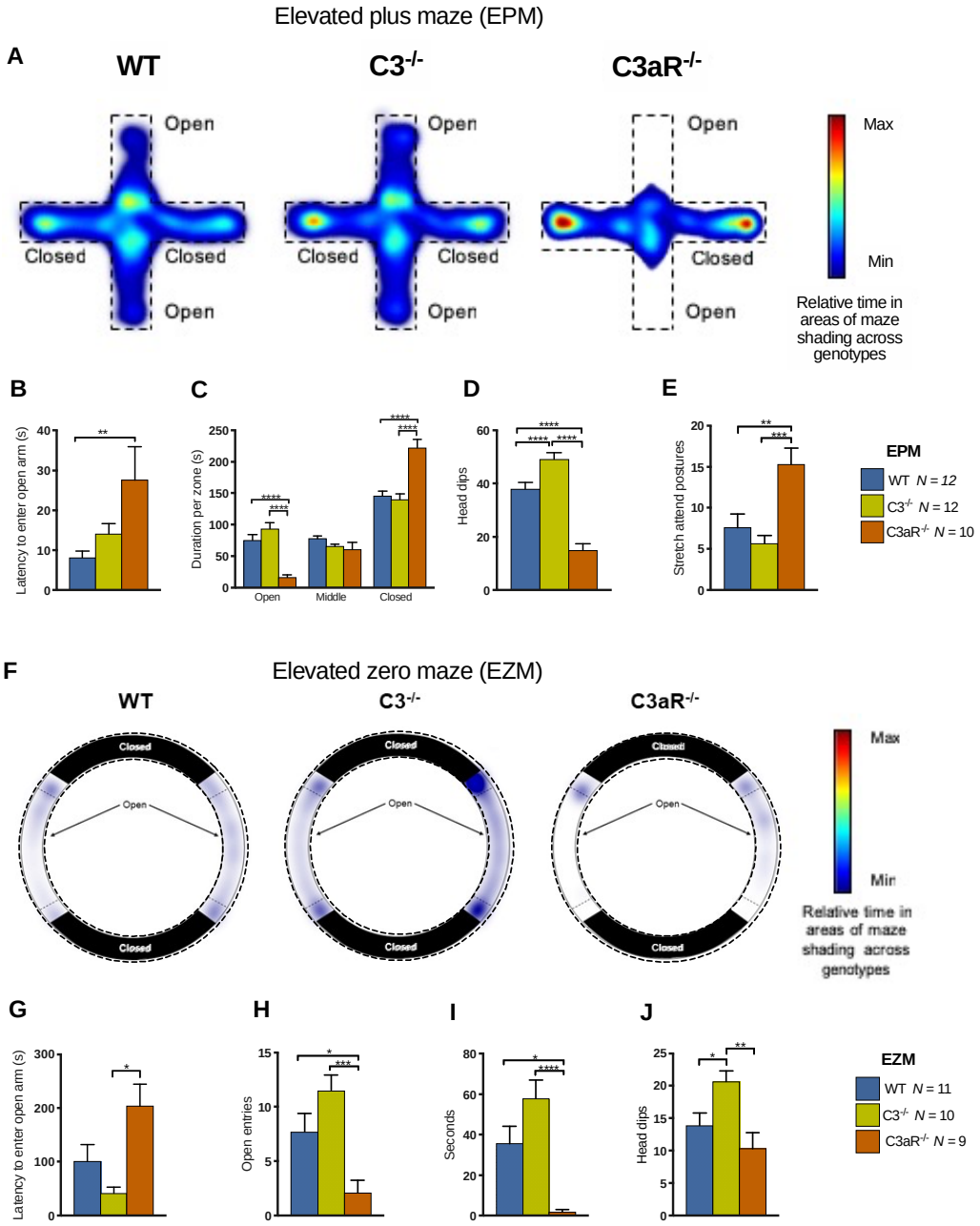


Figure 1. *C3aR*$^{-/-}$, but not *C3*$^{-/-}$ mice show increased anxiety-like behaviour in the elevated plus maze (EPM;A-E) and elevated zero maze (EZM;F-J). (A) Heatmaps displaying relative time per zone of the EPM across genotypes **(B)** There was a significant difference between genotypes in the latency to first open arm visit

(wildtype 8.21±1.53s, *C3*$^{-/-}$ 14.1±2.52s, *C3aR*$^{-/-}$ 27.6±8.31s, H_2=10.5, p=0.005). *Post hoc* tests demonstrated that *C3aR*$^{-/-}$ mice took significantly longer to first enter the open arms than wildtype mice (p=0.0045). **(C)** *C3aR*$^{-/-}$ mice distributed their time across the EPM differently to wildtype and *C3*$^{-/-}$ mice (GENOTYPE×ZONE, $F_{4,62}$=17.7, p=0.0001) spending less time in the open arms (*C3aR*$^{-/-}$ 16.70±3.73s vs. wildtype 75.78±8.86s, p<0.0001, *C3aR*$^{-/-}$ vs. *C3*$^{-/-}$ 93.86±9.59s p<0.0001) and significantly more time in the closed arms (*C3aR*$^{-/-}$ 221.88±12.06s vs. wildtype 146.01±7.01s, p<0.0001, and *C3*$^{-/-}$ 140.04±8.61s p<0.0001). **(D)** *C3aR*$^{-/-}$ (14.90±2.22) mice performed significantly fewer head dips than wildtype (37.92±2.53, p<0.0001) and *C3*$^{-/-}$ mice (49.17±2.37, p<0.0001), whereas *C3*$^{-/-}$ mice performed significantly more head dips than wildtype mice (p=0.0061; overall ANOVA $F_{2,31}$=48.0, p<0.0001). **(E)** *C3aR*$^{-/-}$ mice performed significantly more stretch attend postures (SAPs; 15.30±1.80) than wildtype (7.58±1.66, p=0.0042) and *C3*$^{-/-}$ mice (5.67±0.94, p=0.0004; overall ANOVA $F_{2,31}$=10.3, p=0.0004). **(F)** Heatmaps displaying relative exploration of the open segments of the elevated zero maze, across genotypes. Note that due to the height of the walls in the closed regions it was not possible to track mice or observe ethological behaviours such as grooming or SAPs. **(G)** There was a significant difference in the latency to first enter the open arms (wildtype 101.00±31.00s, *C3*$^{-/-}$ 42.00±2.52s, *C3aR*$^{-/-}$ 204.00±40.40s, H_2=8.13, p=0.0171). *Post hoc* tests revealed that *C3aR*$^{-/-}$ mice took significantly longer than *C3*$^{-/-}$ mice to initially enter the open region (p=0.0140). **(H)** The number of entries made to open regions differed between genotypes (wildtype 7.69±1.69, *C3*$^{-/-}$ 11.5±1.43s, *C3aR*$^{-/-}$ 2.10±1.15, $F_{2,30}$=8.96, p=0.0009). *C3aR*$^{-/-}$ mice made significantly fewer entries to the open areas than wildtype (p=0.0324) and *C3*$^{-/-}$ mice (p=0.0006) and **(I)** spent

significantly less time on the open arms (1.77±1.29) compared to wildtype (35.7±8.43s, p=0.0132) and *C3*$^{-/-}$ (57.7±9.32s, p<0.0001; overall Kruskal-Wallis test H_2=19.2, p<0.0001). **(J)** *C3*$^{-/-}$ mice performed significantly more head dips (20.7±1.62) than wildtype (13.9±1.89, p=0.048) and *C3aR*$^{-/-}$ mice (10.3±2.42, p=0.0034; overall ANOVA $F_{2,27}$=6.86,p=0.0039). Data are mean ± S.E.M. *, **, *** and **** represent p≤0.05, p≤0.01, p≤0.001 and p≤0.0001 for *post-hoc* genotype comparisons, respectively.

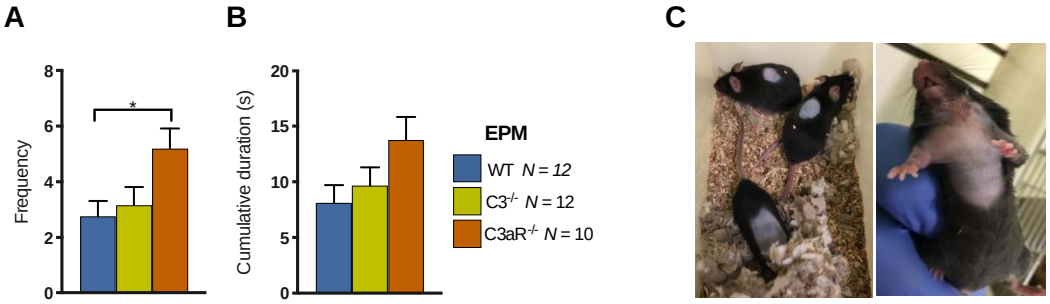


Figure 2. Excessive grooming in *C3aR$^{-/-}$* mice (A) There were significant differences in the frequency of grooming bouts during the 5-minute elevated plus maze (EPM) test (wildtype 2.75±0.55, *C3aR$^{-/-}$* 5.20±0.72, *C3$^{-/-}$* 3.17±0.64, $F_{2,31}$=3.94, p=0.0298). *C3aR$^{-/-}$* mice engaged in grooming significantly more frequently than wildtype mice (p=0.0320). **(B)** There was also a trend towards a greater duration of time spent grooming in *C3aR$^{-/-}$* mice on the EPM, however this did not reach significance ($F_{2,31}$=2.59, p=0.0909, wildtype 8.11±1.61s, *C3aR$^{-/-}$* 13.8±2.10, *C3$^{-/-}$* 9.70±1.63s). **(C)** Representative images of the effects of chronic homecage excessive grooming in *C3aR$^{-/-}$* mice resulting in bald patches. By 5 months of age, 80% of *C3aR$^{-/-}$* mice displayed such signs of excessive grooming. In contrast, no wildtype or *C3$^{-/-}$* mice demonstrated signs of excessive grooming. This phenotype was observed in all cohorts of *C3aR$^{-/-}$* mice assessed. Data are mean + S.E.M. * represents p≤0.05 for *post-hoc* genotype comparisons.

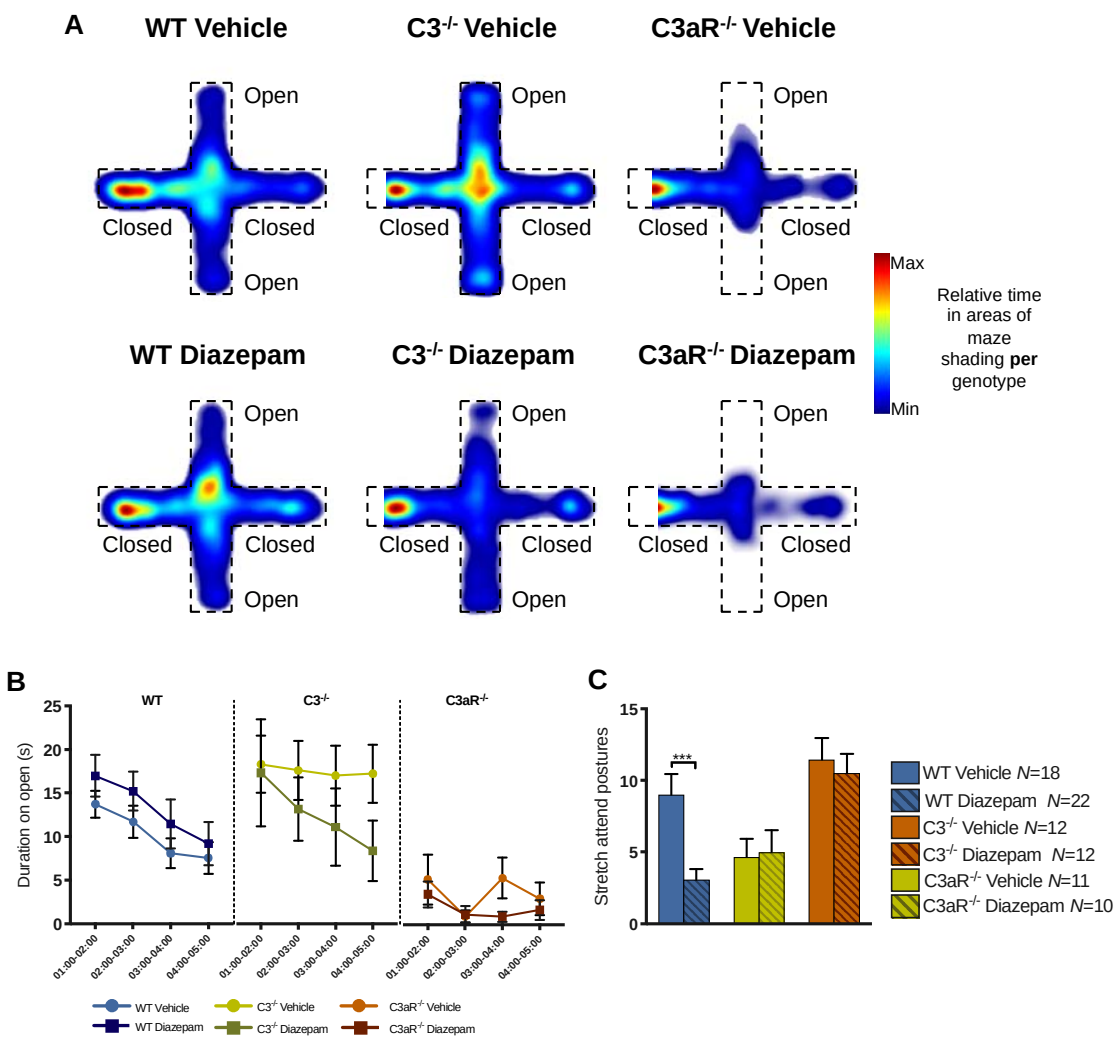


Figure 3. Altered sensitivity to diazepam in *C3aR*$^{-/-}$ and *C3*$^{-/-}$ mice. Behaviourally naïve mice were treated with either diazepam (2mg/kg, i.p) or vehicle injections once daily for 2 days and then 30 minutes prior to testing. (**A)** Heatmaps demonstrating duration spent in zones of the maze by vehicle treated and diazepam treated animals **(B)** Plots showing duration spent on open arms in 1-minute time bins (start-01:00 was excluded due to effect of diazepam in delaying initial entry to open arms, see Supplementary Figure 4a). There was a trend for wildtype diazepam treated animals to spend more time on the open arms throughout the task although this did not reach significance (main effect of DRUG, $F_{1,38}$=1.41, p=0.2462). In *C3*$^{-/-}$ mice

there was a strong tendency for drug treated animals to explore the open arms less than vehicle treated *C3*$^{-/-}$ mice (main effect of DRUG, $F_{1,22}$=1.25, p=0.2764). A similar, though less pronounced pattern was seen in *C3aR*$^{-/-}$ mice (main effect of DRUG, $F_{1,19}$=1.55, p=0.2284) **(C)** There were genotype differences in SAPs (main effect of GENOTYPE, $F_{2,79}$=10.7, p<0.0001), a main effect of DRUG ($F_{1,79}$=4.13, p=0.0454) and a significant GENOTYPE × DRUG interaction ($F_{2,79}$=4.64, p=0.0138). *Post hoc* tests showed that diazepam significantly reduced the number of SAPs in wildtype mice only (wildtype vehicle 9.00±1.44 vs. wildtype diazepam 3.09±0.71, p=0.0006, *C3*$^{-/-}$ vehicle 4.67±1.24 vs. *C3*$^{-/-}$ diazepam 5.00±1.51, p=0.9975, *C3aR*$^{-/-}$ vehicle 11.45±1.49 vs. *C3aR*$^{-/-}$ diazepam 10.50±1.34, p=0.9558). Data are mean + S.E.M. *, **, and *** represent p≤0.05, p≤0.01 and p≤0.001 for *post-hoc* genotype comparisons, respectively.

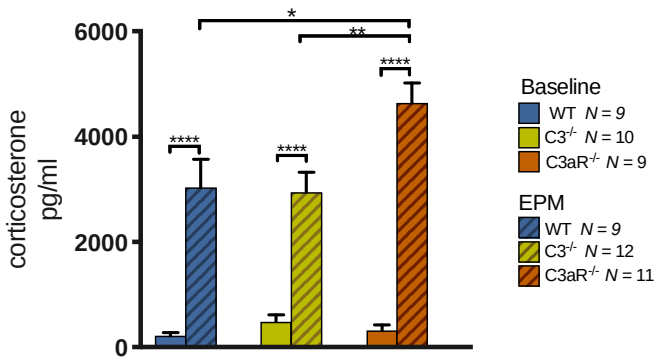


Figure 4. Elevated corticosterone response to the elevated plus maze in *C3aR*$^{-/-}$ mice. Exposure to the EPM significantly elevated corticosterone in all genotypes (main effect of CONDITION, $F_{1,54}$=143, p<0.0001; baseline 344.66±63.70 vs. EPM 3553.84±274.13). There was a significant GENOTYPE × CONDITION interaction ($F_{2,54}$=4.64, p=0.0138). *Post hoc* analysis showed that after the EPM, *C3aR*$^{-/-}$ mice demonstrated significantly higher corticosterone levels (4640.27±376.13) than wildtype (3033.78±535.06, p=0.0127) and *C3*$^{-/-}$ mice (2948.00±374.87, p=0.0032). *Post hoc* tests also indicated that there were no baseline differences between genotypes (wildtype 216.54±63.2 vs. *C3aR*$^{-/-}$ 316.17±111.60 p>0.9999, wildtype vs. *C3*$^{-/-}$ p=0.9927, and *C3*$^{-/-}$ 485.60±130.35 vs.*C3aR*$^{-/-}$ mice p=0.9992). Data are mean + S.E.M. *, **, and **** represent p≤0.05, p≤0.01 and p≤0.0001 for *post-hoc* genotype comparisons, respectively.

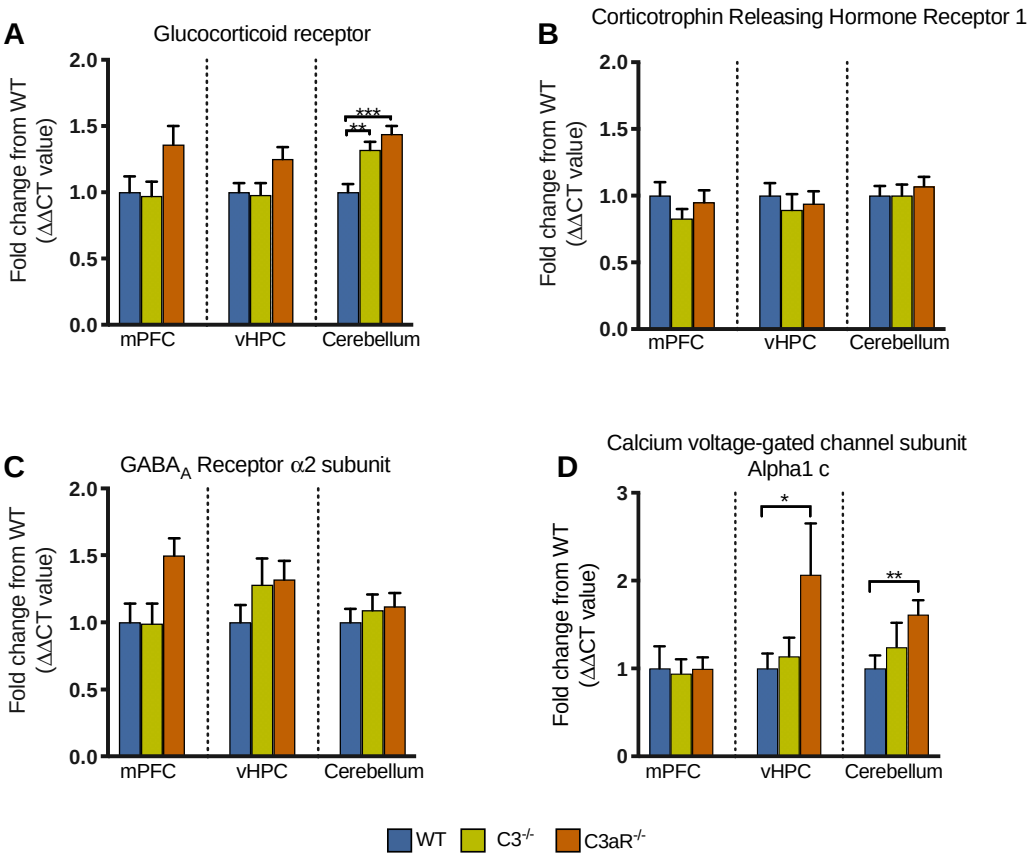
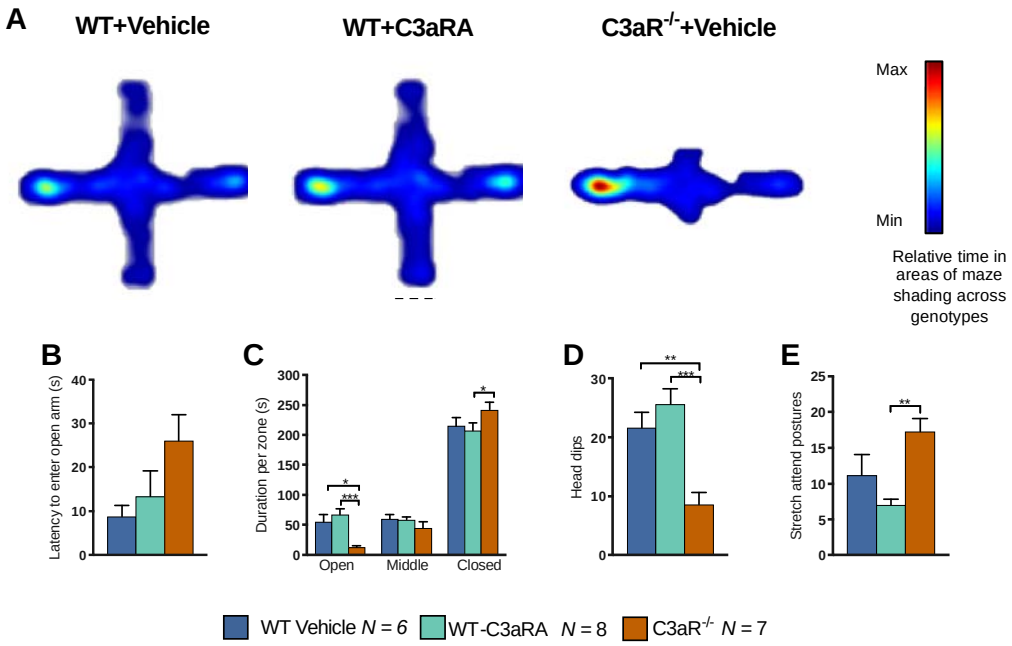


Figure 5. mRNA expression of stress and anxiety related genes. The medial prefrontal cortex (mPFC), ventral hippocampus (vHPC) and cerebellum were dissected from behaviourally naïve mice and were subjected to quantitative PCR analysis for the transcript expression of candidate genes associated with anxiety and the stress response. **(A)** There were trends towards increased expression of the glucocorticoid receptor gene *Nr3c1* in the mPFC and vHPC of *C3aR*$^{-/-}$ mice but these were not significant (mPFC; $F_{2,56}$=1.33, p=0.2723, N wildtype=20, *C3*$^{-/-}$=20, *C3aR*$^{-/-}$= 19 vHPC; $F_{2,62}$=1.11, p=0.3345, N wildtype=23, *C3*$^{-/-}$=20, *C3aR*$^{-/-}$= 22). Expression of the glucocorticoid receptor (*Nr3c1*) was significantly increased in the cerebellum of both *C3*$^{-/-}$ and *C3aR*$^{-/-}$ groups however ($F_{2,61}$=10.3, p=0.0002, *C3*$^{-/-}$ vs. wildtype p=0.0023, *C3aR*$^{-/-}$ vs. wildtype p=0.0002, N wildtype=19, *C3*$^{-/-}$=20, *C3aR*$^{-/-}$= 15). **(B)** There were no significant differences in the expression of Corticotrophin releasing

hormone receptor 1 (*Crhr1*) in any region, across genotypes (mPFC $F_{2,53}$=0.587, p=0.5597, N wildtype=20, *C3*$^{-/-}$=17, *C3aR*$^{-/-}$= 19; vHPC $F_{2,49}$=0.169, p=0.8450, N wildtype=20, *C3*$^{-/-}$=15, *C3aR*$^{-/-}$= 17; cerebellum $F_{2,47}$=0.0482, p=0.8346, N wildtype=19, *C3*$^{-/-}$=17, *C3aR*$^{-/-}$= 14). **(C)** There were also no significant changes in the expression of the $GABA_A$ receptor α2 subunit (*Gabra2*) in any region, across genotypes (mPFC H_2=1.04, p=0.5939, N wildtype=20, *C3*$^{-/-}$=19, *C3aR*$^{-/-}$= 16; vHPC $F_{2,49}$=0.721, p=0.4914, N wildtype=20, *C3*$^{-/-}$=13, *C3aR*$^{-/-}$= 19; cerebellum $F_{2,47}$=0.221, p=0.8026, N wildtype=18, *C3*$^{-/-}$=17, *C3aR*$^{-/-}$= 15). **(D)** Expression levels of the Calcium voltage-gated channel subunit Alpha 1c (*Cacna1c*) were significantly increased in *C3aR*$^{-/-}$ mice in a regionally specific manner in the vHPC ($F_{2,47}$=3.20, p=0.0496, *C3*$^{-/-}$ vs. wildtype p=0.6895, *C3aR*$^{-/-}$ vs. wildtype p=0.0295, N wildtype=21, *C3*$^{-/-}$=13, *C3aR*$^{-/-}$= 16) and the cerebellum ($F_{2,54}$=5.84, p=0.0051, *C3*$^{-/-}$ vs. wildtype p=0.1613, *C3aR*$^{-/-}$ vs. wildtype p=0.0024, N wildtype=20, *C3*$^{-/-}$=20, *C3aR*$^{-/-}$= 17). There were no significant changes in the mPFC ($F_{2,52}$=1.04, p=0.5939, N wildtype=20, *C3*$^{-/-}$=19, *C3aR*$^{-/-}$= 16). Data represent fold change from wildtype + SEM.



855

856 **Figure 6. Semi-chronic pharmacological C3aR blockade in wildtype mice does**
857 **not recapitulate *C3aR*$^{-/-}$ phenotypes in the elevated plus maze.** SB290157
858 (C3aRA) was administered twice daily for five days (i.p. 10mg/kg). **(A)** Heatmaps
859 demonstrating duration spent in zones of the maze **(B)** There was a trend for greater
860 latencies to first open arm visit in *C3aR*$^{-/-}$ mice (26.06±15.73s vs. wildtype vehicle
861 8.75±2.50s, wildtype C3aRA 13.38±6.70s) approaching significance ($F_{2,18}$=2.73,
862 p=0.0923). **(C)** *C3aR*$^{-/-}$ mice again distributed their time across the EPM differently to
863 wildtype-vehicle and wildtype-C3aRA mice (GENOTYPE×ZONE, $F_{4,54}$=5.67,
864 p=0.0007) spending less time in the open arms (*post hoc:* wildtype-vehicle
865 55.39±12.11 vs. *C3aR*$^{-/-}$ 12.85±5.69s p=0.0157, wildtype-C3aRA 67.14±10.86 vs.
866 *C3aR*$^{-/-}$ p=0.0007), and significantly more time in the closed arms than wildtype-
867 C3aRA mice (*C3aR*$^{-/-}$ 242.21±37.72 vs. wildtype-C3aRA 207.18±14.55 p=0.0363,
868 wildtype-vehicle 214.99±13.97s vs. *C3aR*$^{-/-}$ p=0.1667). **(D)** *C3aR*$^{-/-}$ mice made fewer
869 head dips (8.57±5.50) than wildtype-vehicle (21.67±2.62, p=0.0057) and wildtype-

C3aRA mice (25.63±3.04, p=0.0002, overall ANOVA $F_{2,18}$=13.4, P = 0.0003) and **(F)** displayed more SAPs (17.29±4.72) than wildtype-vehicle (13.22±5.19, p=0.0853) and wildtype-C3aRA treated mice (7.45± 2.28, p=0.0018, overall ANOVA $F_{2,18}$=8.50, P = 0.0025). For all measures, there were no significant differences between wildtype-vehicle and wildtype-C3aRA mice. Data are mean + S.E.M. *, **, *** and **** represent p≤0.05, p≤0.01, p≤0.001 and p≤0.0001 for *post-hoc* genotype comparisons, respectively.

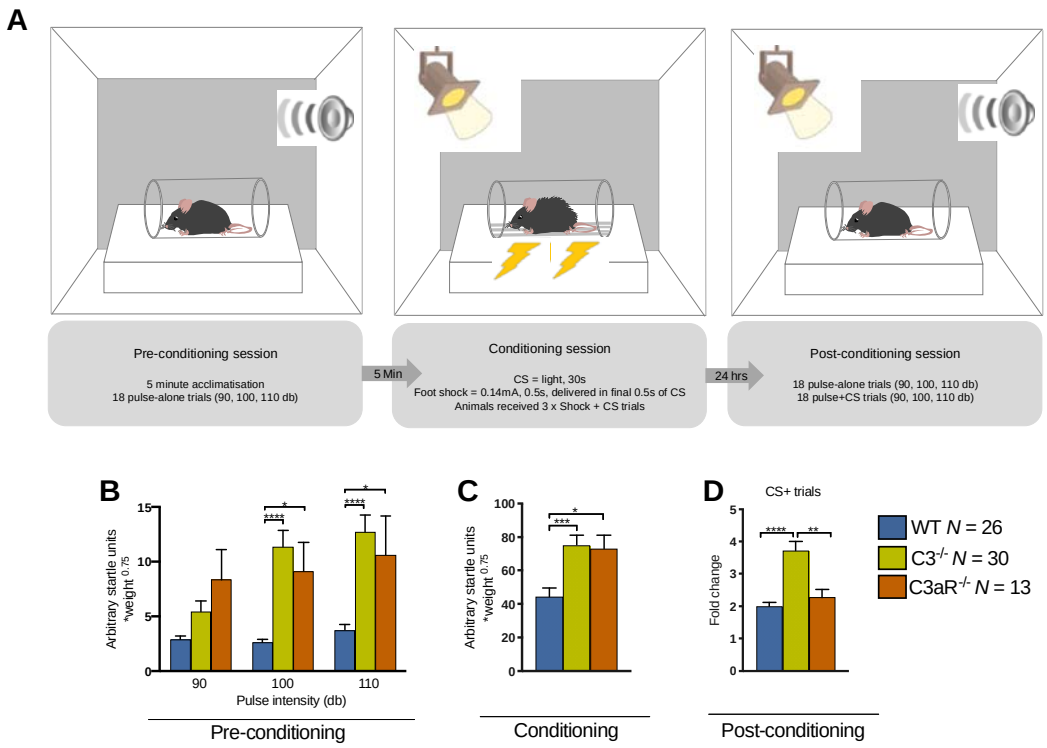


Figure 7. Enhanced fear-potentiated startle in *C3*$^{-/-}$ but not *C3aR*$^{-/-}$ mice. (A) Flow chart depicting the FPS protocol used, which took place in three separate sessions over two consecutive days. Baseline startle reactivity to a range of pulse intensities was assessed in the pre-conditioning session, immediately preceding the conditioning session in which a visual stimulus (light) was paired with 3 weak foot shocks. 24 hours later, subjects were re-introduced to the same chamber and startle reactivity was compared between pulse-alone trials and pulse+CS trials to determine the degree of FPS. On all trials, the peak startle response was recorded and normalised for body weight differences using Kleiber's 0.75 mass exponent[74], and fold-changes calculated. **(B)** There was a significant main effect of GENOTYPE ($F_{2,66}$=9.04, p=0.0003) and a significant GENOTYPE × STIMULUS INTENSITY interaction ($F_{4,132}$=7.55, p<0.0001).

C3$^{-/-}$ and *C3aR*$^{-/-}$ mice demonstrated increased levels of startle responding relative to wildtype mice at 100dB (*C3*$^{-/-}$ 11.34±1.51 vs. wildtype 2.63±0.26, p<0.0001, *C3aR*$^{-/-}$ 9.12±2.63 vs. wildtype p=0.0174) and 110dB (*C3*$^{-/-}$ 12.69±1.55 vs. wildtype 3.74±0.50, p<0.0001, *C3aR*$^{-/-}$ 10.58±3.58 vs. wildtype p=0.0111) **(C)** *C3*$^{-/-}$ and *C3aR*$^{-/-}$ mice also showed increased startle responding to the footshock+CS (*C3*$^{-/-}$ 75.18±5.73, *C3aR*$^{-/-}$ 73.14±7.78) pairings relative to wildtype mice (44.34±5.29, *C3*$^{-/-}$ vs. wildtype p=0.0006, *C3aR*$^{-/-}$ vs. wildtype p=0.0137, overall ANOVA $F_{2,66}$=8.7, p=0.0004), although it should be noted that responses were much greater to these stimuli in all mice than to the startle stimuli in the pre-conditioning session. **(D)** In the post-conditioning session, all mice demonstrated increases to the pulse+CS stimuli in comparison to pulse-alone stimuli, as demonstrated by the fold-change increase in startle responding, however, this effect was significantly increased in *C3*$^{-/-}$ mice (3.72±0.27) relative to wildtype (1.99±0.11, p<0.0001) and *C3aR*$^{-/-}$ mice (2.27±0.24, p=0.0056, overall Kruskal-Wallis test H_2=27.7, p<0.0001). Data are mean + S.E.M. *, **, *** and **** represent p≤0.05, p≤0.01, p≤0.001 and p≤0.0001 for *post-hoc* genotype comparisons, respectively.

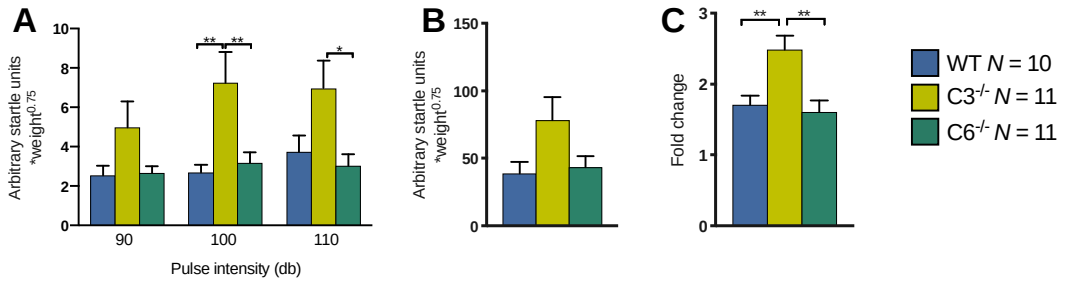


Figure 8. Enhanced fear-potentiated startle in *C3*$^{-/-}$ but not *C6*$^{-/-}$ mice. A separate cohort of wildtype and *C3*$^{-/-}$ mice were assessed alongside *C6*$^{-/-}$ mice. **(A)** There was a significant main effect of GENOTYPE ($F_{2,20}$=5.87, p=0.0098). *C3*$^{-/-}$ mice (90 db: 4.98±0.79; 100 db: 7.24±0.94; 110 db: 6.97±0.84) demonstrated elevated startle responding at all intensities relative to wildtype (90 db: 2.55±0.30 p=0.1846; 100 db: 2.69±0.24 p=0.0042; 110 db: 3.74±0.51 p=0.0545) and *C6*$^{-/-}$ mice (90 db: 2.68±0.31 p=0.2023; 100 db: 3.19±0.48 p=0.0098; 110 db: 3.03±0.544 p=0.0123). **(B)** *C3*$^{-/-}$ mice demonstrated a trend towards greater startle reactivity (78.35±10.25) to the footshock+CS pairings during the conditioning phase of training (wildtype 38.50±5.41, *C6*$^{-/-}$ 43.49±7.57, H_2=4.66, p=0.0971). **(C)** Post-conditioning, *C3*$^{-/-}$ mice showed the greatest fold change (2.48±0.12) in startle responding between pulse-alone stimuli and pulse+CS stimuli, which was significantly greater than that shown by wildtype (1.70±0.08, p=0.0094) and *C6*$^{-/-}$ mice (1.60±0.15, p=0.0025, overall ANOVA $F_{2,29}$=8.08, p=0.0016). There were no differences in responding between *C6*$^{-/-}$ and wildtype mice on any measures. Data are mean + S.E.M. * and ** represent p≤0.05 and p≤0.01 for *post-hoc* genotype comparisons, respectively.