Complement C3 and C3aR differentially impact on learned fear and innate anxiety

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Abbreviations: C3, complement component 3; C3aR, Complement C3a Receptor; C3aRA, C3a Receptor antagonist; C6, Complement component 6; EPM, elevated plus maze; EZM, elevated zero maze; FPS, fear potentiated startle; HPA, hypothalamic-pituitary-adrenal (axis); SAP, stretch attend posture; WT, wildtype.

Keywords: Complement system, Anxiety, Fear, Stress response

Abstract

Recent findings implicate complement, a key component of the innate immune system, in brain function and risk for neuropsychiatric disorders. We reveal novel functions and unexpected dissociations of complement pathways impacting on emotional behaviours. First, we showed profound anxiety phenotypes, indexed by behaviour and physiological measures, in *C3aR*$^{-/-}$ mice but not *C3*$^{-/-}$ mice across several tests of innate anxiety. Administration of the C3aR antagonist SB290157 did not phenocopy *C3aR*$^{-/-}$ mice, consistent with *C3aR* knockout influencing adult behaviour via developmental mechanisms. In contrast to the findings on innate anxiety, *C3*$^{-/-}$ mice, but not *C3aR*$^{-/-}$ mice, showed behaviour indicative of greater learned fear. We probed downstream complement pathways responsible for the C3-specific effects on learned fear and excluded contributions from the terminal pathway, C6 to C9. Our data provide evidence for dissociable effects of complement pathways in aspects of affective function relevant to psychiatric disorder.

54 Recent evidence suggests that the complement system has unexpected versatility
55 beyond canonical immune functions[1]. An implication of these findings is that separate
56 pathways and proteins of the complement system may have unique, non-immune
57 roles in different contexts. A prominent example of this functional promiscuity is the
58 role of complement in neurodevelopment where distinct pathways such as the
59 C3a/C3aR and the C5a/C5aR axes (Figure 1) differentially influence neuronal
60 progenitor proliferation and migration[2]. In the postnatal brain, complement partakes in
61 activity-dependent synaptic refinement via the C3b/CR3 axis[3,4] and the C3a/C3aR axis
62 has a role in regulating neurogenesis in the adult hippocampus[5].

63 [Figure 1 about here]

64 Complement has been implicated in the pathogenesis of both neurodegenerative and
65 psychiatric conditions. In Alzheimer's disease, genetic variants in complement related
66 loci have been associated with increased disease risk[6,7], and in preclinical models
67 complement knockout mice exhibit reduced age-related synapse loss[8] and
68 neuropathology[9]. Complement system alterations have also been reported in the
69 serum of patients with schizophrenia[10], major depressive disorder[11], bipolar disorder[12]
70 and post-traumatic stress disorder[13]. In the case of schizophrenia, an important finding
71 comes from elegant genetic work demonstrating that structural variation in the *C4*
72 locus is associated with greater risk of developing the disease[14]. C4 cleavage
73 generates C4b fragments that, in collaboration with cleaved C2, activate C3, yielding
74 the fragments C3a and C3b (Figure 1). Given the known roles for C3b/CR3 in synaptic
75 pruning[3,4], it has been suggested that *C4* variants may impact on psychiatric risk via
76 this mechanism, leading to abnormal connectivity and disruption of neural networks[14].
77 Recent *in vitro* evidence has supported this idea, showing that *C4A* variants that

78 increase C4A protein levels contribute to increased C3 deposition and greater synaptic
79 elimination[15].

80 Altered emotional function, in particular anxiety, is a pervasive and clinically important
81 symptom that is comorbid across several DSM-5 and ICD-11 defined disorders with
82 growing social, economic and health consequences[16]. In schizophrenia, anxiety can
83 precede the onset of psychotic symptoms; hence, it has been suggested that anxiety
84 may be part of the pathogenesis of the disorder rather than simply a comorbidity[17].
85 While the links between anxiety and inflammation have been well documented [18], there
86 has been little investigation of the role of complement in anxiety disorders in humans.
87 In animal models, prenatal manipulations increasing C3 deposition in the fetal brain
88 are associated with anxiety phenotypes in adulthood[19]. Additionally, complement
89 inhibitors reduce anxiety in models of Alzheimer's disease[20] and aged *C3* deficient
90 mice exhibit reduced anxiety[8]. These studies suggest that complement activation can
91 influence anxiety; however, the precise relationship between signalling within discrete
92 pathways of the complement system and their relationship to dissociable aspects of
93 anxiety-related behaviours and underlying brain function is unknown.

94 Here we use knockout mouse models to manipulate C3 and C3aR signalling in order
95 to functionally parse anxiety-related phenotypes. We focused on C3 due to its central
96 role within the complement system and the requirement for C3 breakdown products to
97 propagate complement activation downstream (Figure 1). In the homozygous *C3*
98 knockout mice[21] (Figure 2) complement cannot be activated beyond C3, and therefore
99 these animals lack activation fragments (C3a, C3b) and thus cannot activate the
100 terminal complement pathway. Phenotypic effects in this model could therefore be the
101 result of disturbance to any downstream effector molecule(s). We compared the $C3^{-/-}$
102 model with homozygous *C3aR* knockout mice[22] (Figure 2). In these mice the

103 complement system is intact apart from the capacity for C3a to bind to its canonical
104 receptor C3aR. Here, we tested the extent to which phenotypic effects were the result
105 specifically of C3a/C3aR axis signalling. *A priori*, because C3a is an obligate cleavage
106 fragment of C3 we hypothesised that any phenotypes dependent on the binding of
107 C3a to C3aR would be apparent in both *C3*$^{-/-}$ and *C3aR*$^{-/-}$ models.

108 [Figure 2 about here]

109 Interestingly, we found completely different effects of the two models on aversive
110 behaviours. *C3aR*$^{-/-}$ mice displayed a profound innate anxiety phenotype across
111 several tests that was lacking in the *C3*$^{-/-}$ model, a dissociation that was mirrored by
112 plasma levels of the stress hormone corticosterone. Furthermore, the anxiety
113 phenotype in the *C3aR*$^{-/-}$ mice was resistant to systemic treatment with diazepam at
114 doses with an anxiolytic effect in wildtype mice. Moreover, acute administration of the
115 selective C3aR antagonist SB290157[23] did not phenocopy *C3aR*$^{-/-}$ mice, consistent
116 with the absence of *C3aR* influencing adult behaviour via developmental effects. When
117 we examined learned fear, we found that the dissociation was reversed, with the *C3*$^{-/-}$
118 mice exhibiting an enhanced response to a conditioned cue, but no difference between
119 wildtype and *C3aR*$^{-/-}$mice. We probed downstream complement pathways responsible
120 for the C3-specific effects on learned fear and excluded contributions from the terminal
121 pathway by using *C6*$^{-/-}$ mice. These findings point to a hitherto unrecognized
122 complexity of complement effects on brain function and behaviour and inform debate
123 regarding complement in psychopathology.

124

125

126 **Results**

127 **High levels of unconditioned anxiety in *C3aR*$^{-/-}$ but not *C3* $^{-/-}$ mice**

128 We first assessed emotional reactivity in the elevated plus maze (EPM), a well-
129 established task that assays the conflict between motivation to explore novel
130 environments and the innate anxiety evoked by open, brightly lit spaces[24,25]. Wildtype
131 animals showed the typical pattern of behaviour in exploring all areas of the maze, but
132 with relatively less exploration of the open arms compared to the closed arms.
133 Comparison of the heat maps in Figure 3a, indexing overall maze exploration,
134 demonstrated that whilst the *C3*$^{-/-}$ animals showed a similar pattern of behaviour to
135 wildtype animals, *C3aR*$^{-/-}$ mice were markedly different in essentially avoiding the open
136 arms completely (see Supplementary File 1 for representative videos). The breakdown
137 of the individual behavioural measures taken on the maze (Figure 3 b-g) revealed that
138 in comparison to wildtype and *C3*$^{-/-}$ mice, *C3aR*$^{-/-}$ mice took longer to first enter the
139 open arms (Figure 3b) and spent less time there per entry (Figure 3c), leading to a
140 markedly reduced overall open arm duration (Figure 3d). Ethological parameters
141 (head dips and stretch attend postures; SAPs) were also different in *C3aR*$^{-/-}$ mice
142 (Figure 3e, f), exhibiting a significant reduction in the former and significant increase
143 in the latter, a pattern consistent with anxiogenic effects[26]. During the task, *C3aR*$^{-/-}$
144 mice more frequently engaged in grooming which may be considered a displacement
145 behaviour when displayed in conflict situations[27] (Supplementary Figure 1a, b). We
146 also noted that in comparison to wildtype and *C3aR*$^{-/-}$ animals, there was a significantly
147 increased prevalence of head dipping in *C3*$^{-/-}$ mice (Figure 3e) which may indicate
148 anxiolysis[28]. These initial data were consistent with an anxiogenic phenotype present
149 in *C3aR*$^{-/-}$ mice but absent in *C3*$^{-/-}$ mice. As might be predicted from a heighted state
150 of anxiety in the EPM, we observed reduced activity in *C3aR*$^{-/-}$ mice (Figure 3g). Given

151 that several measures were dependent on movement around the maze it was
152 important to eliminate potential locomotor confounds. To address this issue, we
153 measured activity independently in a non-anxiety provoking environment and found
154 no group differences (Figure 3h) indicating the EPM measures were not influenced by
155 movement confounds.

156 [Figure 3 about here]

157 Next it was important to establish that the anxiety phenotype was not task-specific, i.e.
158 confined to a single behavioural task. Using a separate cohort of animals, we
159 assessed behaviour in the elevated zero maze (EZM), another established assay of
160 anxiety related-behaviour which differs from the EPM in the absence of a central zone,
161 allowing a more definitive quantification of behaviour that is confined solely to open
162 and closed regions[29]. Heat maps showing overall exploratory behaviour on the EZM
163 are shown in Figure 4a. These data recapitulated the pattern of findings seen in the
164 EPM with the *C3aR*$^{-/-}$ mice avoiding the open arms of the maze to a much greater
165 extent than the wildtype and *C3*$^{-/-}$ animals. Quantification of the maze behaviour
166 confirmed the specificity of the effects to the *C3aR*$^{-/-}$ mice in showing that these
167 animals took longer to first enter the open regions (Figure 4b) and made fewer entries
168 to the open regions (Figure 4c). On the few occasions they did enter, *C3aR*$^{-/-}$ mice
169 spent less time per visit (Figure 4e) and consequently spent considerably less time
170 overall in the open regions (Figure 4d). In contrast to the EPM, there was no difference
171 in head dips between the *C3aR*$^{-/-}$ and wildtype animals, but again *C3*$^{-/-}$ mice performed
172 significantly more head dips (Figure 4f). We carried out additional analyses in the open
173 field test of emotional reactivity and noted, consistent with the maze data, indices of
174 enhanced anxiety in the *C3aR*$^{-/-}$ mice (Supplementary Figure 2). Finally, we observed
175 bald patches due to an excessive-grooming phenotype in the *C3aR*$^{-/-}$ mice

176 (Supplementary Figure 1c) that was present in all *C3aR*$^{-/-}$ cohorts. Over-grooming has
177 been associated with enhanced stress and anxiety[23,25] and has previously been linked
178 to the immune system and microglial dysfunction[30].

179 [Figure 4 about here]

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

181 In a separate cohort of animals we repeated the EPM experiment and in addition
182 tested sensitivity of anxiety-related behaviours to 2mg/kg diazepam, an established
183 clinically effective anxiolytic drug[24,31]. Vehicle treated animals replicated the initial
184 findings across all behavioural indices of anxiety, again showing a dissociation
185 between the *C3aR*$^{-/-}$ and *C3*$^{-/-}$ mice (Figure 5a). Challenge with diazepam led to a
186 complex pattern of effects. Apart from a drug-induced increase in the latency to initially
187 enter the open arms that was common to all genotypes (Figure 5b), the drug was
188 essentially without effects in *C3aR*$^{-/-}$ mice (Figure 5a, c, d & e) whereas there was
189 evidence of an anxiogenic effect of diazepam in *C3*$^{-/-}$ mice (Figure 5c & e). In
190 diazepam-treated wildtype animals there was a trend for increased time on the open
191 arms (Figure 5c), an effect often held to index reduced anxiety[24,32,33], however these
192 effects did not reach statistical significance. In contrast, we did observe effects of
193 diazepam on SAPs, specifically a highly significant reduction in wildtype animals
194 consistent with the anticipated reduction in anxiety. This reduction in SAPs was
195 completely absent in *C3aR*$^{-/-}$ mice (Figure 5d). Differences in the sensitivity of open
196 arm and ethological measures to benzodiazepines has been noted previously[26] and it
197 has been suggested that SAPs offer a more sensitive indication of anxiolysis[34].
198 Locomotor activity monitored across all areas of the maze indicated that the drug
199 effects in wildtype and *C3aR*$^{-/-}$ mice were unlikely to have been influenced by sedation

200 (Figure 5e). In *C3*$^{-/-}$ mice however, activity was significantly suppressed under drug
201 conditions indicating a possible sedative effect, consequently, *C3*$^{-/-}$ diazepam effects
202 should be interpreted with caution.

203 [Figure 5 about here]

204 **Enhanced corticosterone response in *C3aR*$^{-/-}$ compared to *C3* $^{-/-}$ and wildtype**
205 **mice**

206 In a sub-cohort of wildtype, *C3*$^{-/-}$ and *C3aR*$^{-/-}$ mice treated with vehicle in the diazepam
207 study, we assayed plasma corticosterone 30 minutes after exposure to the EPM and
208 compared levels to those of a group of animals who remained in their home-cages
209 (baseline condition). There were no genotype differences in basal levels of
210 corticosterone (Figure 6), however, being placed on the EPM increased plasma
211 corticosterone 6-15 fold in all genotypes, suggesting that the EPM was a potent
212 stressor. *Post hoc* analyses indicated a significantly greater EPM-evoked
213 corticosterone response in the *C3aR*$^{-/-}$ animals. Given the relationship between
214 behavioural indices of anxiogenesis and activation of the hypothalamic-pituitary
215 adrenal (HPA) axis[35,36], these data were consistent with the behavioural findings
216 showing markedly increased levels of unconditioned anxiety in the *C3aR*$^{-/-}$ animals.

217 [Figure 6 about here]

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

220 The specific effects of *C3aR* knockout on unconditioned anxiety could have been due
221 to developmental changes leading to enduring effects on behaviour and/or effects
222 mediated acutely in the adult brain. To distinguish between these possibilities we

223 examined the extent to which systemic administration of the C3aR antagonist
224 SB290157[23] to wildtype mice at doses which have been shown to influence brain
225 phenotypes[5,37] would phenocopy *C3aR*$^{-/-}$ mice. Two dosing regimens were used; a
226 single injection of SB290157 (i.p,10mg/kg) 60 minutes prior to testing on the EPM and
227 a chronic regime of twice daily injections (i.p, 10mg/kg) for 5 days with EPM testing 24
228 hours after the last treatment. Again, the finding of enhanced anxiety was replicated
229 in *C3aR*$^{-/-}$ mice in both acute (Supplementary Figure 3) and chronic regimens (Figure
230 7). However, neither the acute nor chronic dosing with SB290157 had any effects on
231 behaviour in the EPM. *A priori*, these data were most consistent with *C3aR* knockout
232 influencing adult behaviour via developmental effects resulting in long term effects in
233 the adult brain.

234 [Figure 7 about here]

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

236 We next assessed the effects of *C3/C3aR* manipulations on conditioned, (i.e., learnt)
237 fear in a separate group of animals using the fear potentiated startle (FPS)
238 paradigm[38,39]. In the FPS task a conditioned stimulus (CS, in our case a 30sec light
239 stimulus previously associated with a mild foot shock) presented simultaneously with
240 a startle (pulse-alone) stimulus (Figure 8a) leads to an augmented or 'potentiated'
241 startle response. The index of learning is generated by the difference between the
242 startle response to pulse-alone trials relative to pulse+CS trials and is an established
243 means of assaying conditioned fear employed extensively in human and animal model
244 investigations of fear learning[40,41]. In the pre-conditioning session, pulse-alone trials
245 indicated increased basal startle reactivity in both *C3aR*$^{-/-}$ and *C3*$^{-/-}$ animals relative to
246 wildtype (Figure 8b). Increased reactivity was also demonstrated by both knockouts

247 with regard to the effects of the unconditioned foot shock stimulus (in the absence of
248 any startle stimulus) during the conditioning session, as shown in Figure 8c. The
249 potentiation of the startle response by the CS in the post-conditioning session is
250 illustrated in Figure 8d. Here, in contrast to the previous assays of unconditioned
251 anxiety an opposite pattern of effects was observed, with *C3*$^{-/-}$ animals showing a
252 selective enhancement of conditioned fear relative to the *C3aR*$^{-/-}$ and wildtype animals.
253 It is important to note that the enhanced fear learning in *C3*$^{-/-}$ mice was completely
254 dissociated and therefore not confounded by the underlying effects on basal startle
255 reactivity and/or sensitivity to the foot shock alone, since these effects were common
256 to both knockouts yet only *C3*$^{-/-}$ animals showed the effect on fear learning. These data
257 showing opposing effects of *C3*$^{-/-}$ and *C3aR*$^{-/-}$ on innate anxiety-inducing stimuli and
258 learned anticipatory fear suggested differential impact of the knockouts on associated
259 brain circuitries.

260 [Figure 8 about here]

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

262 The demonstration of enhanced fear learning in *C3*$^{-/-}$, but not *C3aR*$^{-/-}$ mice suggested
263 that the effects in *C3*$^{-/-}$ mice were mediated by molecules downstream of C3,
264 independent from C3a/C3aR. As *C3*$^{-/-}$ mice cannot activate complement beyond C3
265 and therefore lack terminal pathway (C6-C9) activation, it is possible that the indirect
266 loss of function of these molecules may contribute to the fear learning phenotype. We
267 therefore compared *C3*$^{-/-}$ with *C6*$^{-/-}$ mice and hypothesised that should *C6*$^{-/-}$ mice show
268 comparable behaviour to *C3*$^{-/-}$ mice then the phenotype would likely be due to a loss
269 of terminal pathway activity. On the other hand, if *C6*$^{-/-}$ mice demonstrated no fear
270 learning phenotype, this would confine the likely mediating molecules to either C3b or

271 the downstream protein C5 and its breakdown products. Using a separate cohort of
272 mice, we replicated the previous *C3*$^{-/-}$ finding (Figure 9) demonstrating that relative to
273 wildtype mice, *C3*$^{-/-}$ mice showed enhanced basal startle responses (Figure 9a),
274 increased reactivity to the foot shock alone (Figure 9b) and a significant enhancement
275 in conditioned fear (Figure 9c). However, across all these measures the *C6*$^{-/-}$ mice
276 were not different to wildtypes, a pattern of effects consistent with the involvement of
277 C3b/CR3 or C5a/C5aR signalling in the *C3*$^{-/-}$ phenotype. Additionally, we did not
278 observe any innate anxiety-like phenotypes in *C6*$^{-/-}$ mice (Supplementary Figure 4).

279 [Figure 9 about here]

280 **Discussion**

281 We have used a number of *in vivo* knockout models manipulating specific complement
282 proteins to reveal dissociable effects on innate and conditioned emotional behaviours.
283 A main finding was a profound innate anxiety phenotype in the *C3aR*$^{-/-}$ mice manifest
284 across independent behavioural tasks, and repeated assays, that was absent in the
285 *C3*$^{-/-}$ animals. The specificity of the anxiety phenotype exhibited by *C3aR*$^{-/-}$ mice at the
286 behavioural level was paralleled by EPM-evoked corticosterone levels, confirming the
287 validity of the EPM as an index of anxiety. A second main finding was a double
288 dissociation in terms of learned fear. Here, using potentiated startle, we observed the
289 opposite pattern of effects whereby *C3*$^{-/-}$ and not *C3aR*$^{-/-}$ mice displayed enhanced fear
290 reactivity to a conditioned light cue. Taken together, these data are strongly suggestive
291 of distinct functional effects mediated by closely related elements of the complement
292 system. The mechanistic basis of these distinct effects is unknown at present, but they
293 suggest fundamentally different impacts of C3 and C3aR signalling on the brain
294 mechanisms underlying innate anxiety and learned fear.

295 A major implication of our findings relates to the unexpected specificity of the effects
296 of C3aR on unconditioned anxiety and corticosterone levels. Since C3a can only be
297 produced via C3 cleavage and C3aR is the canonical receptor for C3a, *a priori* we
298 expected that any phenotypes dependent on the binding of C3a to C3aR would be
299 apparent in both *C3*$^{-/-}$ and *C3aR*$^{-/-}$ models. Given the divergence in innate anxiety
300 observed, one explanation is that the *C3aR*$^{-/-}$ phenotypes are C3a independent and
301 instead mediated by an alternative ligand. A potential molecular promiscuity of C3aR
302 arises from its long extracellular loop, unique among GPCRs[42] but so far, only one
303 alternative ligand has been identified, a cleavage fragment of the neuropeptide
304 precursor protein VGF (non-acronymic), TLQP-21[43,44]. This peptide has highly
305 pleiotropic roles including in the stress response[45], though few of these roles have, to
306 date, been directly attributed to C3aR signalling. VGF is widely expressed throughout
307 the CNS with high levels found in the hypothalamus, a region associated with stress
308 reactivity and abundant C3aR expression[46,47]. Interestingly, there is further potential
309 for interaction between complement and TLQP-21 in that C3a stimulates nerve growth
310 factor (NGF) expression by glial cells[48], which in turn triggers VGF synthesis, the
311 source of TLQP-21[49]. TLQP-21 may also bind the Complement component 1q
312 receptor, gC1qR[50]. TLQP-21 will therefore be an important target in future
313 investigations of the mechanistic basis of C3aR dependent anxiety phenotypes.

314 Evidence of elevated plasma corticosterone levels in *C3aR*$^{-/-}$ mice after exposure to
315 the EPM, but not at baseline, suggests the anxiety phenotype observed corresponds
316 to 'state' rather than 'trait' anxiety. State anxiety occurs acutely and is enhanced by
317 anxiogenic stimuli, whereas trait anxiety is an enduring characteristic[51]. We were
318 interested in the stress response not only due to the inherent link between stress and
319 anxiety, but also due to the known synergy between the immune and endocrine

320 systems[52]. C3aR is expressed at particularly high levels in endocrine tissues and
321 dosing of C3a stimulates hormone secretion via C3aR[46,47], suggesting it is highly likely
322 that the phenotypes observed in *C3aR*$^{-/-}$ mice involve perturbations in the HPA axis.

323 We probed mechanisms underpinning the *C3aR*$^{-/-}$ phenotype on innate anxiety by
324 assessing the effects of the anxiolytic diazepam on EPM behaviour. Under our test
325 conditions, measures of open arm exploration were insensitive to diazepam treatment
326 but there were specific effects on SAPs, whereby a dose of diazepam that was
327 anxiolytic (i.e., reduced SAPs) in wildtype mice had no effects in *C3aR*$^{-/-}$ mice. SAPs
328 are highly sensitive to pharmacological manipulation[28,53] and in agreement with our
329 own findings, diazepam has been shown to specifically decrease SAPs in the absence
330 of effects on open arm exploration[54]. Importantly, *C3aR*$^{-/-}$ mice consistently displayed
331 the greatest number of SAPs compared to other genotypes, and therefore the lack of
332 drug effects were not due to floor effects. Hence, we propose that SAPs constitute a
333 valid, sensitive index of drug action and that our findings of altered reactivity to
334 diazepam in the *C3aR*$^{-/-}$ are most likely indicative of altered underlying neurobiology.
335 Since benzodiazepine action is dependent on $GABA_A$ receptor function[55], the
336 expression of $GABA_A$ receptor subunits in C3aR$^{-/-}$ mice would be a high priority for
337 future work.

338 Another question relating to potential mechanisms was whether anxiety phenotypes
339 seen in the *C3aR*$^{-/-}$ mice were a result of acute, ongoing effects of *C3aR* deletion in
340 the adult brain or instead the consequence of neurodevelopmental impacts of C3aR
341 deficiency. At the outset, both were possibilities on the basis of previous findings
342 implicating C3aR in developmental neurogenesis[56] and acute changes in C3aR
343 expression in the adult brain after behavioural manipulations[57]. The C3aR antagonist
344 SB290157 had no effects on behaviour in wildtype animals in either dosing regimen

345 used and hence the data were most consistent with *C3aR* knockout influencing adult
346 behaviour via developmental mechanisms, resulting in enduring effects in the adult
347 brain. It should be noted that SB290157 has attracted criticism due to evidence
348 suggesting agonist activity at certain doses and in particular cell types[58]. However, the
349 negative results seen here were not consistent with agonist effects, which one may
350 hypothesize would lead to reduced anxiety in the EPM.

351 Previously, aged *C3*$^{-/-}$ mice have been reported to demonstrate less anxiety in the
352 EPM[8]. While we did not test aged mice, we did observe a subtle anxiolytic effect of *C3*
353 deficiency. In maze-based tasks, *C3*$^{-/-}$ mice consistently performed the greatest
354 number of head dips, and the least SAPs. We also saw a trend towards increased
355 open arm exploration (see Figure 4c, d and vehicle-treated *C3*$^{-/-}$ mice in Figure 5c)
356 and greater duration in the central zone of the open field (Supplementary Figure 2d).
357 It is possible that these effects would be more pronounced if aged animals had been
358 tested, and again suggests opposing effects of *C3* and *C3aR* knockout on innate
359 anxiety. However, we also observed an anxiogenic effect of *C3*$^{-/-}$ on baseline acoustic
360 startle responses (Figure 8b), a phenotype that was shared with *C3aR*$^{-/-}$ mice. While
361 it may be expected that animals displaying greater anxiety-like behaviour in a maze-
362 based task may also display enhanced baseline startle reactivity, as seen in *C3aR*$^{-/-}$
363 mice, a disconnect between exploration-based tasks and startle responses has been
364 reported in rats selectively bred for low or high-anxiety like behaviour[59]. Our results
365 suggest that C3 may have differential effects upon brain circuitries that mediate
366 approach/avoidance behaviours versus those that mediate reflexes such as the startle
367 response.

368 In contrast to innate anxiety, we observed a specific effect of *C3* knockout in regard to
369 conditioned fear. Lack of a comparable phenotype in *C6*$^{-/-}$ mice confirmed that the

370 effects were independent of terminal pathway activation and membrane attack
371 complex formation (see Figure 1). Similarly, the lack of effects in *C3aR*$^{-/-}$ mice relative
372 to wildtype indicated that the phenotype was unlikely to be C3a/C3aR dependent.
373 These observations lead to three possibilities regarding the mediating pathways.
374 Firstly, effects could be mediated via altered C3b/CR3 dependent synaptic
375 elimination[3,4], suggesting a developmental mechanism. In support, complement
376 mediated synaptic pruning has now been demonstrated in brain regions relevant to
377 fear learning such as the amygdala[60] and alterations in glutamatergic synapses within
378 the hippocampus have been reported in *C3*$^{-/-}$ mice[61]. The C3b/ CR3 pathway could
379 also be involved acutely in learning within the adult brain, as *C3* mRNA is upregulated
380 during discrete stages of fear learning[57] and microglial CR3 is implicated in long term
381 depression[62]. The second possibility is that C5a/C5aR, which have a range of
382 developmental roles[56], mediate enhanced fear-learning phenotypes in *C3*$^{-/-}$ mice,
383 although there is less evidence currently for a role of C5a/C3aR in this realm. As a
384 third possibility, there is also evidence for interaction between C3a breakdown product
385 (C3aDesArg) and C5L2, a receptor sharing similar sequence homology with C5aR and
386 C3aR whose function has not yet been fully elucidated[63]. Further investigation of the
387 signalling pathways contributing to the conditioned fear phenotype will be a priority for
388 future research. It will also be necessary to determine whether the phenotype applies
389 to aversive stimuli specifically or whether *C3*$^{-/-}$ mice show enhanced learning generally,
390 irrespective of motivation.

391 The functional consequences of complement are dependent on the location of
392 complement activation and receptor expression. Whether differential expression of C3
393 and C3aR in the brain circuitries and cell types underlying innate anxiety and learned
394 fear contribute to the behavioural dissociations we observe is difficult to ascertain at

395 present. C3aR is expressed primarily by neurons but is also found in astrocytes,
396 microglia and oligodendrocytes [64,65] and C3 is synthesized by most cell types at low
397 constitutive levels throughout the CNS[66,67]. The neural substrates of innate anxiety
398 behaviours overlap to an extent with learned fear circuits[68]. Given there is currently no
399 systematic data comparing and contrasting C3 with C3aR expression across these
400 circuits, an unbiased approach may be the most immediately informative. For
401 example, neuronal activity markers such as immediate early genes could be used to
402 assess circuit and cellular activation, at baseline and subsequent to aversive challenge
403 in *C3*$^{-/-}$ and *C3aR*$^{-/-}$ mice, where the prediction would be that the patterns of activation
404 would be different in the two models despite exposure to identical aversive stimuli.

405 While our experiments using *C3*$^{-/-}$ and *C3aR*$^{-/-}$ mouse models have focused on aspects
406 of anxiety and learned fear, these endophenotypes are commonly comorbid with
407 depression, and recent work has suggested a role for C3a/C3aR in the
408 pathophysiology of major depressive disorder resulting from chronic stress[69]. Given
409 the common co-occurrence of anxiety and depression, our findings of enhanced
410 anxiety in *C3aR*$^{-/-}$ mice might seem at odds with the reported resilience of *C3aR*$^{-/-}$ mice
411 to chronic-stress induced depressive behaviour[69]. However, Crider et al (2018) used
412 a chronic unpredictable-stress paradigm[69] likely to evoke significant inflammation, and
413 therefore the extent to which our data in acutely stressed animals can be compared is
414 questionable.

415 Finally, it is of interest to consider the degree to which our findings relate to the genetic
416 data implicating *C4* in the pathogenesis of schizophrenia[14]. The *C4* variants identified
417 were predicted to increase C4 expression and therefore increase activation of C3[15],
418 and thus our data from *C3*$^{-/-}$ mice is not immediately applicable. Nonetheless, it is
419 possible that both excessive or reduced complement activation could lead to

420 dysfunction, implicating complement as a potential mediator of abnormal fear learning
421 in schizophrenia [70]. Similarly, it is difficult at present to draw links between *C4* and our
422 results demonstrating a role of C3aR in innate anxiety, firstly because of the
423 independence of the phenotype to C3 signalling and secondly due to the current lack
424 of data linking other elements of the complement system beyond C4 to psychiatric
425 disorder. Irrespective of the exact mechanisms that link C3/C3aR and their
426 dissociable effects on emotionality, our findings highlight novel functions of these
427 complement pathways on innate anxiety and conditioned fear that are of relevance to
428 core clinical symptoms of psychiatric disease. Abnormal reactivity to innate anxiety-
429 provoking stimuli and conditioned aversive cues are common and clinically important
430 psychiatric symptoms[17,71] and our data suggest a hitherto unrecognized complexity of
431 complement in mediating these distinct aspects of emotionality.

432 **Author's contributions**

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

438 **Acknowledgements**

439 The authors thank Professor Craig Gerard for providing the *C3aR*$^{-/-}$ mice, and Rhys
440 Perry and staff at JBIOS for their invaluable animal care and husbandry. This work
441 was supported by a Wellcome Trust Integrative Neuroscience PhD Studentship
442 awarded to LJW (099816/Z/12/Z), a Waterloo Foundation Early Career Fellowship
443 awarded to LJW, a Hodge Centre for Neuropsychiatric Immunology Seed Corn and

Project grant awarded to LJW and a Wellcome Trust Strategic Award 100202/Z/12/Z (DEFINE) held by the Neuroscience and Mental Health Research Institute at Cardiff University.

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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671 **Methods**

672 **Animals.** All animals were provided by B. Paul Morgan and Timothy R. Hughes of
673 Cardiff University, UK. Wildtype and *C3*$^{-/-}$ strains were originally sourced from The
674 Jackson Laboratory (strain B6.PL-Thy1^{a}/CyJ stock #000406 and B6;129S4-
675 C3tm1Crr/J stock #003641 respectively), whereas the *C3aR*$^{-/-}$ model was provided by
676 Craig Gerard of Boston Children's Hospital, USA, as a collaboration via B. Paul
677 Morgan and Timothy R. Hughes. *C3*$^{-/-}$ and *C3aR*$^{-/-}$ strains were maintained via
678 homozygous breeding on a C57Bl/6J background.

679 **Husbandry** Male mice between 3-8 months of age were used in the experiments and
680 were kept in littermate groups through the duration of study (2-5 mice per cage).
681 Animals were kept in a temperature and humidity-controlled vivarium (21 ± 2°C and
682 50 ± 10%, respectively) with a 12-hour light-dark cycle (lights on at 07:00 hours/lights
683 off at 19:00 hours). Home cages featured dust-free woodchip bedding and were
684 environmentally enriched with cardboard tubes, soft wood blocks and nesting
685 materials. Standard rodent laboratory chow and water were available *ad libitum*. All
686 strains were kept in one room under the same housing conditions (conventional, open
687 top caging) within a standard vivarium, although they were isolated from other mice to
688 limit exposure to potential pathogens. All procedures were performed in accordance
689 with the requirements of the UK Animals (Scientific Procedures) Act (1986).

690 **General behavioural methods.** Testing took place between the hours of 09:00 and
691 17:00, with random distribution of testing for subjects of different genotypes throughout
692 the day. Mice were habituated to the test rooms for 30 min prior to testing. Each of
693 the test methods used involved individual testing of mice, hence apparatus was
694 cleaned thoroughly with a 70% ethanol solution and antimicrobial cleanser (VetTech

695 Solutions Ltd UK) between subjects. Data for the EPM and EZM were collected using
696 EthoVision XT software (Noldus Information Technology, Netherlands) via a video
697 camera mounted above the centre of each piece of apparatus. Tracking of each
698 subject was determined as the location of the greater body-proportion (12 frames/s) in
699 the specific virtual zones of each piece of apparatus (see Figure 3 for EPM and Figure
700 4 for EZM). Tracking was calibrated for each piece of apparatus prior to testing using
701 mice of the same body size and fur colour as the experimental subjects.

702 **The elevated plus maze (EPM).** The maze, positioned 300 mm above the floor and
703 illuminated evenly at 15 lux, was constructed of opaque white Perspex and consisted
704 of two exposed open arms (175 x 78 mm^2, length x width, no ledges) and two equally
705 sized enclosed arms, which had 150 mm high walls [72]. Equivalent arms were arranged
706 opposite one another (see Figure 3). Subjects were placed at the enclosed end of a
707 closed arm and allowed to freely explore for 5 min. Data from each pair of arms were
708 combined to generate single open and closed arm values (number and duration of arm
709 entries and latency of first entry to each arm). In addition, the following parameters
710 were manually scored: number of stretch-attend postures (SAPs; defined as an animal
711 keeping its hindquarters in the closed arms but stretching forwards onto an open arm),
712 number of head dips from the open arms (looking over the edge of an open arm) and
713 time spent grooming.

714 **The elevated zero maze (EZM).** The maze, positioned 520 mm above the floor and
715 illuminated evenly at 15 lux, was constructed of wood and consisted of two exposed
716 open regions (without ledges; 52 mm wide) and two equally sized enclosed regions
717 (also 52 mm wide), which had 200 mm high grey opaque walls. The diameter of the
718 maze was 600mm. Equivalent regions were arranged opposite one another (see
719 Figure 4). Subjects were placed at the border of one of the open and closed regions

720 and allowed to freely explore for 5 min. Data from each pair of regions were combined
721 to generate single open and closed region values (number and duration of region
722 entries and latency of first entry to each region). In addition, the number of head dips
723 (as above) and time spent grooming were measured. Due to the high walls of the
724 enclosed sections of the maze, subjects were not visible to the experimenter when in
725 the closed regions and therefore these parameters were scored only when a subject
726 was on the open regions.

727 **Locomotor activity (LMA).** LMA was measured in an apparatus consisting of twelve
728 transparent Perspex chambers (each 210 x 360 x 200 mm, width x length x height).
729 Two infrared beams were embedded within the walls of each chamber, which crossed
730 the chamber 30 mm from each end and 10 mm from the chamber floor. Individual
731 subjects were placed in a designated chamber for a 120 min duration on three
732 consecutive days. Beam breaks were recorded as an index of activity, using a
733 computer running custom written BBC Basic V6 programme with additional interfacing
734 by ARACHNID (Cambridge Cognition Ltd, Cambridge, UK). Data were analysed as
735 the total number of beam breaks per session per day.

736 **Assessment of fear potentiated startle (FPS).** FPS was assessed using startle
737 chamber apparatus which consisted of a pair of ventilated and soundproofed SR-LAB
738 startle chambers (San Diego Instruments, CA, USA) each containing a non-restrictive
739 Plexiglas cylinder (35 mm in diameter), mounted on a Perspex plinth, into which a
740 single mouse was placed. The motor responses of subjects to white noise stimuli
741 (generated from a speaker 120 mm above the cylinder) were recorded via a
742 piezoelectric accelerometer, attached centrally below the Plexiglas cylinder, which
743 converted flexion plinth vibration into electrical signals[73]. The peak startle response,
744 within 200ms from the onset of each startle presentation, in each trial, was normalized

745 for body weight differences using Kleiber's 0.75 mass exponent[74]. A computer running
746 SR-Lab software (Version 94.1.7.48) was used to programme trials and record data.
747 A foot shock grid connected to a shock generator (San Diego Instruments, CA, USA)
748 was inserted into the Plexiglas cylinder before conditioning sessions.

749 FPS consisted of three separate sessions presented over a two-day period (see Figure
750 8a). On the first day, mice were given a pre-conditioning session immediately followed
751 by the conditioning session. The pre-conditioning session started with a 5 min
752 acclimatisation phase followed by presentation of 3 no-stimulus trials, and then a block
753 of pulse-alone trials presented at 90, 100 and 110dB (5 of each at 40 ms duration).
754 Trials were randomly distributed throughout the session and presented with a 60 s
755 random interval (range 36 s to 88 s). After the pre-conditioning session was complete,
756 mice were removed from the startle chambers, restraint tubes cleaned, and shock
757 grids placed into the Plexiglas cylinders prior to commencing the conditioning session.
758 The mice were then returned to the startle chambers and subjected to a session
759 consisting of a 5 min acclimatisation phase followed by 3 CS+shock trials, with 3 no
760 stimulus trials before and after, presented with a 2min random interval (range 1.5 to
761 3min). The scrambled 0.14 mA, 0.5 s foot shock was delivered in the final 0.5 s of the
762 30 s visual CS. Following a 24hr delay, subjects were assessed for FPS in the post-
763 conditioning session. This session followed the same format as the pre-conditioning
764 session (5 min acclimatisation phase followed by presentation of 3 no-stimulus trials,
765 and then a block of pulse-alone trials presented at 90, 100 and 110dB, with 5 of each
766 at 40 ms duration) however the final block of trials also included pulse+CS trials at 90,
767 100 and 110 dB (5 of each), with the startle pulse presented in the final 40 ms of the
768 CS. FPS was determined as the fold change between pulse-alone trials and pulse+CS
769 trials within the post-conditioning session.

770 **Pharmacological manipulations**

771 For each drug treatment, separate groups of wildtype, *C3*$^{-/-}$ and *C3aR*$^{-/-}$ were used and
772 were randomly assigned to either vehicle or drug conditions within each genotype.

773

774 ***Diazepam*** A three-day dosing regimen of diazepam (2 mg/kg, i.p., Hameln
775 Pharmaceuticals, UK) or an equivalent volume of vehicle (0.1 M phosphate buffered
776 saline, pH 7.4) was used. Following 2 days of pre-treatment, diazepam or vehicle was
777 administered 30 min prior to testing on the EPM on the 3rd day. A subset of mice
778 receiving vehicle injections in the diazepam experiment were used for assaying
779 plasma corticosterone (see below).

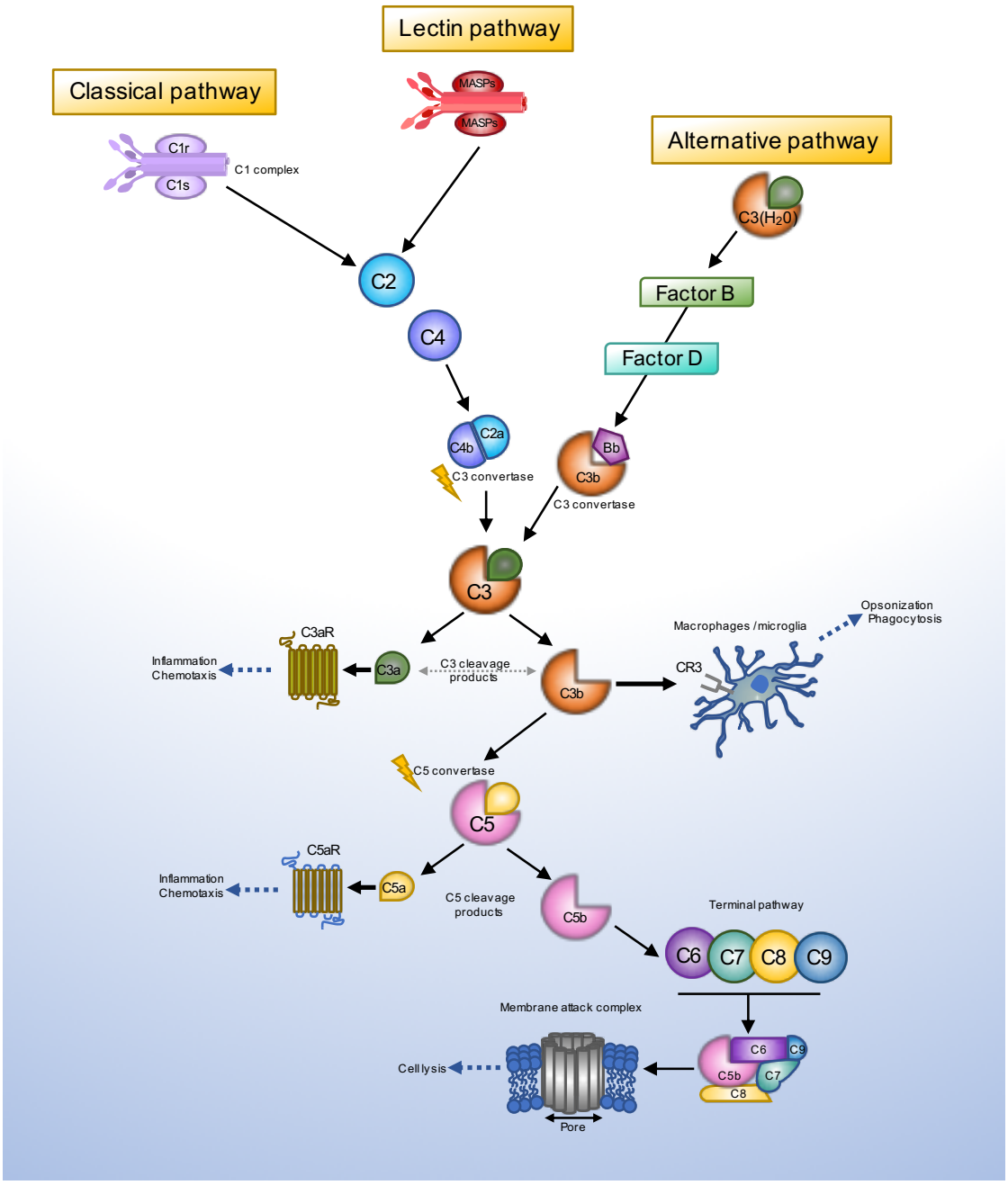
780 ***C3aR antagonist SB290157*** Two dosing regimens were used to assess acute and
781 chronic effects of SB290157[23] (N2-[2-(2,2-Diphenylethoxy)acetyl]-L-arginine
782 trifluoroacetate salt; 10 mg/kg, i.p, Sigma Aldrich, UK) or equivalent volume of vehicle
783 (0.1 M phosphate buffered saline, pH 7.4, with 1% DMSO) on EPM behaviour. In the
784 acute experiment, SB290157 or vehicle was administered 1 hour prior to testing on
785 the EPM. In the chronic experiment, the drug or vehicle was injected twice daily for
786 five days and subjects were tested on the EPM on the 6th day.

787

788 **Corticosterone measurements in mice subjected to the EPM.** Testing took place
789 between the hours of 10:00 and 14:00 to account for the diurnal pattern of
790 corticosterone release[75]. Mice were allowed to freely explore the EPM for 5 min, after
791 which they were placed in a holding cage for a further 20 min before being culled by
792 cervical dislocation. Control mice were removed from their home cage and
793 immediately culled. There was an equal distribution of subjects of different genotypes,
794 counterbalanced between the two test conditions and throughout the testing period.

795 Trunk blood was collected into heparin tubes (Becton Dickinson, USA), and
796 immediately centrifuged at 4000 rpm for 10 min, and the supernatant removed and
797 frozen at -80°c until required. A corticosterone ELISA was performed according to
798 manufacturer's instructions (ADI-900-097, Enzo Life Sciences, UK) and analysed
799 using a four-parameter logistic curve plug in (https://www.myassays.com/four-
800 parameter-logistic-curve.assay).
801
802 **Statistical analysis.** All statistical analyses were carried out using GraphPad Prism
803 8.0 (GraphPad Software, CA, USA). Data was assessed for equality of variances
804 using the Brown-Forsythe test and then appropriate parametric (1-way or 2-way
805 ANOVA) or non-parametric (Kruskal-Wallis) tests used. The main between subjects'
806 factor for all 1-way ANOVA analyses was GENOTYPE (WT, *C3*$^{-/-}$, *C3aR*$^{-/-}$, or *C6*$^{-/-}$).
807 For the EPM, LMA and FPS experiments, there were within subject factors of ZONE
808 (open, closed, middle), DAY (1,2,3) and STIMULUS INTENSITY (90, 100, 110 dB)
809 respectively. Analysis of plasma corticosterone by 2-way ANOVA included an
810 additional between subject factor of CONDITION (baseline, EPM), and for the acute
811 and chronic diazepam experiments, there was an additional between subject factor of
812 DRUG (diazepam, vehicle). A between subject factor of TREATMENT (WT+vehicle,
813 WT+C3aRA and *C3aR*$^{-/-}$+vehicle) and within subject factor of ZONE (open, middle,
814 closed) was used to analyse the data from the C3aR antagonist experiment. *Post hoc*
815 pairwise comparisons were performed using the Tukey or Dunn's tests for parametric
816 or non-parametric analyses, respectively. Alpha values of <0.05 were regarded as
817 significant. Exact p values were reported unless p<0.0001. All p values were
818 multiplicity adjusted[76]. Data are expressed as mean + s.e.m.
819

820 **Figures and legends**



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822 **Figure 1. Overview of the complement system.** The complement system consists
823 of over 30 circulating and cell-surface proteins, cell surface receptors and regulatory
824 proteins, which interact via a cascade of enzymatic activation, ultimately leading to the
825 destruction of pathogens and elimination of cellular debris. The classical pathway is

826 activated upon recognition of antibody-antigen complexes by the C1 complex,
827 whereas the lectin pathway is activated by recognition of carbohydrate residues on
828 pathogen surfaces. The alternative pathway is continually activated in a 'tick-over'
829 manner by spontaneous hydrolysis of the internal thioester within C3. Upon activation,
830 the three pathways lead to assembly of C3 convertases that can cleave the central
831 molecule C3, generating C3b and C3a activation products. C3a is an anaphylatoxin
832 that exerts pro and anti-inflammatory actions upon binding to its canonical receptor,
833 the G-protein coupled C3a Receptor (C3aR). The larger cleavage fragment, C3b, acts
834 as an opsonin which attracts phagocytic cells such as macrophages and microglia
835 when deposited on cell surfaces. C3b also contributes to assembly of the C5
836 convertase which subsequently cleaves C5, resulting in formation of cleavage
837 fragments C5a and C5b, the former of which is an anaphylatoxin that binds to the C5a
838 receptor (C5aR). C5b triggers the terminal complement pathway by binding proteins
839 C6 and C7, inducing conformational change that leads to recruitment of C8 and C9.
840 These molecules aggregate to form the membrane attack complex (MAC) which
841 embeds into the lipid bilayer to create a pore in the target cell membrane, resulting in
842 rapid lysis and cell death.

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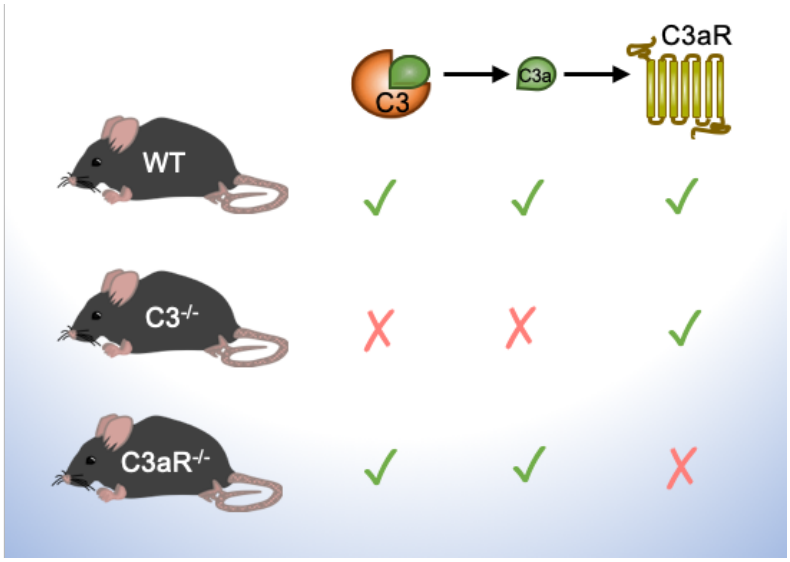

848 **Figure 2. Schematic of knockout mouse models.** C3 activation yields the cleavage
849 fragment C3a, which binds to its canonical receptor C3aR, a member of the GPCR
850 superfamily, characterised by seven transmembrane domains and a large second
851 extracellular loop. This signaling pathway is preserved in wildtype (WT) mice, whereas
852 homozygous *C3* knockout mice lack a functional C3 molecule. Therefore, C3 cannot
853 be cleaved by C3 convertase and thus complement cannot be activated beyond C3,
854 and C3a cannot be generated. While *C3*$^{-/-}$ mice have a functional C3aR, there is no
855 C3a present to bind the receptor. On the other hand, homozygous *C3aR* knockout
856 mice lack only the C3aR and therefore have a functional complement system other
857 than the ability for C3a to bind to C3aR.

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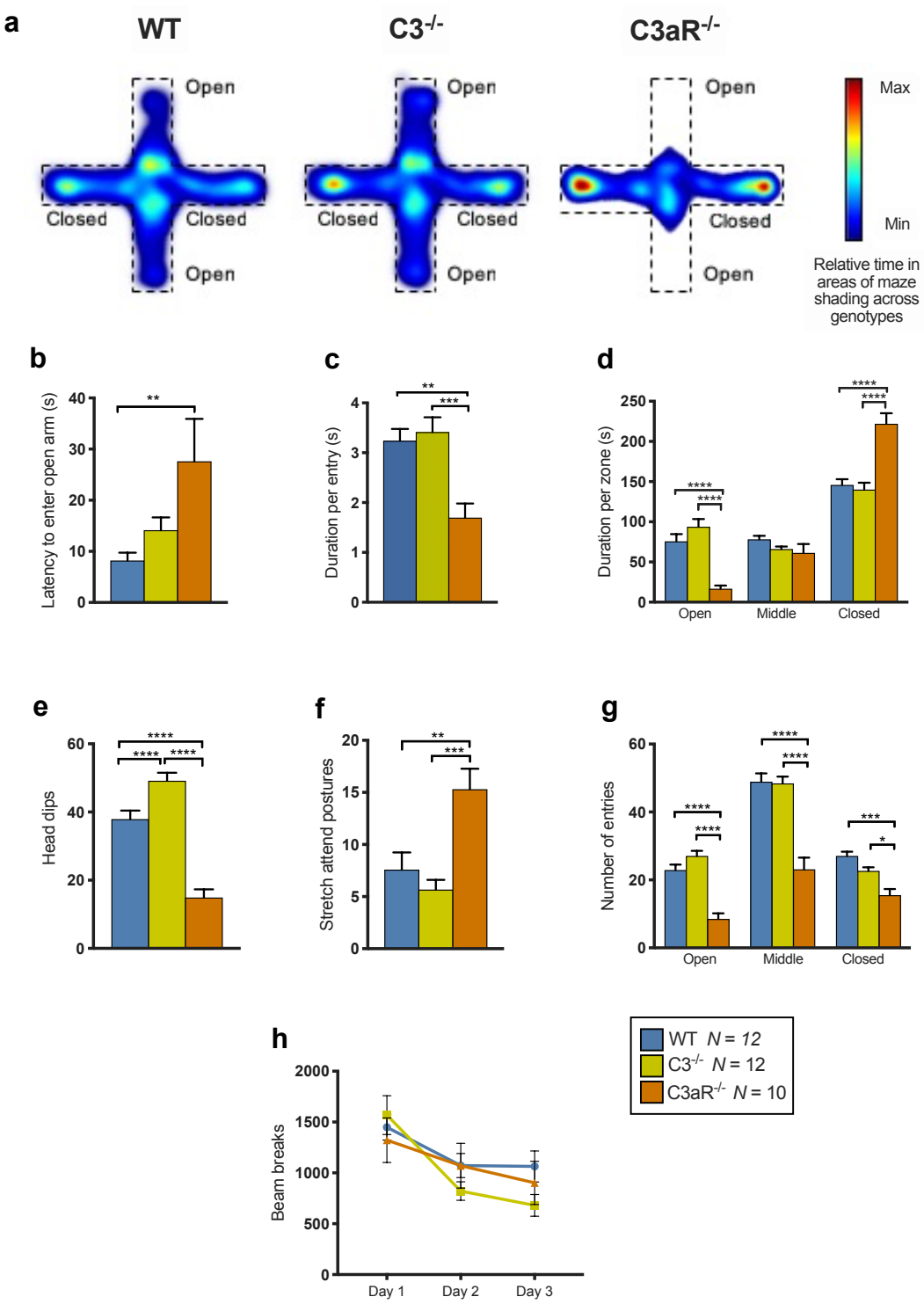

Figure 3. *C3aR*$^{-/-}$, but not *C3*$^{-/-}$ mice show increased anxiety-like behaviour in the elevated plus maze. (a) Heatmaps, displaying relative time per zone across genotypes, illustrate that *C3aR*$^{-/-}$ mice spent less time exploring the open arms than

wildtype and *C3*$^{-/-}$ mice. (**b)** *C3aR*$^{-/-}$ mice were slower to enter the anxiety-provoking open arms than wildtype mice (H_2=10.5, p=0.005) and spent significantly less time on the open arms per entry **(c)** than wildtype and *C3*$^{-/-}$ mice ($F_{2,31}$=11.1, p=0.0002). **(d)** *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 (*post hoc:* wildtype vs. *C3aR*$^{-/-}$ p<0.0001, *C3*$^{-/-}$ vs. *C3aR*$^{-/-}$ p<0.0001), and significantly more time in the closed arms (*post hoc:* wildtype vs. *C3aR*$^{-/-}$ <0.0001, *C3*$^{-/-}$ vs. *C3aR*$^{-/-}$ p<0.0001). **(e)** *C3aR*$^{-/-}$ mice made fewer head dips over the edge of the open arms, a further measure of increased anxiety, than wildtype and *C3*$^{-/-}$ mice, whereas *C3*$^{-/-}$ mice performed more head dips than wildtype and *C3aR*$^{-/-}$ mice ($F_{2,31}$=48.0, p<0.0001). **(f)** *C3aR*$^{-/-}$ mice performed significantly more SAPs than wildtype (p=0.0042) and *C3*$^{-/-}$ mice (p=0.0004) **(g)** *C3aR*$^{-/-}$ mice were less active in the EPM than wildtype and *C3*$^{-/-}$ mice, making significantly fewer entries to all of the zones of the maze (main effect of GENOTYPE, $F_{2,31}$=29.4, p<0.0001), however demonstrated a similar pattern to the other groups with increased transitions through the middle zone (main effect of ZONE, $F_{2,62}$=384.0, p<0.0001). **(h)** To demonstrate that the reduced activity in the EPM was task/situation specific, locomotor activity was measured in a separate non-anxiety provoking environment, over three days. There were no differences between wildtype, *C3aR*$^{-/-}$ and *C3*$^{-/-}$ mice in total activity in these sessions (main effect of GENOTYPE, $F_{2,31}$=0.344, p=0.712), and all mice demonstrated normal habituation to the test environment between sessions (main effect of DAY, $F_{2,62}$ =61.9, p<0.001). 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.

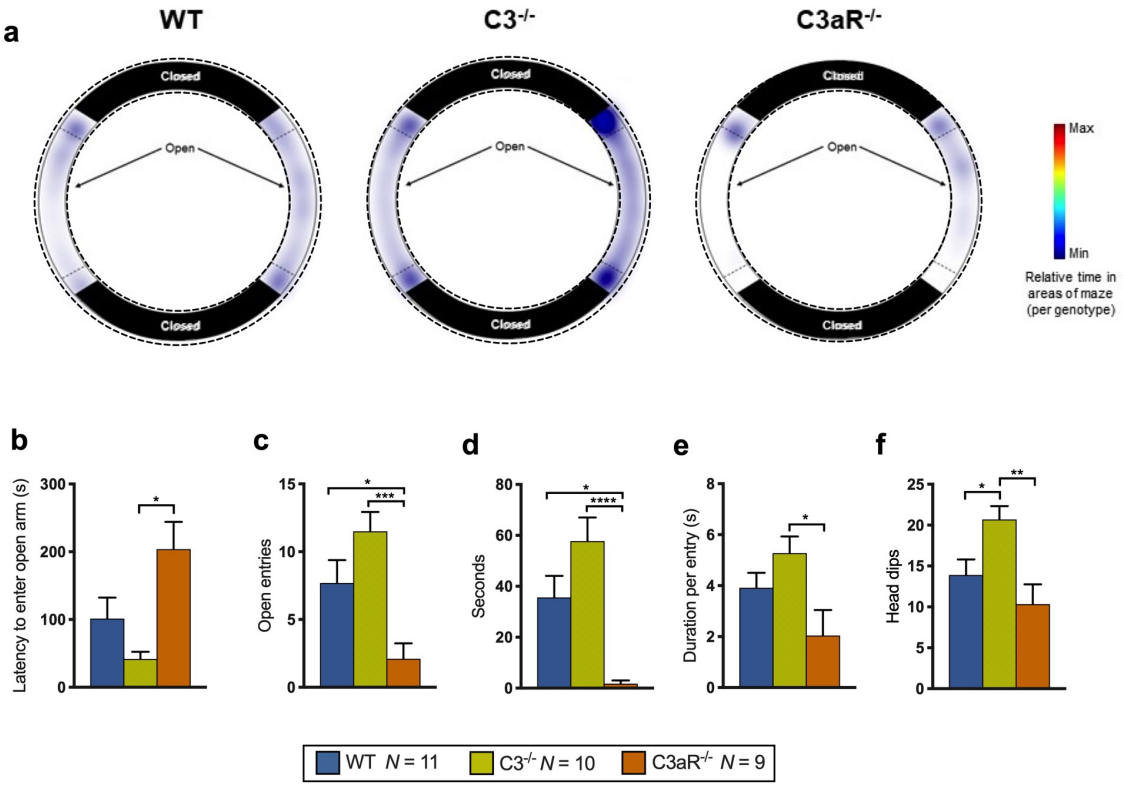

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Figure 4. The elevated zero maze elicited anxiety-like behaviour in *C3aR*$^{-/-}$, but not *C3*$^{-/-}$ mice. (a) Heatmaps, displaying relative exploration of the open segments across genotypes, illustrate that the *C3aR*$^{-/-}$ mice explored these regions less than *C3*$^{-/-}$ and wildtype mice. Note: due to the height of the walls in the closed regions it was not possible to track the mice or observe ethological behaviours such as grooming or SAPs. **(b)** *C3aR*$^{-/-}$ mice were slower than *C3*$^{-/-}$ and wildtype subjects to enter the open areas of the EZM (H_2=8.13, p=0.0171), but only reaching significance in comparison with the *C3*$^{-/-}$ mice (*post hoc* tests wildtype vs. *C3aR*$^{-/-}$, p=0.2012 and *C3*$^{-/-}$ vs. *C3aR*$^{-/-}$ p=0.0140). **(c)** *C3aR*$^{-/-}$ mice made significantly fewer entries to the open areas ($F_{2,30}$=8.96, p=0.0009), spent less time on the open arms **(d)** throughout the 5 minute test than wildtype or *C3*$^{-/-}$ mice (H_2=19.2, p<0.0001) and less time per open arm visit **(e)** than *C3*$^{-/-}$ subjects ($F_{2,26}$=4.56, p=0.0201). **(f)** As in the EPM, *C3*$^{-/-}$ mice performed more head dips than wildtype or *C3aR*$^{-/-}$ mice ($F_{2,27}$=6.86,p=0.0039).

Data are mean + S.E.M. *, **, ***, and **** represent $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$and $p \leq 0.0001$ for *post-hoc* genotype comparisons, respectively.

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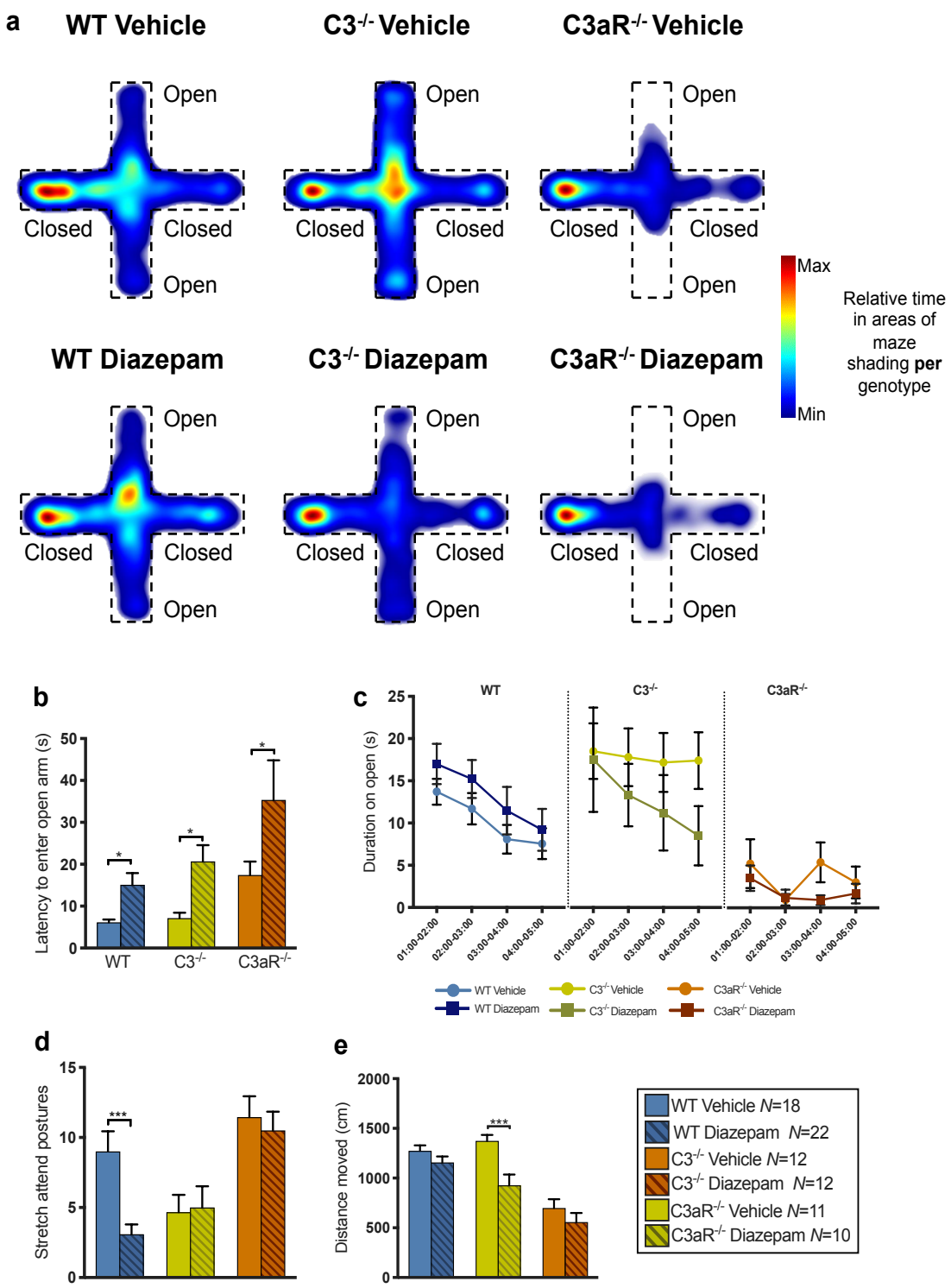

Figure 5. Altered sensitivity to diazepam in *C3aR*$^{-/-}$ and *C3*$^{-/-}$ mice. A separate cohort of behaviourally naïve mice were treated with either diazepam (2mg/kg, i.p) or vehicle injections once daily for 2 days and then 30 minutes prior to testing. (**a**) Heatmaps demonstrating duration spent in zones of the maze by vehicle treated and

871 diazepam treated animals **(b)** Across genotypes, diazepam treatment significantly
872 increased latency to first open arm visit compared to vehicle treated animals (main
873 effect of DRUG, $F_{1,73}$=23.6, p<0.0001). As previously, *C3aR*$^{-/-}$ mice took significantly
874 longer than other groups to initially enter the open arm (main effect of GENOTYPE,
875 $F_{2,69}$=10.6, p<0.0001). **(c)** Plots showing duration spent on open arms in 1-minute time
876 bins (start-01:00 excluded due to initial effect of diazepam in delaying initial entry to
877 open arms). There was a trend for wildtype diazepam treated animals to spend more
878 time on the open arms throughout the task although this did not reach significance
879 (main effect of DRUG, $F_{1,38}$=1.41, p=0.2462). In *C3*$^{-/-}$ mice there was a strong tendency
880 for drug treated animals to explore the open arms less than vehicle treated *C3*$^{-/-}$ mice
881 (main effect of DRUG, $F_{1,22}$=1.25, p=0.2764). A similar, though less pronounced
882 pattern was seen in *C3aR*$^{-/-}$ mice (main effect of DRUG, $F_{1,19}$=1.55, p=0.2284) **(d)**
883 There were genotype differences in SAPs (main effect of GENOTYPE, $F_{2,79}$=10.7,
884 p<0.0001) and a main effect of DRUG ($F_{1,79}$=4.13, p=0.0454). Diazepam significantly
885 reduced the number of SAPs in wildtype mice only (GENOTYPE × DRUG interaction,
886 $F_{2,79}$=4.64, p=0.0138, *post hoc* tests wildtype vehicle vs. wildtype diazepam
887 p=0.0006). There were also genotype differences in distance moved around the maze
888 **(e)** (main effect of GENOTYPE, $F_{2,79}$=32.1, p <0.0001) and a GENOTYPE × DRUG
889 interaction approaching significance ($F_{2,79}$=2.85, p =0.0640). Diazepam treatment
890 significantly reduced distance moved in *C3*$^{-/-}$ mice only (*post hoc C3*$^{-/-}$ vehicle vs. *C3*$^{-/-}$
891 diazepam p=0.0006). Data are mean + S.E.M. *, **, and *** represent p≤0.05, p≤0.01
892 and p≤0.001 for *post-hoc* genotype comparisons, respectively.

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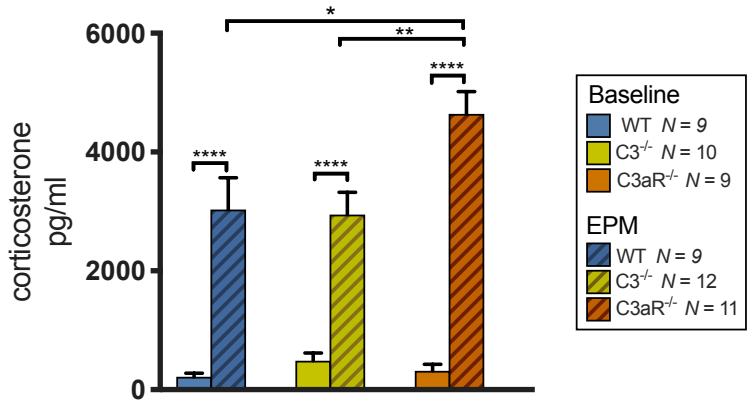

Figure 6. Elevated corticosterone response to the elevated plus maze in *C3aR*$^{-/-}$ mice. Corticosterone was measured in plasma, using sandwich ELISA, from previously behaviourally naïve mice that were either tested on the EPM for 5 minutes and culled 30 minutes later, or were culled swiftly after being removed from their home cage (baseline group). Exposure to the EPM significantly elevated corticosterone in all genotypes (main effect of CONDITION, $F_{1,54}$=143, p<0.0001), but was more pronounced in *C3aR*$^{-/-}$ mice (GENOTYPE × CONDITION interaction, $F_{2,54}$=4.64, p=0.0138). *Post hoc* analysis showed that in the EPM condition, *C3aR*$^{-/-}$ mice demonstrated significantly higher corticosterone levels than wildtype (p=0.0127) and *C3*$^{-/-}$ mice (p=0.0032). *Post hoc* tests also indicated that there were no baseline differences between the groups of mice (wildtype and *C3aR*$^{-/-}$ p>0.9999, wildtype and *C3*$^{-/-}$ p=0.9927, and *C3*$^{-/-}$ and *C3aR*$^{-/-}$ mice p=0.9992). Data are mean + S.E.M. *, **, and **** represent p≤0.05, p≤0.01 and p≤0.00)1 for *post-hoc* genotype comparisons, respectively.

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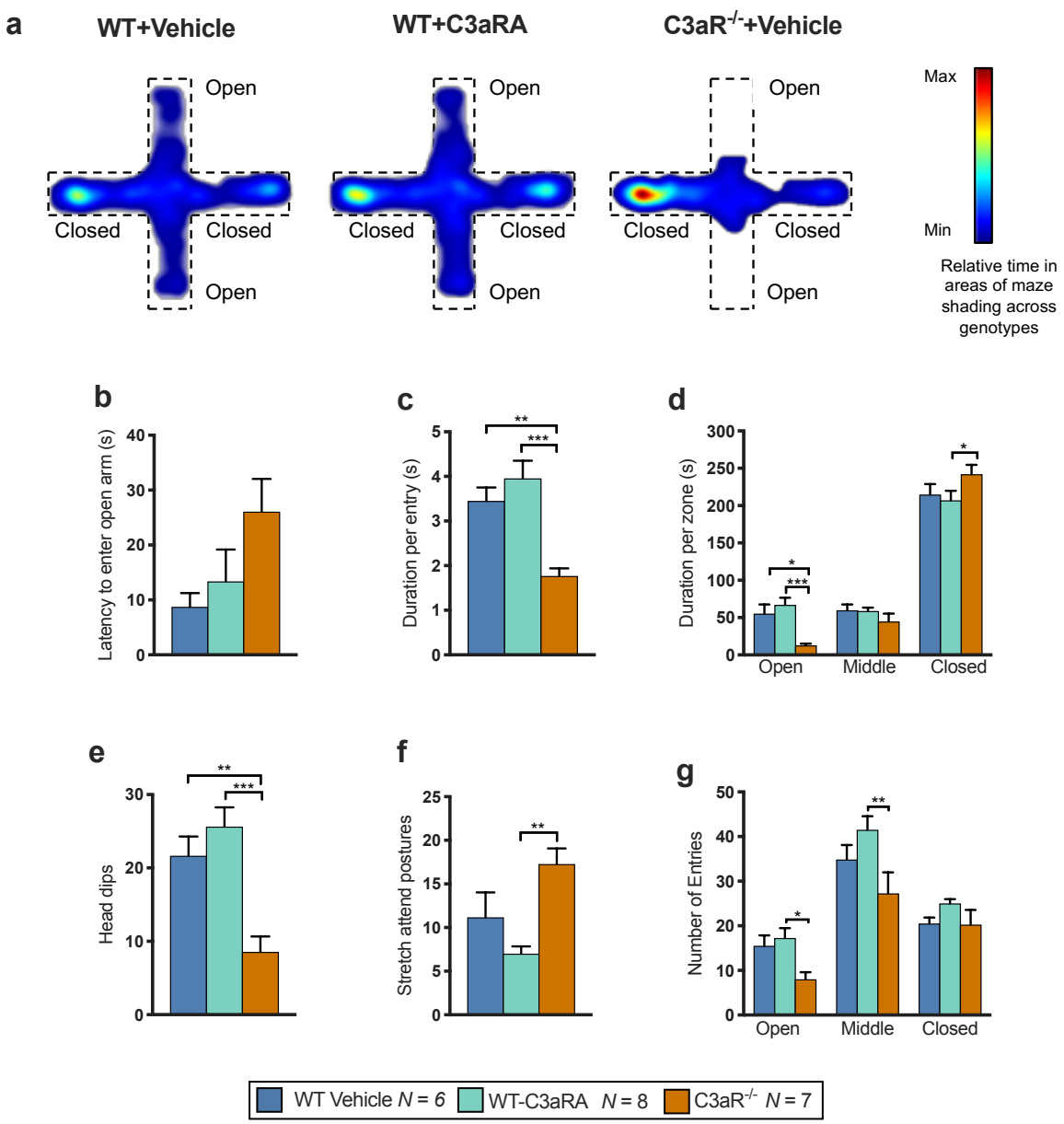

Figure 7. Pharmacological C3aR blockade in wild types does not recapitulate *C3aR*$^{-/-}$ phenotypes in the elevated plus maze. (a) Heatmaps demonstrating a further replication of the previously shown anxiety phenotype in *C3aR*$^{-/-}$ mice but no effect of chronic administration of the C3aR antagonist (C3aRA; SB290157, i.p. 10mg/kg, twice daily for five days) in wildtype mice (Note: *C3aR*$^{-/-}$ mice received vehicle injections). **(b)** There was a trend for greater latencies to first open arm visit

in *C3aR*$^{-/-}$ ($F_{2,18}$=2.73, p=0.0923). **(c)** *C3aR*$^{-/-}$ mice spent significantly less time on the open arms per entry than wildtype-vehicle and wildtype-C3aRA treated mice ($F_{2,18}$=13.4, p=0.0003) **(d)** *C3aR*$^{-/-}$ mice again distributed their time across the EPM differently to wildtype-vehicle and wildtype-C3aRA (GENOTYPE×ZONE, $F_{4,54}$=5.67, p=0.0007) spending less time in the open arms (*post hoc:* wildtype-vehicle vs. *C3aR*$^{-/-}$ p=0.0157, wildtype-C3aRA vs. *C3aR*$^{-/-}$ p=0.0007), and significantly more time in the closed arms than wildtype-C3aRA mice (*post hoc:* wildtype-C3aRA vs. *C3aR*$^{-/-}$ p=0.0363, wildtype-vehicle vs. *C3aR*$^{-/-}$ p=0.1667). *C3aR*$^{-/-}$ mice made fewer head dips **(e)** ($F_{2,18}$=13.4, P = 0.0003) and displayed more SAPs **(f)** ($F_{2,18}$=8.50, P = 0.0025) than wildtype-vehicle and wildtype-C3aRA treated mice. **(g)** There was a significant GROUP×ZONE interaction, ($F_{4,36}$=3.35, p=0.0199) which demonstrated that *C3aR*$^{-/-}$ mice made fewer entries to the open arm than wildtype-C3aRA mice (p=0.456). They also made fewer entries to the middle region than wildtype-C3aRA mice (p=0.0012). 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.

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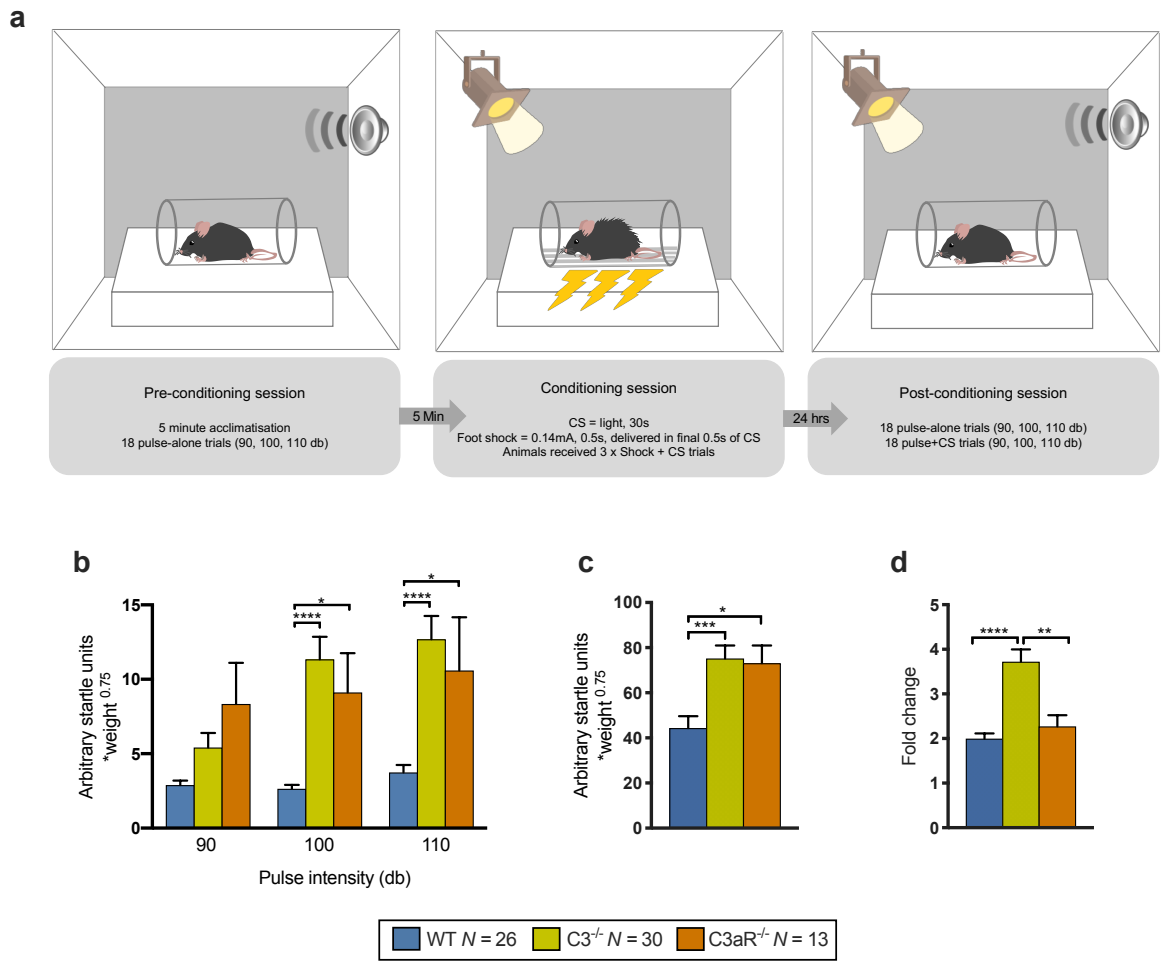

Figure 8. Enhanced fear-potentiated startle in *C3*$^{-/-}$ but not *C3aR*$^{-/-}$ mice. (a) Flow chart depicting the FPS protocol used, which took place in three separate sessions over two consecutive days. Baseline startle reactivity to a range of pulse intensities was assessed in the pre-conditioning session, immediately preceding the conditioning session in which a visual stimulus 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)** Both *C3*$^{-/-}$ and *C3aR*$^{-/-}$ mice demonstrated increased levels of startle responding relative to wildtype mice at all pulse intensities

(main effect of GENOTYPE, $F_{2,66}$=9.04, p=0.0003), although this was greatest at 100dB and 110dB (GENOTYPE × STIMULUS INTENSITY interaction, $F_{4,132}$=7.55, p<0.0001). **(c)** *C3*$^{-/-}$ and *C3aR*$^{-/-}$ mice also showed increased startle responding to the footshock+CS pairings relative to wildtype mice ($F_{2,66}$=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 relative to wildtype and *C3aR*$^{-/-}$ subjects (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.

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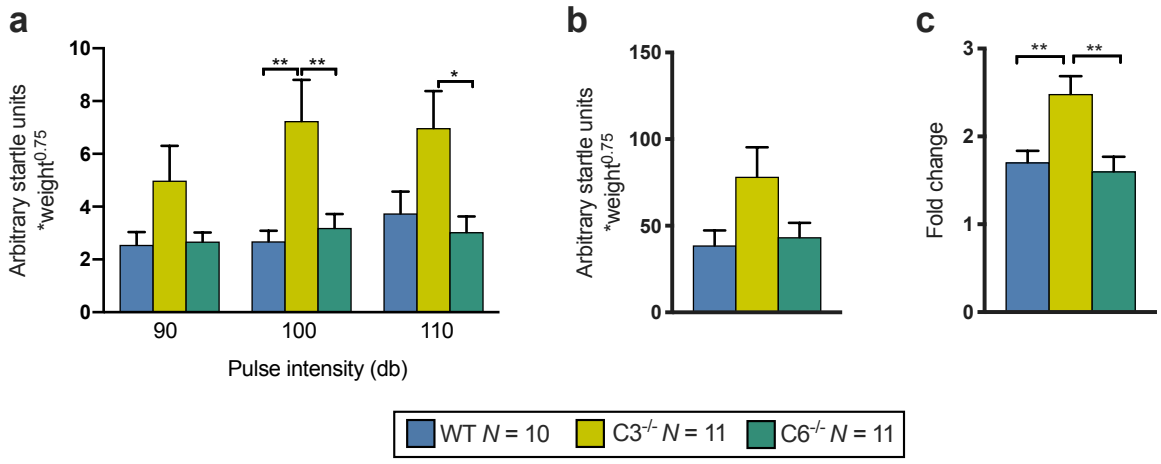

Figure 9. Enhanced fear-potentiated startle in *C3*$^{-/-}$ but not *C6*$^{-/-}$ mice. Using the same FPS protocol as before (Figure 9), a separate cohort of wildtype and *C3*$^{-/-}$ mice were assessed in comparison with a group of *C6*$^{-/-}$ mice. **(a)** *C3*$^{-/-}$ mice demonstrated elevated startle responding relative to wildtype, as shown previously, and also *C6*$^{-/-}$ mice at all pulse-alone intensities used (main effect of GENOTYPE, $F_{2,20}$=5.87, p=0.0098). **(b)** Although not significant, *C3*$^{-/-}$ mice also demonstrated a trend towards greater startle reactivity to the footshock+CS pairings during the conditioning phase of training (H_2=4.66, p=0.0971). **(c)** Post-conditioning, *C3*$^{-/-}$ mice showed the greatest fold change in startle responding between pulse-alone stimuli and pulse+CS stimuli, which was significantly greater than that shown by wildtype and *C6*$^{-/-}$ mice ($F_{2,29}$=8.08, p=0.0016). In each of phase of this experiment, there were no differences in responding between *C6*$^{-/-}$ and wildtype 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.

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