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9 **Dual-component structural plasticity mediated by**
10 **α CaMKII-autophosphorylation on basal dendrites of**
11 **cortical layer 2/3 neurones**

12
13 by

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55

56

57 **Abstract**

58

59 Sensory cortex exhibits receptive field plasticity throughout life in response to changes in sensory
60 experience and offers the experimental possibility of aligning functional changes in receptive field
61 properties with underpinning structural changes in synapses. We looked at the effects of two
62 different patterns of whisker deprivation in male and female mice; 'Chessboard deprivation', which
63 causes functional plasticity and 'All deprived', which does not. Using 2-photon microscopy and
64 chronic imaging through a cranial window over the barrel cortex, we found that layer 2/3 neurones
65 exhibit robust structural plasticity, but only in response to whisker deprivation patterns that cause
66 functional plasticity. Chessboard pattern deprivation caused dual-component plasticity in layer 2/3
67 by (1) increasing production of new spines that subsequently persisted for weeks and (2) enlarging
68 spines-head sizes in the pre-existing stable spine population. Structural plasticity occurred on
69 basal dendrites but not apical dendrites. Both components of plasticity were absent in α CaMKII-
70 T286A mutants that lack LTP and experience-dependent potentiation in barrel cortex, implying that
71 α CaMKII auto-phosphorylation is not only important for stabilisation and enlargement of spines but
72 also for new spine production. These studies therefore reveal the relationship between spared
73 whisker potentiation in layer 2/3 neurones and the form and mechanisms of structural plasticity
74 processes that underly them.

75

76

77

78 **Significance Statement**

79

80 This study provides a missing link in a chain of reasoning that connects LTP to experience-
81 dependent functional plasticity *in vivo*. We found that increases in dendritic spine formation and
82 spine enlargement (both of which are characteristic of LTP) only occurred in barrel cortex during
83 sensory deprivation that produced potentiation of sensory responses. Furthermore, the dendritic
84 spine plasticity did not occur during sensory deprivation in mice lacking LTP and experience-
85 dependent potentiation (α CaMKII auto-phosphorylation mutants). We also found that the dual-
86 component dendritic spine plasticity only occurred on basal dendrites and not on apical dendrites,
87 thereby resolving a paradox in the literature suggesting that layer 2/3 neurones lack structural
88 plasticity in response to sensory deprivation.

90 Introduction

91

92 Understanding the relationship between functional and structural plasticity requires knowing where
93 in the brain the functional plasticity takes place and then looking for the structural plasticity in that
94 location. This issue is important for understanding processes underlying learning and memory.
95 However, it is usually not possible to know where to look in the brain when plasticity is induced
96 during learning because memories are distributed across networks of neurones within single brain
97 structures and even relatively simple learned behaviours involve multiple brain regions, any of
98 which could house the sought after structural changes (Hoffman and McNaughton, 2002; Josselyn
99 and Frankland, 2018). From this view-point, understanding plasticity's structure-function
100 relationship is more tractable when studied in sensory cortex and when induced by sensory
101 deprivation because, in this case, the location of the functional plasticity is often well characterised.
102

103

104 Sensory deprivation causes functional plasticity in layer 2/3 in visual and somatosensory cortex
105 (Fox and Wong, 2005). Layer 2/3 neurones increase their responses to sensory inputs spared from
106 the deprivation and decrease their responses to sensory inputs that are deprived. Following
107 whisker trimming in a chessboard pattern, layer 2/3 neurones increase their responses to spared
108 whisker stimulation and decrease their responses to deprived whisker stimulation (Wallace and
109 Fox, 1999b). These changes are known to be cortical rather than subcortical and to depend on
110 cortical activity (Fox, 1994; Wallace et al., 2001). Potentiation of the spared whisker response
111 depends on auto-phosphorylation of CaMKII (Hardingham et al., 2003), which is a key step in
112 induction of LTP (Giese et al., 1998; Chang et al., 2017). Depression of the deprived response is
113 known to depend on GluA1 and to occlude LTD (Hardingham et al., 2008; Wright et al., 2008).
114 These findings and others have implicated Hebbian processes in experience dependent cortical
115 plasticity (Glazewski and Fox, 1996; Glazewski et al., 2000; Wallace et al., 2001; Dachtler et al.,
116 2011).

117

118 Although a great deal of work has been conducted on functional plasticity in layer 2/3 cells, to date
119 most studies on spine dynamics and structural plasticity in the cerebral cortex have been carried
120 out on layer 5 apical dendrites (Lendvai et al., 2000; Holtmaat et al., 2006; Wilbrecht et al., 2010;
121 Keck et al., 2013). This can partly be explained by the availability of Thy-1 GFP lines, where the
122 fluorophore is very conveniently expressed sparsely in a subset of layer 5 neurones and partly by
123 the relative ease of imaging apical dendrites that lie close to the surface of the brain. However,
124 functional plasticity in cortical layer 5 cells is complicated by the differences in plasticity
125 mechanisms present in regular spiking (RS) and intrinsic bursting (IB) cells, whereas layer 2/3
126 neurones appear more uniform in mechanism (Jacob et al., 2012; Greenhill et al., 2015).
Furthermore, it is not clear how structural plasticity of apical dendritic spines might be related to

127 functional changes in receptive fields, when most of the sensory input via thalamic and layer 4
128 projections to layer 5 neurones impinge on the basal not the apical dendrites (Petreanu et al.,
129 2009). Even in layer 2/3 neurones, the basal dendrites tend to receive strong sensory input from
130 VPM and layer 4 while apical dendrites receive the input from motor cortex (Petreanu et al., 2009;
131 Hooks et al., 2011). In this study, we have focused on structural plasticity in layer 2/3 rather than
132 layer 5 and on basal dendrites more than apical in an effort to rebalance these mismatches.

133

134 To understand structural changes related to potentiation mechanisms, we also compared the effect
135 of whisker deprivation on plasticity in wild-types with that in CaMKII auto-phosphorylation mutants
136 that lack cortical and hippocampal LTP (Giese et al., 1998; Hardingham et al., 2003). Our findings
137 elucidate the relationship between structural and functional plasticity in the cortex and demonstrate
138 a pivotal role for CaMKII in both functional and structural plasticity.

139

140

141 **Methods**

142

143 **Animals and rAAV constructs**

144

145 We used Male and female α CaMKII-T286A homozygous mutant mice, which have an Alanine
146 substituted at the Threonine 286 location (Giese et al., 1998), and their wild-type litter-mates for
147 imaging experiments (see Table 1). Animals were social-group housed with *ad libitum* food and
148 water in a 12:12 hour normal light/dark cycle. All animal care and use was performed in
149 compliance with the UK Animals (Scientific Procedures) Act 1986. The rAAVs were purchased
150 from the University of Pennsylvania Vector Core:

151 rAAV2/1.CAG.FLEX.EGFP.WPRE.bGH (Allen Institute 854) and rAAV.CaMKII 0.4.Cre.SV40
152 (Allen Institute).

153

154 **Trans-cranial window implantation and rAAV intracranial virus 155 injection**

156

157 Cranial windows were implanted using methods similar to those published previously (Chen et al.,
158 2000; Mostany and Portera-Cailliau, 2008; Holtmaat et al., 2009). Briefly, mice were injected with
159 dexamethasone (2 mg g⁻¹ body weight), deeply anesthetized with isoflurane and head-fixed on an
160 ultra-precise stereotaxic frame (Kopf model 963). After shaving the hair, a midline incision of the
161 scalp was made by scissors. The periosteum tissue was removed, the outer skin layers adhered to
162 the skull with tissue adhesive (Vetbond), and the surgical steel head-plate was implanted with
163 dental cement (Prestige Dental Super Bond C+B kit). Mice were then head fixed with the steel
164 head-plate, and areas were marked in the designated stereotactic coordinates for the D1 whisker
165 of the barrel field (3.0 mm lateral from midline and 1.5 mm posterior from bregma). A 3mm
166 diameter craniotomy was performed using a micro drill. The skull was removed gently and intact
167 dura was covered with a drop of cortex buffer. Glass pipettes (tip diameter 10–20 μ m connected to
168 a WPI Ultra-microsyringe pump and Micro4 controller (WPI inc. Sarasota USA) were lowered with
169 a micro-positioner (Kopf Instruments) to 200 μ m DV. The virus solution (200nl) was injected slowly
170 (25nl/min) into the barrel cortex and was composed of virus solution (cre-AAV 1:10000 in equal
171 proportion with GFP-Flex 1:10) mixed with 10% Fast Green for visualisation. Sparse labelling was
172 achieved by using low-titre cre-recombinase and high titre floxed GFP. Rois were chosen at the
173 edge of the virus diffusion radius (usually 150 μ m radius). The glass pipette was left for a further 2
174 mins in the brain after injection had finished. In total an injection was completed in 10 mins. A
175 sterile 3mm glass coverslip was placed over the exposed area and sealed with Super Glue and
176 dental cement. Imaging began after a 2- to 3-week recovery period as described previously (Crowe
177 and Ellis-Davies, 2014).

178

179

180

181 **Sensory Manipulation**

182

183 For sensory deprivation experiments, whiskers of the facial pad contralateral to the cranial window
184 were trimmed by a pair of scissors under a dissection microscope while the mice were under
185 transient isoflurane anaesthesia. Whiskers were subsequently trimmed every other day for the
186 duration of the imaging protocol. Whisker trimming for whole whisker pad deprivation involved
187 trimming all whiskers from the contralateral facial pad (Figure 1A,B), while chessboard pattern
188 deprivation was performed with the D1 whisker always deprived and every other whisker cut with a
189 pair of scissors in a chessboard pattern (Figure 1C,D).

190

191 **2-photon imaging**

192

193 For imaging sessions, animals were anesthetized lightly with isoflurane and head fixed via the steel
194 head plate under the objective lens. Two-photon imaging was performed with an Olympus BX68
195 microscope and PrairieView software. All images were taken with 25x water-immersion objective
196 (Olympus W Plan-APOCHROMAT, 1.05 numerical aperture), 6mm galvo mirrors and a beam
197 expander to ensure maximum illumination of the back-aperture. A mode-locked Ti:sapphire laser
198 (Chameleon Vision S; Coherent) was used to generate two-photon excitation (900nm), with power
199 at the back aperture in the range of 10–50 mW. A pixel dwell time of 8 μ s with a frame size of 1024
200 \times 1024 pixels was used. Emission wavelengths were band-passed between 525-570nm and the
201 light path included an IR filter. Layer 2/3 neurones were identified by imaging dendrites a minimum
202 of 120 microns from the brain surface, and where possible, tracing basal dendrites back to the cell
203 soma and noting the depth. Dendritic spines on the basal dendrites of layer 2 and layer 3 cells
204 (average depth of soma below dura: 222 μ m, range: 175-375) were imaged repeatedly every 3 to 4
205 days over a three-week period before and after deprivation. Dendritic spine images were acquired
206 in 1 μ m z-steps. Surface vasculature landmarks in combination with logged coordinates for each
207 region of interest were used for mapping and imaging the same region over the experimental time
208 course. We aimed to image 10 regions of interest from each animal over the period of 3-4 weeks.
209 Two or three baseline images were taken separated by 3 or 4 days (-10, -6, -2 days relative to the
210 day of deprivation at 0). Five post-deprivation time-points were taken at +1,+4, +7, +11 and +14
211 (Figure 1F).

212

213 **Photo-lesions**

214

215 Mice were deeply anaesthetised with isoflurane and head-fixed under the 40x objective lens
216 (Olympus W Plan-APOCHROMAT 0.8 NA water). An optical zoom of x2 was used producing a
217 50 μ m \times 50 μ m field of view. The laser was mode locked to a wavelength of 800nm and the
218 Pockels cell adjusted to deliver approximately 50-64 mW power. 2-photon excitation was focused

219 400 μm below the dura to lesion layer 4. The galvos were centred and the shutter opened for a
220 period of 10-12 mins. Mice were then perfused under terminal anaesthesia and brain sections
221 were stained for cytochrome oxidase to visualise the barrel field and photo-lesions demarcating the
222 imaging field (Figure 1E). Photo-lesions could be seen against the barrel field in horizontal section
223 in layer 4. In more superficial sections the effect was apparent as regions of bleached
224 fluorescence.

225
226

227 **Image analysis**

228
229 ImageJ was used to analyse all images. Raw image stacks were deconvolved using Fiji
230 Deconvolution Lab plugin for Image J from point spread functions taken for the microscope and
231 objective lens used. Images were only analysed where the signal to background intensity was at
232 least 4. For dendritic spine analysis, dendritic spines were classified as a protrusion from the
233 dendritic shaft at least 0.4 μm (Holtmaat et al., 2009). The numbers of spines and dendrites
234 imaged for each genotype and deprivation method can be found detailed in Table 1. Spine
235 formation and elimination rates were calculated by counting the number of gained spines, lost
236 spines, and total spines between each imaging session, per day for each dendrite (Figure 1G,H).
237 Formation rate was calculated by dividing the number of gained spines at each time point by the
238 number of spines present at the first time point. The number formed per day was then calculated
239 based on the interval between observation points. Elimination rate was calculated in an analogous
240 way.

241

242 Bifurcating dendrites were chosen randomly in so far as they were not originally sought during
243 image acquisition and were found to be the only ones in our sample that were relatively parallel to
244 the field of view and satisfied our criterion for a bifurcation rather than a smaller offshoot branch.
245 Dendritic width was measured at 3 points way from the bifurcation point and averaged. Where the
246 two branch widths differed by less than 15% we counted them as an even pair of branches.

247

248 Spine head size, neck width and neck length were measured for each spine and used to classify
249 spine types. Spine head width was taken as the greatest diameter across the spine head in the
250 image in which it was in focus. Spines were only counted if they protruded at least 0.4 μm from the
251 dendrite. Spine head size distributions approximated a log-normal distribution when measured this
252 way (Kolmogorov test) similar to the finding with other methods (Loewenstein et al., 2011). To
253 estimate the error in measuring spine size we took images of dendrites 30 minutes apart and
254 cross-correlated the measures. The method assumes that the spines do not change size greatly
255 over this time period. The average difference in size between observations was less than 0.5% and
256 ranged from 0-11% (mean \pm SD; 0.04% + 0.10%, n=17). The difference in size measured over 30
257 minutes was therefore approximately 20 times smaller than the average size increase seen with

258 deprivation. The sum of the residuals for a linear regression fit ($y = 1.013x - 0.03$) was almost zero
259 (6.2×10^{-3}) suggesting no difference in the population.

260

261 We also classified spines according to the major types reported before. Mushroom spines were
262 defined as having a head size >1.15 times the neck width plus a neck length $< 0.9 \mu\text{m}$. Thin spines
263 were counted as those having a head size >1.15 times the neck width and a neck length $>0.9\mu\text{m}$.
264 Stubby spines had a neck length < 0.9 , and a head size <1.15 times the neck width (in practice
265 very similar neck and head width). We also saw a smaller number of filopodia which were
266 classified as having head size <1.15 times the neck width, but neck length $>0.9\mu\text{m}$. Filopodia were
267 not included in the spine analysis except where stated in the spine classification sections.

268

269 **Electrophysiology**

270

271 Six C57BL/6J mice aged between P87 and P132 (average P104) were deprived of all their
272 whiskers on one side of the snout for 1 day and 4 mice aged between P80 and P152 (average
273 P111) were similarly whisker deprived for 7 days. In addition, 6 mice were deprived in a
274 chessboard pattern for 1 day (P84-97, average P91) and 6 for 7 days (P92-117, average P103). A
275 further 6 undeprived mice were recorded as controls (P75 -P200, average P97). Animals were
276 prepared for spike recording using carbon fibre micro-electrodes under urethane anaesthesia as
277 described before (Armstrong-James and Fox, 1987). Whiskers were acutely trimmed from the
278 spared side of the snout and glued onto the whisker stubs on the deprived side using
279 cyanoacrylate glue. Principal whisker responses were evoked by deflecting the whisker with a fast
280 piezo-electric bimorph stimulator by a standard 1 degree deflection (10ms). Responses were
281 averaged over 50 stimuli and defined as spikes produced during a 3-53ms following stimulation.
282 Details of recording methods can be found elsewhere (Fox, 1992; Fox et al., 2018). Mice were
283 perfused with para-formaldehyde and cryo-protected with sucrose before the brains were flattened
284 for sectioning using a freezing microtome. Sections were reacted for cytochrome oxidase to view
285 the electrolytic lesions made after each recording penetration and thereby establish the principal
286 barrel for each recording penetration and the depth of recording for each cell. Neurones were
287 identified as layer 2/3 or layer 4 and the ratio of the average layer 2/3 to layer 4 response was
288 calculated for each animal. Group averages were calculated for 1 day deprived and 7 day deprived
289 animals and compared with published values for young animals (P28-53) receiving all whisker
290 deprivation for 1 or 7 days.

291

292 **Experimental Design and Statistical Analysis**

293

294 The experimental design was longitudinal for spine imaging studies comprising 2 or 3 baseline time
295 points followed by 5 time points over a further two weeks of repeatedly imaging the same locations.
296 This allowed us to apply paired t-tests to compare all possible baseline and post-deprivation time

297 point combinations. Three variants of this statistical approach were planned; one to study another
298 genotype, CaMKII-t286a mice using chessboard deprivation; the other two, to study the effects of
299 whisker deprivation, namely undeprived mice with “chessboard deprived” and “all whisker
300 deprived” mice. Male and female mice were studied for all groups. The ratio of male to female mice
301 was approximately 3:2 respectively in the final sample, due to slightly fewer female mice in the
302 CaMKII-t286a group reaching the weight required for recovery surgery (as stipulated by the animal
303 care legislation under which we operate). We planned to image 10 regions of interest (Roi) for
304 each animal (see Table 1 for summary statistics). However, due to the long period of imaging and
305 the fact that basal dendrites were located deeper than those conventionally studied on apical
306 dendrites, not all Rois remained clear over the full 3 week period. On average, approximately 3
307 Rois remained clear per animal over the full 3 week period (7 or 8 observations for each Roi)

308

309 Spine size changes were analysed using matched pair t-tests as described in the Results section
310 and, where unmatched populations were studied, by ANOVA methods. Spine head sizes were
311 found to be log-normal as described before (Loewenstein et al., 2011), and were therefore log-
312 transformed before using parametric methods. In one case (transient spines in CaMKII-T286A
313 mice), the data was not normally or log-normally distributed and non-parametric tests were used.
314 Spine categorisation analysis and spine lifetime measures were analysed using non-parametric
315 tests (Wilcoxon signed rank and Chi squared methods). Cross-correlations were assessed using
316 linear regression analysis. Data was analysed using JMP software (SAS, Marlow, Bucks UK).

317

318 Precautions were taken against unintended bias: the images were either (a) analysed blind to the
319 hypothesis and/or (b) analysed by more than one person and cross-checked and/or (c) analysed
320 blind to the genotype. In addition, in all cases, a different person to the one collecting and
321 measuring the images performed statistical analysis on the data.

322

323 Electrophysiological data was analysed by averaging neuronal responses to standard whisker
324 deflections for all cells in a given layer for each animal and then averaging values across animals
325 within the treatment/time-point group. Comparisons between groups were then made using
326 ANOVA followed by post-hoc t-tests where appropriate. Population data for formation and
327 elimination rates were also analysed using ANOVA followed by post-hoc t-tests where effects were
328 detected.

329

330 Results

331

332 1. The effect of whisker deprivation pattern on receptive 333 field plasticity

334

335 We compared the effects of chessboard pattern deprivation (CWD) and all-whisker deprivation
336 (AWD) on receptive field plasticity in layers 2/3 of the barrel cortex in young adult mice (average
337 age P100).

338

339 All whisker deprivation

340

341 Depriving all the whiskers uniformly for 1 or 7 days did not cause potentiation of any surround
342 receptive field whisker ($F_{(2,2)}=1.16$, $p=0.32$), nor indeed change any receptive field component at all
343 (Figure 2A,B). While depriving all the whiskers can cause depression of deprived whisker
344 responses in younger animals (Glazewski et al., 2017), we found it did not produce any change in
345 the receptive fields of the older animals studied here (average age 107 days, range 80-152). The
346 principal whisker response appeared to decrease marginally (to 90% of undeprived values), but
347 was not found to be significantly different from control values ($F_{(1,16)}=1.44$, $p=0.25$).

348

349 Chessboard pattern deprivation

350

351 In contrast, chessboard pattern deprivation did cause substantial potentiation of spared whisker
352 responses, both in the barrel-columns where the principal whisker had been deprived ($F_{(2,2)}=18.66$,
353 $p<0.001$, Figure 2C) and in the spared barrel-columns where the principal whisker had been
354 spared ($F_{(2,2)}=5.26$, $p<0.01$; Figure 2D). In deprived barrels, the three strongest surround receptive
355 field whisker responses potentiated two to three fold after a single day of deprivation (S1, $\times 2.23$;
356 S2, 2.14; S3, 3.03) and increased further by 7 days (S1, $\times 2.75$; S2, 3.16; S3, 3.53). In spared
357 barrels, there was a delay to the potentiation, which occurred after 7 days, again for the three
358 strongest surround receptive field whiskers (S1, $\times 2.62$; S2, $\times 3.18$ S3, $\times 2.91$). We also found that
359 principal whisker responses fell to 65% of control values 1-7 days following chessboard pattern
360 deprivation and were significantly different from responses in control undeprived mice ($F_{(1,20)} =$
361 6.18, $p<0.03$).

362

363 The difference in effects of CWD and AWD are summarised in Figure 3 (A and D) which show
364 principal whisker responses and the strongest surround whisker responses (S1) for control, 1 day
365 and 7 day deprived mice.

366

367

368

369 **2. The effect of whisker deprivation pattern on spine** 370 **formation and elimination** 371

372 To determine whether structural plasticity occurred in layer 2/3 neurones and to see whether it was
373 related to receptive field plasticity observed in layer 2/3 neurones, we repeated the two whisker
374 deprivation patterns in mice prepared with cranial windows for imaging dendritic spines.

375

376 **All whisker deprivation**

377

378 We compared the rate of spine formation and elimination in AWD mice with their pre-deprivation
379 baseline rates and found that formation and elimination were unchanged 24 hours after deprivation
380 (baseline versus formation at day 1: $t_{(10)} = 0.45$, $p < .65$; baseline versus elimination at day 1: $t_{(9)} =$
381 0.40 , $p < .69$; paired t-tests) (Figure 3B,C). Similarly, formation and elimination rates were not
382 different from those seen in undeprived animals at any time-point (no effect of deprivation on
383 formation $F_{(1,137)} = 0.068$, $p=0.79$, or elimination $F_{(1,130)}=0.77$, $p=0.38$; 2-way ANOVA). This finding
384 is consistent with the lack of functional plasticity found with this deprivation pattern at these ages
385 (Figure 3A) and suggests that spine dynamics are unaffected by a general loss of afferent drive.

386

387 **Chessboard pattern deprivation**

388

389 We compared rates of dendritic spine formation and elimination in wild-type mice that had their
390 whiskers deprived in a chessboard pattern with their pre-deprivation baseline rates. We found that
391 formation and elimination increased significantly following 24 hours of deprivation (formation:
392 baseline versus 24h deprivation: $t_{(17)} = 8.75$, $p < .0001$; elimination baseline versus 24h
393 deprivation: $t_{(17)} = 5.10$, $p < 0.0001$; paired t-tests) (Figure 3E). To quantify the effect we compared
394 baseline formation and elimination rates in mice without whisker deprivation over a similar period of
395 time. In undeprived mice at this age (70-125 days), we found that baseline formation and
396 elimination rates were evenly matched, comprising approximately 4% of the original spines per day
397 (Figure 3E). The effect of whisker deprivation was to increase transiently the formation rate to 18%
398 and the elimination rate to 12%. The formation rate then remained elevated above baseline over
399 the succeeding 14 days, though at a far lower rate than that observed on the first day (Figure
400 3E,F). Repeated measures ANOVA showed a significant two-way interaction between time and
401 deprivation for spine formation in wild-type mice ($F_{(5,163)} = 31.35$, $p < .0001$). When analysed per
402 time-point, the formation rate was significantly elevated on day 1, 4, and 11 ($F_{(1,32)} = 55.93$, $p <$
403 $.0001$ on day 1, $F_{(1,31)} = 13.15$, $p < .001$ on day 4, $F_{(1,25)} = 13.51$, $p = .005$ at day 11) (Figure 3E).

404

405 Elimination rates also remained elevated during CWD, meaning that only a small net gain in spines
406 occurred over the two-week period (Figure 3E). Once again, a repeated measures ANOVA showed
407 a significant two-way interaction between time and deprivation for wild-type mice ($F_{(5,160)} = 6.52$, p
408 < 0.0001). Analysed per time-point, spine elimination was significantly elevated 1, 4, 7 and 11 days
409 following deprivation, ($F_{(1,32)} = 22.91$, $p < .0001$ on day 1, $F_{(1,31)} = 4.77$, $p < .05$ on day 4, $F_{(1,30)} =$
410 7.34 , $p < .05$ at day 7, $F_{(1,22)} = 9.51$, $p < .01$ at day 11)(Figure 3E). These results show that whisker
411 deprivation patterns that cause functional plasticity (CWD) also cause structural plasticity in layer
412 2/3 neurones, while whisker deprivation patterns that do not cause functional plasticity (AWD),
413 leave no trace of structural plasticity.

414
415 Previous studies have demonstrated that new spines tend to form on a particular subset of
416 dendritic branches that exhibit a naturally high formation rate (Yang et al., 2009). We therefore
417 looked for instances of bifurcating dendrites within our data set. Evenly dividing bifurcations were
418 defined as two daughter branches that differed in width by 15% or less, (average width difference
419 4%) to distinguish them from minor branches protruding from a main dendrite. We found that both
420 high formation branches (HFB) and low formation branches (LFB) showed significant increases in
421 spine formation 24 hours after chessboard deprivation (HFB $t_{(6)}=3.33$, $p<0.02$; LFB $t_{(6)} = 3.94$,
422 $p<0.01$, paired t-test), although the increase appeared larger for the HFBs (18.7% increase above
423 baseline versus 8.6%), (Figure 4). We compared the behaviour of the HFB and LFB located at
424 bifurcations with individual dendrites that we paired randomly. The HFBs in the random pairs again
425 showed significant increases in spine formation with deprivation (HFB random $t_{(6)}=4.05$, $p<0.01$
426 LFB random $t_{(6)} = 3.32$, $p<0.02$), paired t-tests), but the difference between HFB and LFB
427 formation rates was smaller than with the natural bifurcating pairs (11.8% increase versus
428 9.1% increase). Taken across all time-points following deprivation, spine formation was greater in
429 the HFB than the LFB for the bifurcation pairs ($t_{(28)} = 3.42$, $p<0.002$, paired t-test), but was not
430 different for the randomly assigned pairs ($t_{(26)} = 1.3$, $p=0.2$, paired t-test). These findings suggest
431 that while baseline formation rate is predictive of a larger response to deprivation, a particular
432 relationship exists between high and low formation pairs of dendrites at a bifurcation point. In
433 concert with this finding, we found that the absolute rate of spine formation 24 hours after
434 deprivation was moderately well correlated with baseline spine rate for bifurcating pairs of
435 dendrites ($r^2=0.45$) but not at all for randomly paired dendrites ($r^2=0.002$) (Figure 4E,F).

436
437 Previous studies had not found structural plasticity in layer 2/3 neurones in response to sensory
438 deprivation (Hofer et al., 2009; Ma et al., 2016), but most studies in this area have looked at the
439 apical dendrites rather than the basal dendrites. Apical and basal dendrites receive different
440 afferent input on balance (Petreanu et al., 2009) as shown in Figure 5A. We therefore checked to
441 see whether CWD had similar effects on the apical dendrites compared to the basal dendrites
442 (Figure 5B). We found that 24 hours after deprivation formation and elimination rates were

443 unaffected by CWD (Figure 5C). Baseline formation rates were similar to that seen on basal
444 dendrites 4.7% (see Table 1) and did not increase significantly following deprivation ($t_{(3)} = 0.54$,
445 $p=0.63$, paired t-test). Similarly, elimination rates were similar to those of basal dendrites at 6.1%,
446 and while they appeared slightly higher following deprivation at 8.3%, were not significantly
447 different from baseline measures ($t_{(3)} = 1.5$, $p=0.22$, paired t-test). Our results are therefore
448 consistent with previous reports concerning apical dendrites, but additionally show that basal and
449 apical dendrites behave differently under chessboard pattern deprivation.

450

451 **3. Spine formation and elimination in α CaMKII-T286A** 452 **mutants**

453

454 To test whether the increase in spine formation we observe in chessboard deprived wild-type mice
455 is dependent on a cortical LTP-like process, we trimmed whiskers in a chessboard pattern in
456 α CaMKII-T286A point mutants, which have an Alanine substituted at the Threonine 286 location;
457 these animals lack CaMKII auto-phosphorylation (Miller and Kennedy, 1986; Giese et al., 1998)
458 and both cortical LTP in the layer 4 to 2/3 pathway (Hardingham et al., 2003) and cortical
459 experience-dependent potentiation in layer 2/3 (Glazewski et al., 2000). We found that spine
460 formation was unchanged 24 hours following deprivation compared to their baseline pre-
461 deprivation rates (baseline versus formation at day 1: $t_{(11)} = 0.177$, $p < 0.86$) (Figure 6). Similarly,
462 there was no difference between formation rates in deprived versus undeprived α CaMKII-T286A
463 mice ($F_{(1,145)} = 1.02$, $p=0.314$).

464

465 Independent of deprivation, baseline formation and elimination rates were elevated in α CaMKII-
466 T286A mice. Comparison of undeprived animals across all time-points revealed formation rates of
467 3.8% for wild-types and 4.9% for α CaMKII-T286A mice and these values were significantly
468 different ($t_{(148)}=12.71$, $p<0.0005$). Similarly, elimination rates were higher in α CaMKII-T286A mice
469 at an average of 4.1% in wild-types versus 4.9% in α CaMKII-T286A mice ($t_{(145)}=10.87$, $p<0.002$). In
470 these cases, as with others we studied, formation and elimination were closely matched over a
471 timespan of several days, though the equilibrium could be temporarily interrupted by whisker
472 deprivation. However, a striking exception to this rule was found with deprivation of the α CaMKII-
473 T286A mice. Chessboard deprivation increased spine elimination in a similar fashion to that seen
474 in wild-types (compare Figures 3E and Figure 6B, negative values). Spine elimination increased to
475 15%, 24 hours following deprivation compared to baseline ($t_{(11)} = 3.99$, $p<0.002$; paired t-test),
476 though no other time-point was significantly different from undeprived cases. In the absence of
477 spine formation, this transient period of spine elimination produced a net loss of spines that were
478 not replaced over the period of observation.

479

480 We also compared formation and elimination rates across wild-type and α CaMKII-T286A mice
481 following chessboard deprivation. We found a significant interaction between time and genotype
482 ($F_{(4,122)} = 9.06$, $p < 0.0001$) due to a higher formation rate in the wild-types at 1 day and 4 days
483 following deprivation (compare Figures 3E and 6B), ($F_{(1,29)} = 26.0$, $p < 0.001$ for 1 day and $F_{(1,28)} =$
484 6.54 , $p < 0.02$ at 4 days). However, ANOVA analysis showed that elimination rates were not
485 different between the two genotypes ($F_{(1,26)} = 0.07$, $p = 0.78$), even though elimination appeared to
486 last a shorter period after deprivation in α CaMKII-T286A mice. These results show that
487 experience-dependent formation of new spines is dependent on CaMKII auto-phosphorylation,
488 while elimination is not.

489
490

491 **4. Spine persistence, spine head size and spine** 492 **morphology in wild-types** 493

494

495 **Spine persistence** 496

497 The new spines that appear on the first day of whisker deprivation in chessboard deprived wild-
498 type mice may either disappear quite quickly or last for some period of time and, in the latter case,
499 they may be capable of forming the substrate for experience-dependent potentiation. To
500 investigate the persistence of new spines, we plotted the rate of spine loss for newly formed spines
501 (i.e. those spines not present in the baseline time period, but which first appeared 24 hours after
502 whisker trimming) (Figure 7A).

503

504 Spine lifetimes for new spines were bi-phasicly distributed, with transient spines (observed for
505 just a single time-point) and new persistent spines (lasting at least 13 days) dominating the
506 distribution. In undeprived animals, 57% of new spines were transient and just 29% persistent. This
507 pattern was reversed in CWD mice where 29% were transient and 45% persistent. Consequently,
508 the average lifetime of a new spine increased significantly following whisker deprivation ($X^2_{(1)} = 12.7$,
509 $p < 0.0005$, $n = 188$, Wilcoxon test). When coupled with the increased production of spines one day
510 following deprivation, this led to a substantial increase in the proportion of new persistent spines.
511 Over the observation period, approximately 8% of new spines were persistent in chessboard
512 deprived animals compared to less than 1% in undeprived animals (Figure 7A).

513

514 Chessboard whisker deprivation creates a mosaic pattern of barrels in the cortex where a barrel
515 that has lost its principal whisker input due to whisker trimming sits next to several barrels with
516 intact principal whisker input (Figure 1D). Electrophysiological measurements of evoked whisker
517 responses showed that potentiation of responses to spared whisker stimulation occurs in deprived
518 barrels and spared barrels (Figure 2C,D). In other words, the spared whisker components of

519 surround receptive fields are potentiated in general by CWD. In concert with this finding, we
520 observed that the (increased) lifetime of newly formed spines following CWD was identical in the
521 deprived and spared barrels ($X^2_{(1)} = 0.74$, $p=0.38$, $n=73$, Wilcoxon test).

522
523 A substantial component of the spines present on the dendrites following deprivation were present
524 in the baseline from the start of observations (Figure 7B). These spines are likely to code for the
525 pre-existing receptive field properties of the neurones, which tend to be dominated by the principal
526 whisker. Given that the principal whisker response decreases following chessboard deprivation,
527 again in deprived and spared barrels (Figure 2) (Wallace and Fox, 1999b), we looked at how spine
528 lifetime was affected by deprivation in this sub-population of spines. We found that whisker
529 deprivation increased the rate of spine loss from the first day of deprivation (Figure 7B). In
530 undeprived animals, the proportion of surviving spines was asymptotic at approximately 65% of the
531 original number after 21 days of observation, suggesting that approximately 65% percent of spines
532 were stable. In chessboard deprived mice, the proportion of surviving spines dropped to 48% over
533 the same observation period, implying an increased loss of at least 17% due to deprivation.
534 Consequently, spine lifetime decreased significantly in chessboard deprived animals for spines
535 already present at the first observation point ($X^2_{(1)} = 10.9$, $p<0.001$, $n=472$, Wilcoxon test) and once
536 again this value was not significantly different between spared and deprived barrels ($X^2_{(1)} = 0.24$,
537 $p=0.62$, $n=310$, Wilcoxon test).

538 539 **Spine head size for new and eliminated spines**

540
541 The lifetime of a spine is normally closely related to the size of the spine head, with larger spines
542 exhibiting longer lifetimes than smaller spines (Yasumatsu et al., 2008). We therefore looked at the
543 distribution of spine head sizes of spines newly formed 24 hours after deprivation that persisted for
544 the duration of the CWD period and compared it with the distribution for spines that were present
545 before deprivation and persisted over the whole observation period. We found that the distribution
546 of spine head sizes for new persistent spines (NPS) after 24 hours (Figure 8A) was not significantly
547 different from that for the stable spines that were present throughout the observation period
548 (always present spines, APS; $F_{(1,173)}=3.13$, $p=0.07$). However, NPS heads were significantly larger
549 than those of transient spines (present for a single time period) ($F_{(1,86)}=5.76$, $p<0.02$). NPS were
550 also larger than newly formed spines that were subsequently lost over the next 13 days (Figure
551 8C,D). A two way ANOVA showed an effect of head size on persistence of newly formed spines at
552 24 hours ($F_{(1,185)} = 3.61$, $p<0.002$), with the difference also apparent at 4, 7 and 11 days following
553 deprivation. These findings suggest that NPS rapidly acquire the same spine head size as the
554 stable population of AP spines after just 24 hours, which prompted us to study spine head size at
555 a briefer 12 hour time-point. We found that spine head sizes for new persistent spines at 12 hours
556 (NPS_{12}) were smaller than those at 24 hours (NPS_{24}) and not different from those of transient
557 spines ($F_{(1,99)} = 5.05$, $p<0.01$). These results suggest that newly formed spines become established

558 somewhere between 12 and 24 hours following deprivation (Figure 8C,D).

559

560 We also looked at the sizes of spines that become eliminated following whisker deprivation. During
561 the deprivation period, spines that were lost had significantly smaller spine heads than those of the
562 baseline AP population of spines ($F_{(1,296)}=18.8$, $p<0.0001$) (Figure 8B).

563

564

565 **Induced changes in spine head size for stable spines**

566

567 We were interested to see whether CWD caused a general increase in spine head size, as this
568 might provide a structural substrate for the potentiation of spared whisker responses in addition to
569 the increased numbers of NPSs. When the overall spine population was considered, which
570 included stable and transient populations of spines, we found little overall change in spine size and
571 no statistically significant effects (Figure 9A,C,E). However, spine sizes vary from one time-point to
572 another, due partly to spontaneous spine fluctuations (Yasumatsu et al., 2008) and due partly to
573 the variety of spine lifetimes (and therefore spine sizes) present in any given sample (Figure 8).
574 The AP sub-population of spines, while still showing spontaneous spine fluctuations, were at least
575 free of the variability in spine size due to transient and intermediate spine lifetimes. We therefore
576 tested whether there was an effect of CWD on the AP population of spines. We found that spines
577 in deprived and spared barrels increased in spine head size following deprivation (Figure 9B,D).
578 Within the general population of AP spines, individual spines increased and others decreased in
579 size, but overall the population increased in size (Figure 9D).

580

581 There was a clear relationship between the size of the spines at baseline at its direction of size
582 change following deprivation (Figure 9F). The small spines tended to show increased head sizes
583 while the larger spines showed decreased head sizes. This effectively provided an apparent
584 homeostatic reaction to the CWD induced enlargement seen in the stable spine population. The
585 increase in the population spine head size was therefore due to many small spines increasing and
586 only being partly compensated by fewer large spines decreasing in head-size.

587

588 The change in spine size was relatively small (on average 10%). Nevertheless, the AP spines
589 represent some 65% of the total spine population at any one time (dependent on age) and the
590 general effect may therefore be physiologically significant. We found no difference in spine size
591 between the control period baseline time-points ($t_{(147)}=1.13$, $p=0.26$), but all the baseline time-
592 points differed from all the post-deprivation time-points (for example at 1 day post-deprivation,
593 $t_{(147)}=4.05$, $p<0.0001$, matched pair t-test; see Figure 9 legend for full statistics).

594

595 We also looked to see if apical dendrites also showed increases in the size of the stable spine
596 population following CWD. We found that unlike basal dendrites, the stable spine population on the

597 apical dendrites showed no change in population spine size 24 hours following deprivation ($t_{(97)}$
598 $=0.76$, $p=0.44$, matched pair t-test) (Figure 5D). It was also apparent that the average size of the
599 apical dendrite spine heads was in general smaller those of basal dendrites when comparing
600 baseline measures with undeprived controls over a similar period of time ($F_{(1, 589)}=11.8$, $p<0.001$).

601

602 We also tested to see whether the AP population of spines changed size in the AWD mice. In
603 contrast to the effect of CWD, we found that AWD produced a small decrease in average spine
604 size (Figure 9B). Overall, AWD reduced AP spine head size to 94% of control values over the
605 deprivation period and this was a significant effect ($F_{(1,1285)} = 4.03$, $p<0.0002$). The effect was
606 clearer from 7 days onward and AP spine head sizes averaged 90% of control values after 14 days
607 of AWD ($t_{(137)}=3.43$, $p<0.0005$, matched pair t-test).

608

609 **Spine Morphology**

610

611 We classified spines into one of four types, mushroom spines, thin spines, stubby spines and
612 filopodia (see Methods) using previously published criteria (Grutzendler et al., 2002; Oray et al.,
613 2006; Rodriguez et al., 2008). In the general population of all spines, we found that most spines
614 were thin (61%), many were mushroom (16%) and a few were filopodia (9%) (see Table 2). The
615 rest were classified as apparently stubby spines, where the neck was short and appeared to be of
616 similar size to the head (14%).

617

618 We found that the NPS population differed in morphology from the general population, even after
619 14 days of CWD, comprising fewer mushroom spines (5% versus 16%) and more stubby spines
620 and filopodia ($X^2_{(9)} = 63$, $p<0.001$; see Table 2). This suggests that it takes longer than 14 day for
621 most of the very largest spine types to become established from genesis. We also looked at the
622 stable population of AP spines and found that they progressively lost mushroom spines over the 14
623 day post-deprivation period from 16% to 2% by day14 ($X^2_{(9)} = 40$, $p<0.001$; see Table 2), being
624 replaced mostly with thin and stubby spine types. If one assumes that the principal whisker
625 probably transmits via mushroom spines in its principal barrel, this finding is in keeping with the
626 physiological data showing that principal whisker responses decrease with chessboard deprivation.
627 It is also in keeping with the general finding that larger spines tend to decrease and smaller spines
628 increase in size with deprivation (Figure 9F). On average, a small increase in spine head size in
629 the AP population occurs with CWD (Figure 9B,D) accompanied by a reduced number of
630 mushroom spines.

631

632

633 **5. Spine persistence, spine head size and spine** 634 **morphology in α CaMKII-T286A mutants** 635

636

637 **Spine persistence**

638

639 Given the relationship between spine lifetime and spine size, we tested whether the higher
640 baseline formation and elimination rates present in α CaMKII-T286A mice resulted in shorter spine
641 lifetimes in general and whether the size of the spines was subsequently different. Indeed, spine
642 lifetimes were found to be briefer in α CaMKII-T286A mutants compared to wild-types (Figure 10A).
643 A two way ANOVA showed an effect of deprivation and genotype on spine lifetime but no
644 interaction between the two ($F_{(3,1059)} = 7.65, p < 0.0001$). In undeprived α CaMKII-T286A mutants,
645 spines that were already present from the first observation point were eliminated at a faster rate
646 than in wild-types (Figure 10A; $X^2_{(1)} = 7.0, p < 0.01, n = 511$, Wilcoxon test) falling to 50% of the
647 original number over 20 days. This is consistent with the observation that baseline spine formation
648 and elimination is higher in α CaMKII-T286A animals than in wild-types. The rate of spine loss was
649 increased further by deprivation (Figure 10A; $X^2_{(1)} = 8.8, p < 0.003, n = 588$, Wilcoxon test) and
650 resulted in just 38% of spines persisting for 20 days. Neither decay curves for surviving spines in
651 deprived nor undeprived animals reached an asymptote over the period of observation (Figure
652 10A). Spine loss was approximately 12% greater in deprived α CaMKII-T286A mice than in
653 undeprived control cases after 14 days of CWD. These observations are consistent with the
654 electrophysiological evidence, which shows that CWD causes depression of deprived whisker
655 responses in α CaMKII-T286A mice but no potentiation of spared whisker responses (Hardingham
656 et al., 2003).

657

658 Spine lifetime for new spines produced 24 hours following deprivation were similar to those of wild-
659 types. However, the number of new spines formed after deprivation were no greater than at any
660 other time-point (Figure 10B), which meant that after 14 days of deprivation, the number of spines
661 formed 24 hours after deprivation was 1.3% of the total and not significantly different from the
662 number expected in undeprived α CaMKII-T286A mutants of 0.8% (Figure 10B).

663

664 **Spine head size for new and eliminated spines**

665

666 We compared new persistent spines (NPS) formed on the first day following deprivation with
667 spines that were stable and always present (AP) throughout the entire observation period in
668 undeprived animals. We found that just as with wild-types, NPSs had the same size spine heads
669 as the AP population in α CaMKII-T286A mice (Figure 10D). However, spine heads of all types
670 were generally smaller than in wild-types. A two way ANOVA showed an effect of genotype but not

671 of spine type (AP versus NP) across wild-types and α CaMKII-T286A mutants ($F_{(3,394)}=4.88$,
672 $p<0.003$). Post hoc test showed that this was because persistent spine heads were significantly
673 smaller in α CaMKII-T286A mutants than in wild-types $t_{(393)}= 3.29$, $p<0.002$. This conclusion was
674 strengthened when we further tested whether spine head sizes were different in undeprived wild-
675 types and α CaMKII-T286A mutants (Figure 10 E,F) and found they were ($t_{(1281)}= 6.89$, $p<0.0001$).

676

677 We also compared the size of transient spines with the persistent spine population and found once
678 again that, as with wild-types, transient spines were significantly smaller than persistent spines (χ^2
679 = 68.75, $p<0.0001$). These findings suggest that spine head size is an important determinant of
680 spine stability in α CaMKII-T286A mutants just as in wild-types, but that the critical size for stability
681 is smaller in α CaMKII-T286A mutants.

682

683

684 **Changes in spine head size for initially present spines**

685

686 As described above, we found that in wild-types, the AP population of spines showed a small but
687 significant increase in spine head size following deprivation. We found no comparable change in
688 α CaMKII-T286A mice however (Figure 11A,B) and the average spine sizes for the population of
689 AP spines were not different from any pair of baseline to post-deprivation comparisons (for
690 example baseline to day 1 $t_{(86)}=1.04$, $p=0.299$; Figure 11). However, just as with the wild-type
691 cases, individual spines in the α CaMKII-T286A mice showed increases and decreases in spine
692 size from one time-point to another (Figure 11C). Consistent with spine fluctuation analysis, the
693 smaller spines tended to increase in size and the larger spines decrease in size (Figure 11D), but
694 overall the spine head size distribution remained unchanged by deprivation. The effect of
695 fluctuations are therefore not dependent on CaMKII auto-phosphorylation. However, because the
696 spontaneous increases in spine size within the population are small compared with those in wild-
697 types (due to a lack of potentiation in these animals), the fluctuation range is also smaller and the
698 spine population settles to a smaller average spine head size (Figure 11A,C,D).

699

700 **Spine Morphology**

701

702 The distribution of spine types found in undeprived α CaMKII-T286A mice was different from that
703 seen in wild-types, with fewer mushroom spines (6.5%), and more thin spines (87%) (see Table 2;
704 $\chi^2_{(3)}=64.5$, $p<0.0001$). This result is in keeping with the general finding that spine head sizes were
705 smaller in α CaMKII-T286A mice than in wild-types, which may be related to their lack of LTP and
706 may thereby give rise to their higher basal levels of spine elimination.

707

708

709

710 **Discussion**

711

712 This study shows that layer 2/3 neurones do undergo structural plasticity in the barrel cortex, but
713 (a) only under conditions that produce functional plasticity of receptive field structure (CWD not
714 AWD) and (b) only on the basal and not the apical dendrites. Why does CWD cause functional and
715 structural plasticity while AWD does not? CWD alters the natural timing of activity in columnar and
716 trans-columnar circuits driven by spared and deprived whiskers and therefore creates the
717 conditions for spike-timing dependent potentiation and depression (Wallace and Fox, 1999a;
718 Celikel et al., 2004). The spared whiskers can also provide activity for non spike-timing forms of
719 LTP in barrel cortex (Gambino and Holtmaat, 2012). Neither of these contingencies are created by
720 AWD, which leads to a uniform decrease in activity levels and consequently little opportunity for
721 Hebbian forms of plasticity. At the ages studied here, neither does AWD cause homeostatic
722 plasticity (compare Figure 2B with (Glazewski et al., 2017)). In common with the visual cortex
723 (Ranson et al., 2012), barrel cortex appears to exhibit homeostatic plasticity in young rather than
724 adult animals.

725

726 Our findings may help to explain earlier studies that did not observe structural plasticity in layer 2/3
727 cortical neurones. Studies in barrel cortex where all the whiskers were deprived uniformly also
728 reported a lack of rapid structural plasticity in layer 2/3 neurones (Zuo et al., 2005; Ma et al., 2016).
729 Studies in visual cortex, where activity was uniformly decreased in the monocular zone by
730 contralateral eye-enucleation, also found a lack of structural plasticity in layer 2/3 (Barnes et al.,
731 2015). One study in binocular visual cortex did use monocular deprivation however, which would
732 be expected to create activity contrasts between ipsi- and contra-lateral eye inputs. In this case, no
733 structural plasticity was found on the layer 2/3 neurones (Hofer et al., 2009), possibly because the
734 apical dendrites were studied rather than the basal dendrites.

735

736 Why do the basal dendrites exhibit plasticity while the apical dendrites do not? A possible
737 explanation may lie in their different inputs. Basal dendrites tend to receive feedforward sensory
738 input from layer 4 and to some extent directly from the thalamus (White, 1978; Petreanu et al.,
739 2009; Hooks et al., 2011; Mao et al., 2011). Apical dendrites tend to receive feedback connections
740 from other cortical areas including motor cortex (Petreanu et al., 2009). Therefore, sensory
741 deprivation is more likely to affect feedforward connections onto basal dendrites while motor tasks
742 are more likely to affect feedback connections onto apical dendrites. In favour of this theory, apical
743 dendritic plasticity does occur in motor tasks requiring mice to move their whiskers accurately to
744 receive a reward (Kuhlman et al., 2014).

745

746 One further level of dendritic specialisation was observed in this study. We found that new spine
747 formation tended to be greater following whisker deprivation at dendritic branches with a naturally
748 higher basal turnover rate, confirming findings of (Yang et al., 2014) and colleagues. This suggests
749 that even among basal dendrites, some are primed to undergo plasticity and some are not.

750

751 **Dual-component structural plasticity**

752

753 Chessboard pattern deprivation causes potentiation of spared whisker responses and depression
754 of deprived whisker responses (Wallace and Fox, 1999b). Spared whisker potentiation correlates
755 with an increase in new persistent spines, but also a small but significant increase in spine head
756 size of the stable (AP) spine population. Most layer 2/3 neurones in the barrel cortex receive multi-
757 whisker input (Armstrong-James and Fox, 1987) and therefore, theoretically, only need to
758 strengthen pre-existing synapses rather than to create new ones. Nevertheless, new spines *are*
759 produced and since they stabilise over a period of two weeks, are thought to make functional
760 synapses (Knott et al., 2006). It is therefore likely that new persistent spines represent the second
761 component of the dual-component structural plasticity mechanism. Neither, AP enlargement nor
762 NPS formation are present in the CaMKII-T286A mutants, which also lack experience-dependent
763 potentiation (Glazewski et al., 2000) and cortical LTP (Hardingham et al., 2003), providing further
764 evidence that functional plasticity depends on the observed structural plasticity. A similar
765 conclusion on NPS formation has been reached before for CWD induced potentiation of spared
766 whisker responses and layer 5IB apical dendrites in barrel cortex (Wilbrecht et al., 2010), however,
767 we believe the CaMKII auto-phosphorylation dependent AP spine enlargement is an entirely new
768 observation.

769

770 **The Effect of Intrinsic Spine Fluctuations**

771

772 Within the stable spine population, we found that smaller spines increased and the larger spines
773 tended to decrease in size between time-points. This provides a self-regulatory homeostatic
774 response to potentiation. Spine fluctuation analysis shows that spine sizes tend to spontaneously
775 change this way in the absence of overt Hebbian processes to direct changes in spine size
776 (Yasumatsu et al., 2008) and indeed lead to the log-normal spine head size distribution observed
777 here and in other studies (Loewenstein et al., 2011). Theoretical studies have shown that Hebbian
778 processes combined with random spine fluctuations creates an intrinsically homeostatic system
779 (Matsubara and Uehara, 2016).

780

781 The increase in size of the stable spine population following CWD is reminiscent of a TNF-alpha
782 dependent homeostatic increase in spine size seen in dendrites that show elevated spine
783 elimination (Barnes et al., 2017). However, two arguments suggest that the size increase we saw

784 is not homeostatic; first, because the AP spine enlargement occurs against a background of
785 increased spine formation rather than a loss of spines, which suggests that there is no loss for the
786 homeostatic mechanism to compensate. Second, the AP spine enlargement was absent in the
787 α CaMKII-T286A point mutants, which lack LTP but not TNF-alpha dependent homeostatic
788 plasticity (Greenhill et al., 2015). This suggests that AP spine enlargement is related to Hebbian
789 addition and input specific potentiation rather than a homeostatic mechanism. This fits with the
790 neurophysiological effect of chessboard deprivation, which is to increase selectively the spared
791 whisker responses rather than homeostatically increase whisker responses in general (Wallace
792 and Fox, 1999b; Hardingham et al., 2008).

793

794 **The role of CaMKII in structural plasticity**

795

796 Spine heads fluctuate in size independent of activity driven increases and decreases in spine size
797 (Yasumatsu et al., 2008). Consequently, spines with small heads are vulnerable to elimination from
798 spontaneous decreases in spine size. New spines are vulnerable to elimination for this reason and
799 we found that they only persist if their heads grow rapidly to the population average size. Spine
800 head size for new persistent spines is indistinguishable from the main population of stable spines
801 after 24 hours in wild-type mice, while new spines that are eliminated are smaller, like transient
802 spines in general. Activity-dependent spine enlargement requires CaMKII (Bosch et al., 2014;
803 Hedrick et al., 2016; Fu and Ip, 2017). The lack of CaMKII auto-phosphorylation in the α CaMKII-
804 T286A mice, presumably prevents sensory directed spine enlargement and stabilisation, therefore
805 new spines tend to be eliminated more frequently in α CaMKII-T286A mice leading to their baseline
806 turnover rate being about 24% higher than in wild-types.

807

808 In addition to the decreased persistence of new spines, we also found that new spines do not form
809 at an elevated rate following CWD in α CaMKII-T286A mice. This suggests that α CaMKII-
810 autophosphorylation is required for the substantial increase in new spine formation itself. In favour
811 of this theory, it has been shown that CaMKII lies at the centre of several signalling pathways in the
812 spine head, one of which leads to production of RhoA, which can diffuse to neighbouring spines
813 and thereby affect the cytoskeleton of new and emerging spines and another that generates local
814 BDNF synthesis, trkB signalling and diffusion of newly activated Rac1 to neighbouring spines with
815 a similar effect (Hedrick et al., 2016). Both Rac1 and RhoA are part of the system that leads to
816 spine enlargement via LIMk translocation to and binding of cofilin to the spine head (Bosch et al.,
817 2014). However, it is not clear at this stage whether this system alters the dendritic cytoskeleton in
818 such a way as to initiate new spine production, rather than increasing the probability of
819 spontaneously occurring new spines becoming stabilised by spine head enlargement.

820

821 **Conclusions**

822
823 We draw a number of conclusions from the present findings; first, that Layer 2/3 neurones do show
824 robust structural plasticity in response to whisker deprivation and therefore the functional plasticity
825 we see in this layer is likely to depend on underpinning structural plasticity. Previous studies may
826 have missed this by looking at other dendritic locations or by using an ineffective whisker
827 deprivation method. Second, that potentiation occurs due to a dual-component enlargement of
828 stable spines plus addition of new spines and CaMKII is central to both. While the role of CaMKII in
829 LTP and spine enlargement is reasonably well understood, the mechanism by which it is involved
830 in spine production is not established at present.

831

832

833 **Figure Legends**

834

835 **Figure 1.** Whisker deprivation patterns and spine tracking.

836 **A:** Unilateral all whisker deprivation (AWD), which produces **B:** uniform deprivation of all barrels in
837 the cortex. **C:** Unilateral Chessboard pattern whisker deprivation (CWD) produces **D:** a chessboard
838 pattern of active and deprived barrels whereby every barrel deprived of its principal whisker (light
839 grey) is surrounded by four barrels that have their principal whisker intact (dark grey) and vice
840 versa. **E:** Photo-lesion are made in layer 4 of the barrel cortex on the last day of imaging (black
841 arrows), to co-register the regions of interest within which spines are imaged with their
842 corresponding home barrels. **F:** Imaging time points relative to deprivation on time-point zero were
843 -10, -6, -2, +1, +4, +7, +11 and +14 days. In some cases 12 and 24 hour time points were taken.
844 **G:** Spines are tracked over a period of days, shown here for 6 days before deprivation (-6), two
845 days before (-2) and 4 days after deprivation (+4). Note that spine number 17 is branched: such
846 cases were counted as one spine. Some spines are eliminated from one time point to the next (red
847 numbering), others are formed anew (green numbering). **H:** Examples of eliminated (red arrows)
848 and newly formed or enlarged spines (green arrows) shown for a dendrite imaged at 2 days before
849 and 7 days after deprivation. Yellow arrow indicates a spine where the spine head shrinks over this
850 period. Calibration bars are 150 μm (E), and 5 μm (G and H).

851

852 **Figure 2.** Effect of deprivation pattern on receptive field properties.

853 **A:** Principal whisker and surround receptive field (SRF) whiskers are plotted against the response
854 evoked in layer 2/3 averaged across animals. SRF responses are ranked for each cell (S1, S2
855 ...S6) before averaging across cells for each animal. Inset: diagram of barrel field indicates all
856 barrels receive principal whisker input (dark grey). **B:** Receptive field properties are unchanged in
857 animals unilaterally deprived of all their whiskers at 1 day (grey) and 7 days (black) post-
858 deprivation. Inset: diagram of barrel field shows all barrels are deprived of principal whisker input
859 (light grey). **C:** Receptive fields in barrels deprived of principal whisker input are altered by
860 chessboard pattern deprivation (CWD). In deprived barrels, spared surround whisker responses
861 (S1-6) increase while principal whisker (PW) responses decrease. Inset: diagram of barrel field
862 shows that barrels deprived of their principal whisker (orange) alternate with barrels with their
863 spared whisker intact (dark grey). **D:** Receptive fields in barrels with spared principal whiskers also
864 show an increase in surround whisker responses at 7 days but not 1 day. Inset: green represents
865 spared barrels and light grey deprived barrels.

866

867 **Figure 3.** Effect of deprivation pattern on spine formation and elimination.

868 **A:** All whisker deprivation (AWD) evenly deprives the barrel field of its principal whisker input and
869 does not significantly alter principal whisker responses (white bars), nor the strongest (S1) spared

870 surround whisker responses (black bars) after 1 or 7 days of deprivation. **B**: Similarly, AWD does
871 not affect spine formation (black bars) or elimination (black bars, plotted as negative values for
872 clarity), which remain constant following deprivation compared with formation and elimination in
873 undeprived animals (white bars). **C**: Therefore, AWD cumulative formation (blue line) and
874 elimination curves (red line) entirely overlap with those for undeprived cases (see key). **D**:
875 Chessboard pattern deprivation (CWD) results in alternate deprived and spared barrels in the
876 cortex (diagram; spared barrels dark grey) and causes potentiation of spared whisker responses in
877 deprived barrels (black bars) and principal whisker responses to depressed (white bars). **E**:
878 Similarly, CWD causes spine formation and elimination to increase significantly 1 day following
879 deprivation and remain elevated for at least 11 days following deprivation compared to undeprived
880 values (** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). **F**: Consequently, cumulative spine formation is
881 increased over 14 days to approximately 90% of the originally present spines (blue line) compared
882 to approximately 40% in undeprived animals (green line). Cumulative spine elimination in CWD
883 (red line) is similar to formation over 14 days and significantly higher than in undeprived animals
884 (purple line).

885

886 **Figure 4.** Effect of basal formation rate on chessboard pattern whisker deprivation induced
887 formation rate in bifurcating dendrites and randomly paired singly assayed dendrites. **A**: Bifurcating
888 dendrites: the high formation branches (HFB, solid lines, black diamonds) from the bifurcation pair
889 are defined from their baseline formation rate and show a greater reaction to deprivation than low
890 formation branches (LFB, dashed lines, open squares). The plot shows the cumulative spine
891 formation with time. **B**: Random pairs: HFBs from randomly paired branches appear to show a
892 greater reaction to deprivation but this is not significantly different from the LFB random pair. **C**:
893 Bifurcating pairs: formation rate plotted in histogram format showing rates assayed per time point
894 for HFBs (black bars) and LFBs (white bars). **D**: Random pairs: formation rates for randomly paired
895 dendrites. **E**: Cross-correlation between basal formation and deprivation induced formation rates in
896 bifurcation dendrite pairs. Basal formation is broadly predictive of deprivation induced formation
897 ($r^2=0.45$) and is highly significant (see Results section). **F**: Basal formation rate is not predictive of
898 deprivation induced formation rate for randomly assigned pairs of dendrites ($r^2=0.00195$).

899

900 **Figure 5.** Lack of effect of chessboard pattern deprivation on measures of synaptic plasticity on
901 apical dendrites in barrel cortex.

902 **A**: Diagram of barrel cortex showing the inputs to apical dendrites in layer I (L1) and the different
903 inputs to basal dendrites in layers 2 (L2) and layer 3 (L3). Inputs to apical dendrites arise from
904 other cortical areas such as secondary somatosensory cortex (S2) and primary motor cortex (M1)
905 as well as the medial part of the posterior thalamic nucleus (POm). Basal input arise from layer 4
906 cells and other layer 2/3 cells as well as some direct VPM input onto layer 3 cells. **B**: (i) Low power
907 image of apical dendrites in L1 (scale bar =) (ii- iv) descending sequence of images from 30-180

908 microns below the dura (scale bar in iv is 30 μ m). **C**: The cumulative spine formation rate is shown
909 for baseline time-points and for 1 day (24 hours) after chessboard whisker deprivation. The plot
910 does not show an increase in slope 1 day after deprivation that would be characteristic of
911 increased spine formation and is seen with CWD for basal dendrites (compare with 3F and 4A,B).
912 **D**: The spine sizes of the stable (AP) population of spines were calculated for each time-point and
913 show no change post-deprivation (compare with Figure 9B for basal dendrites).

914

915 **Figure 6.** Lack of effect of chessboard pattern deprivation on spine formation in α CaMKII-T286A
916 homozygous mice.

917 **A**: Diagrammatic representation of the chessboard deprived pattern. **B**: Chessboard pattern
918 deprivation (black bars) does not cause an increase in spine formation (positive values) above
919 baseline (white bars) following deprivation. However, spine elimination (plotted as negative values
920 for clarity) is increased on the first day following whisker deprivation (black bars) relative to
921 undeprived CaMKII-T2286A (white bars) (* $p < 0.05$). **C**: Cumulative formation curves overlap for
922 deprived (blue line) and undeprived (green line) α CaMKII-T286A mice and are not different, while
923 cumulative spine elimination (red line) increases one day after deprivation but returns to basal
924 rates thereafter.

925

926 **Figure 7.** Effect of chessboard whisker deprivation on lifetime of newly formed and already present
927 spines.

928 **A**: Newly formed spines in CWD wild-type mice (blue line) comprise 18% of initially present spines
929 one day following deprivation. The new spine count decays with time to asymptote at
930 approximately 8% by 14 days of deprivation. New spines in undeprived wild-types only comprise
931 4% of the total on any given day and decay to approximately 1% over the same time period (black
932 line). **B**: Spines already present at the first observation time-point naturally decay over time in
933 undeprived animals (black line) to asymptote at approximately 65% of the population after 20 days.
934 Chessboard pattern deprivation (onset shown by arrow) increases the rate of decay (green line) by
935 approximately 18% over the same period. NB: spines summed across all cases in each group.

936

937 **Figure 8.** Relationship between spine size and lifetime for eliminated, transient and new persistent
938 spines in wild-types.

939 **A**: New spines formed after chessboard deprivation that persist (blue line) have the same spine
940 head size distribution 24 hours after deprivation as the stable spine population (black line). **B**:
941 Spines that are eliminated one time-point following observation of their presence (green line) are
942 smaller than the stable spine population (black line). **C**: The average spine head size of the stable
943 spine population for undeprived wild-types is plotted over a three week period (grey line, mean and
944 sem). Transient spines (present for a single time point) have smaller average spine head sizes (red
945 triangles). Average head size of new persistent spines (blue line) increase rapidly between 12 and

946 24 hours of chessboard whisker deprivation to exceed transient spine head sizes at 24 hours and
947 are indistinguishable from the stable spine sizes after 4 days. **D**: Cumulative distribution functions
948 for the spine head sizes of transient (red), new persistent at 12 hours (light blue), new persistent at
949 24 hours (dark blue) and stable spines (black) shown in **C**.

950

951

952 **Figure 9.** The effect of deprivation pattern on spine size of the stable spine population in wild-
953 types.

954 **A**: The overall spine head size in the general population of all spines does not change with CWD.
955 However, **B**: the average spine head size does increase in the population of always present
956 spines with CWD (blue line), though not AWD (grey line). **C**: Cumulative distribution functions for
957 the general population of all spines before (red) and after deprivation (green) are similar (note that
958 red and green lines correspond to red and green time-points in **A**). **D**: However, the cumulative
959 distribution function for the stable spine population shifts right (larger values) from baseline (red)
960 after chessboard pattern deprivation (green). Log transformed spine size distributions for each time
961 point were compared using matched pair-t-tests. Baseline time-points were not different
962 ($t_{(147)}=1.13$, $p=0.26$), while baseline and day 1, 4, 7, 11 and 14 were different ($t_{(147)}=4.0$, $p<0.0001$;
963 $t_{(147)}=4.44$, $p<0.0001$; $t_{(147)}=3.63$, $p<0.0004$; $t_{(147)}=2.50$, $p<0.013$; $t_{(147)}=2.3$, $p<0.022$) respectively. **E**:
964 The change in spine head size is related to the original size of the spines and is shown for the
965 general population of spines in **E** and for the stable spines only in **F**. Note that newly formed spines
966 appear on the y-axis and eliminated spines appear along $x = -y$. **F**: Spine larger than about 1 μm
967 tend to decrease in size while those smaller than 1 μm increase in size.

968

969

970 **Figure 10.** Effect of chessboard whisker deprivation and the $\alpha\text{CaMKII-T286A}$ genotype on lifetime
971 of newly formed and already present spines.

972 **A**: The survival fraction plot shows that spine lifetimes are briefer in $\alpha\text{CaMKII-T286A}$ mice (black
973 line) compared to wild-types (grey line). Chessboard pattern deprivation decreases spine survival
974 further in $\alpha\text{CaMKII-T286A}$ mice (green line). **B**: Newly formed spines show similar persistence in
975 chessboard deprived and undeprived $\alpha\text{CaMKII-T286A}$ mice. **C**: The distribution of spine head
976 sizes is smaller for spines eliminated at the next time point (green line) compared to stable spines
977 (black line). **D**: Newly formed spines that persist (blue line) have a similar spine size distribution to
978 that of stable spines (black line) in $\alpha\text{CaMKII-T286A}$ mice. **E**: Spine head sizes are smaller in
979 $\alpha\text{CaMKII-T286A}$ mice (red) compared to wild-types (black); data for undeprived animals. **F**:
980 Cumulative distribution function for data shown in **E**. NB: Spines are summed for all cases within
981 each group to form the decay curves.

982

983 **Figure 11.** The effect of deprivation pattern on spine size of the stable spine population in
984 α CaMKII-T286A mice.

985 **A:** Chessboard pattern deprivation leads to an increase in the average spine head size in the
986 stable spine population in wild-types (blue line) but not in the α CaMKII-T286A mutants (green line).

987 **B:** In α CaMKII-T286A mutants, the cumulative distribution functions of spine head size overlap for
988 the stable spine population before (red line) and after chessboard pattern deprivation (blue). **C:**

989 Trajectories of individual spine size changes between baseline and 1 day post chessboard-
990 deprivation. **D:** For the stable population, small spine heads tend to increase in size and large

991 spine heads decrease in α CaMKII-T286A mice, but the overlap in sizes increasing and decreasing
992 is greater in α CaMKII-T286A mice than with wild-types (compare with Figure 9F). Data in **D** is for

993 the same population shown in **C** and **B**.

994

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Genotype	Deprivation	Rois	Mice	Initial Spines	Total Spines	Age range (days)	Baseline formation	Baseline elimination	Peak formation (deprived)	Peak elimination (deprived)
WT	Undeprived	15	5	478	715	70-125	3.78	3.53	-	-
WT	Chessboard	18	8	680	1501	75-107	4.22	4.35	17.87	11.66
WT	12 hour chessboard	4	1	88	180	63	3.86	3.42	31.58	25.72
WT	Chessboard (apical)	7	2	203	317	74-87	4.73	5.83	6.78	8.3
WT	All deprived	12	6	292	595	86-116	4.16	3.55	3.32	3.84
T286A	Undeprived	11	4	438	932	91-104	4.96	4.54	-	-
T286A	Chessboard	13	5	382	787	86-131	5.89	3.83	5.71	15.12

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Table 1. Basic statistics for the different groups of animals studied. The number of Regions of interest (Rois), animals, original spines at the first observation point and total spines (new plus original) are given. The age range is for the start of the observation period and is in days postnatal. Baseline formation and baseline elimination rates are taken from the 2 or 3 baseline time points for the animals that will become deprived or across the entire observation period for undeprived cases. Formation and elimination values are expressed as percentages of the total number of spines present at the first time point and per day. All data for basal dendrites except where stated as apical.

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	Filopodia	Stubby	Thin	Mushroom
Wild-type (all spines) undeprived	9	14	61	16
AP spines (day 1)	14	14	63	9
AP spines (day 14)	25	17	56	2
N spines (day 1)	26	35	31	8
NP spines (day 14)	28	13	54	5
CaMKII-T286A (all spines) undeprived	2	4	87	7

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Table 2. Percentages of basal dendritic spines in different morphological classes by genotype and spine lifetime classification. AP = always persistent spines, either viewed 1 day after chessboard whisker deprivation or at 14. N = new spines produced on the first day of deprivation (day 1) and day 14. CamKII-T286A mice in the last row and wild-types in the first row were undeprived and the general population were classified independent of spine lifetime.

Figure 1

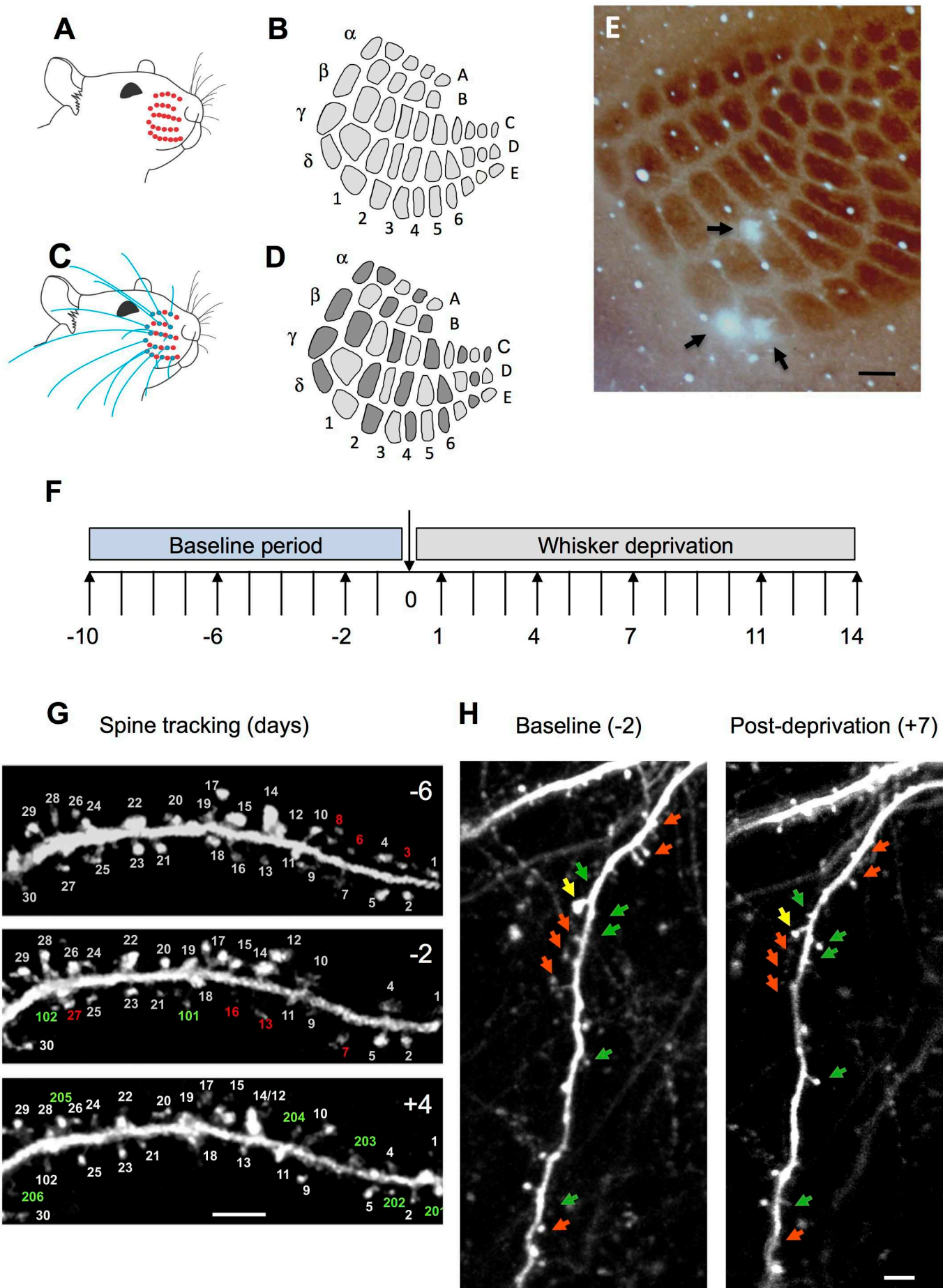


Figure 2

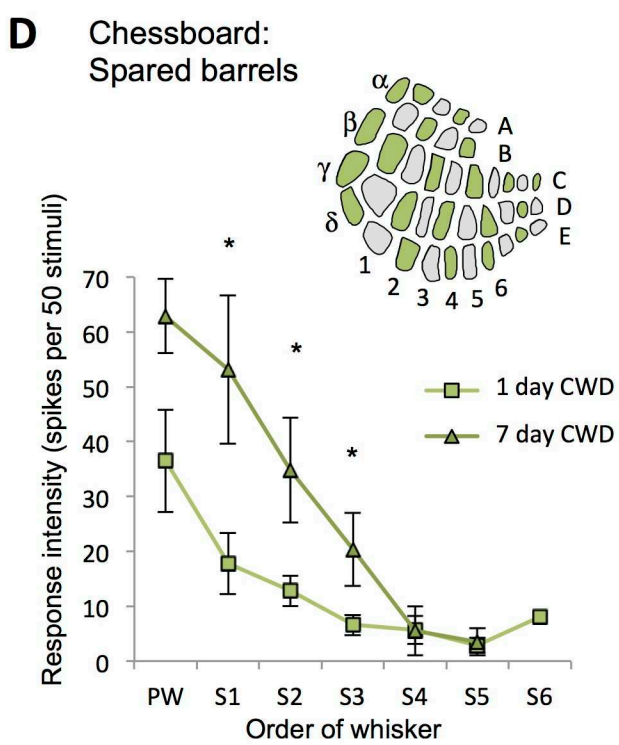
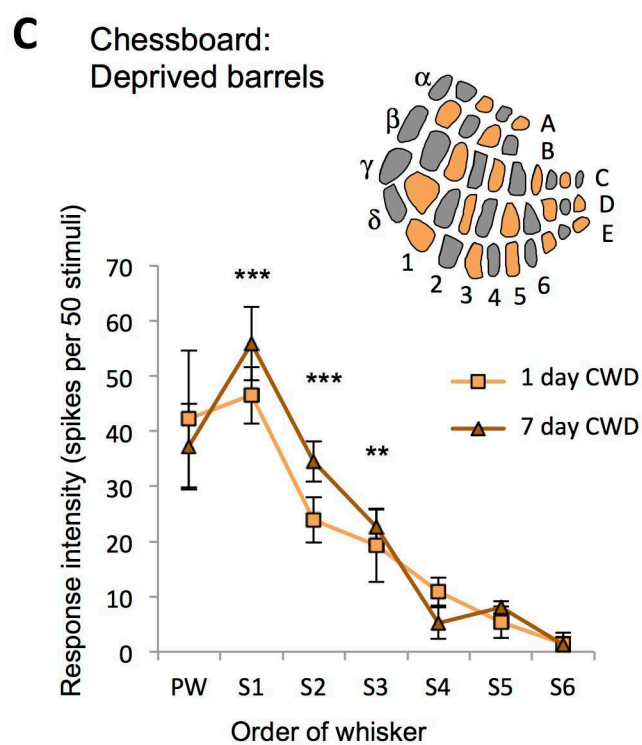
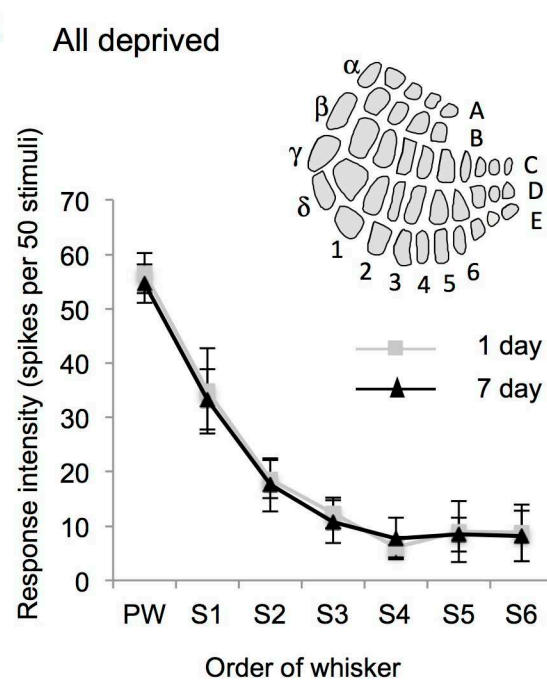
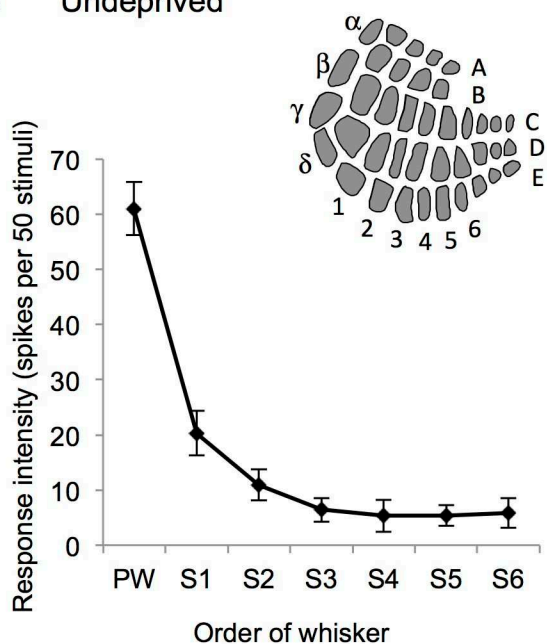
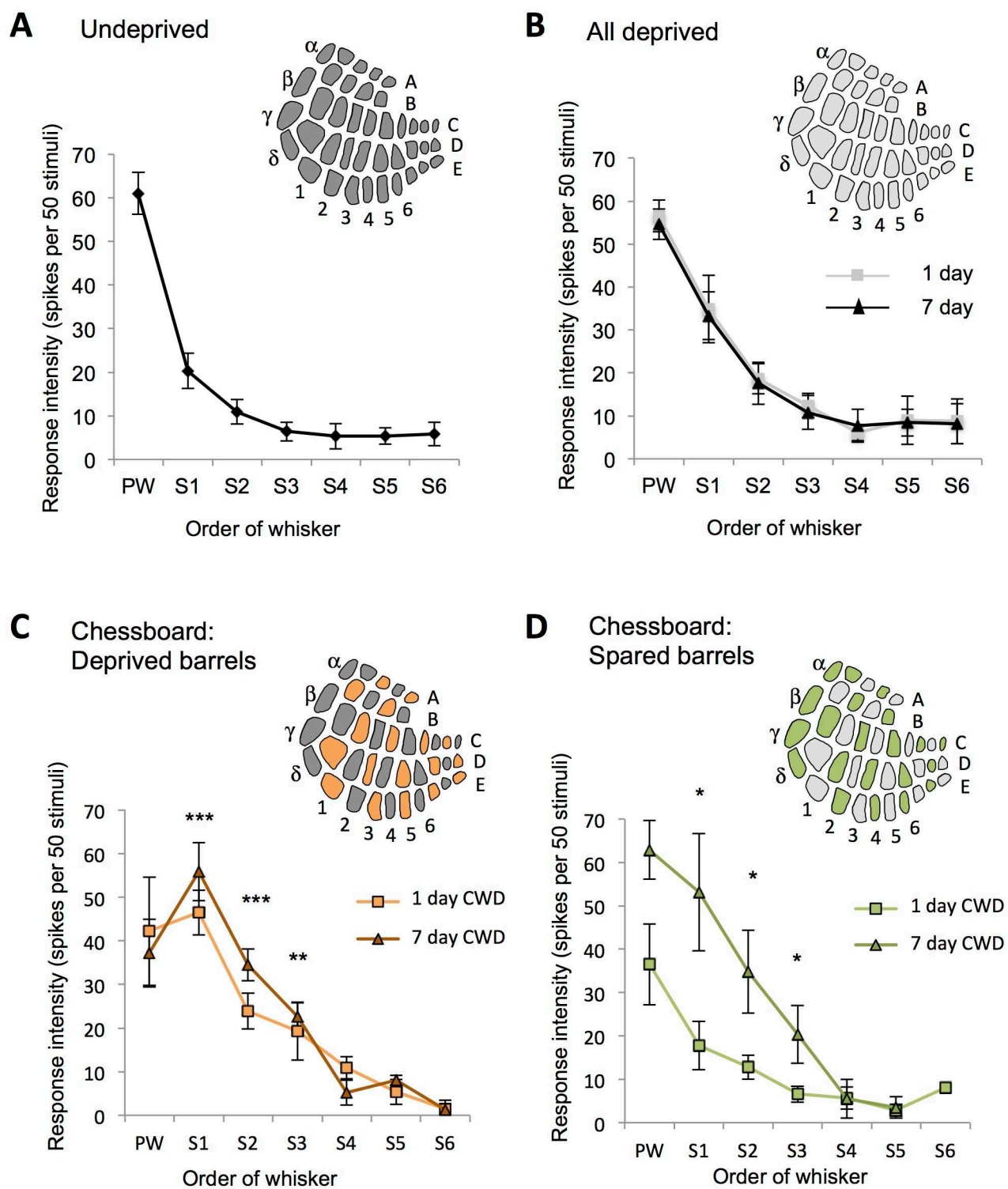


Figure 3

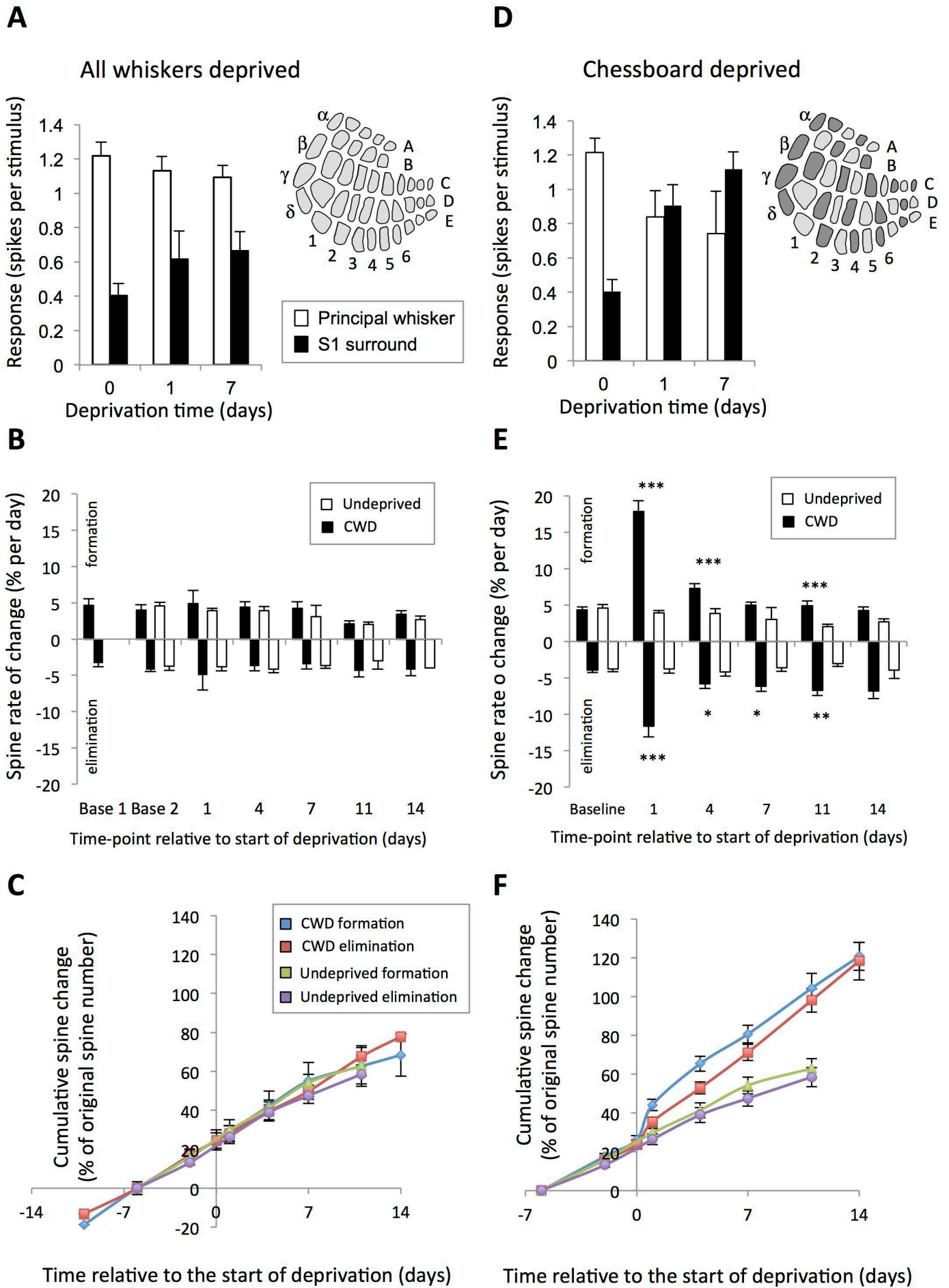
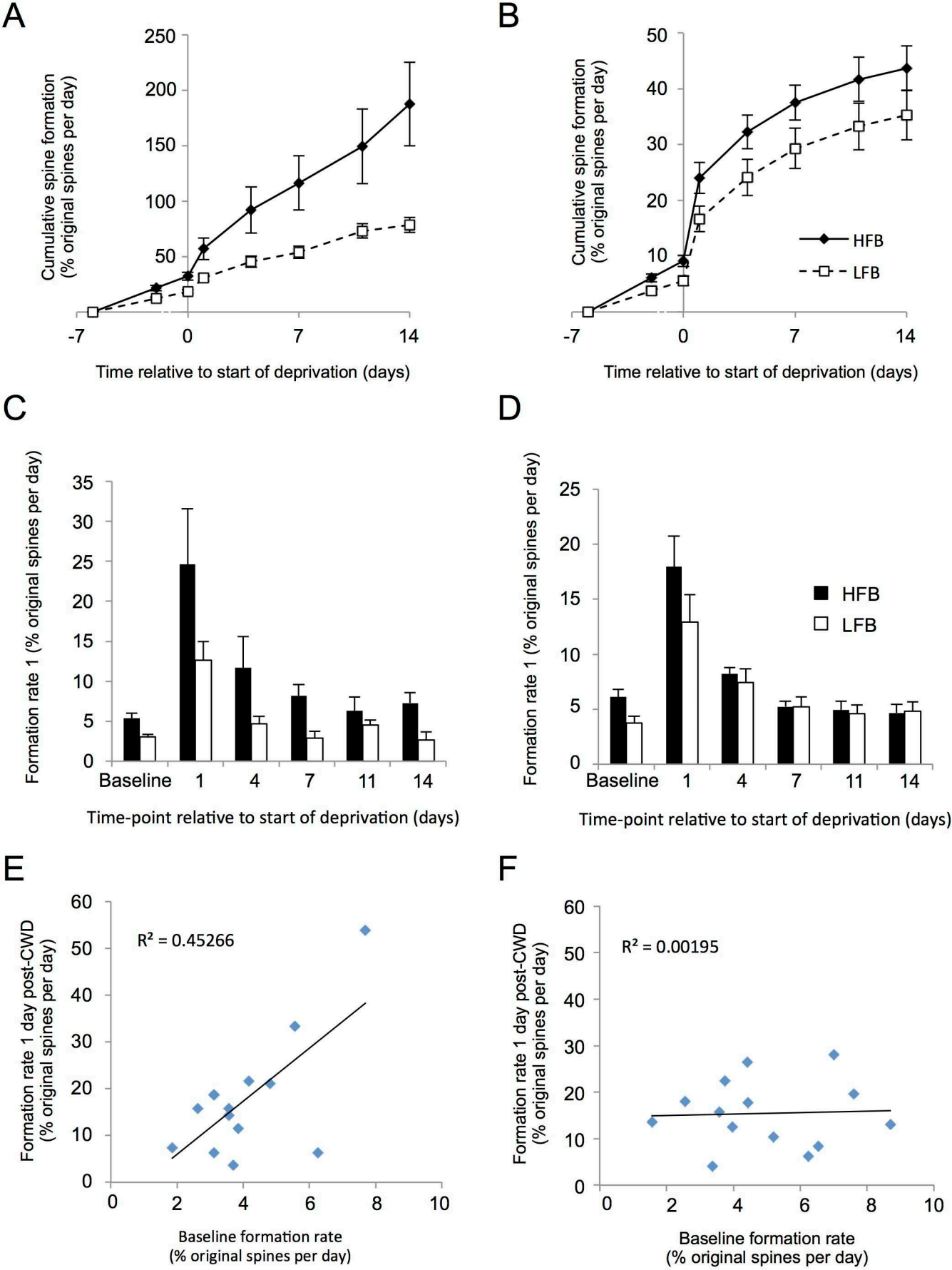
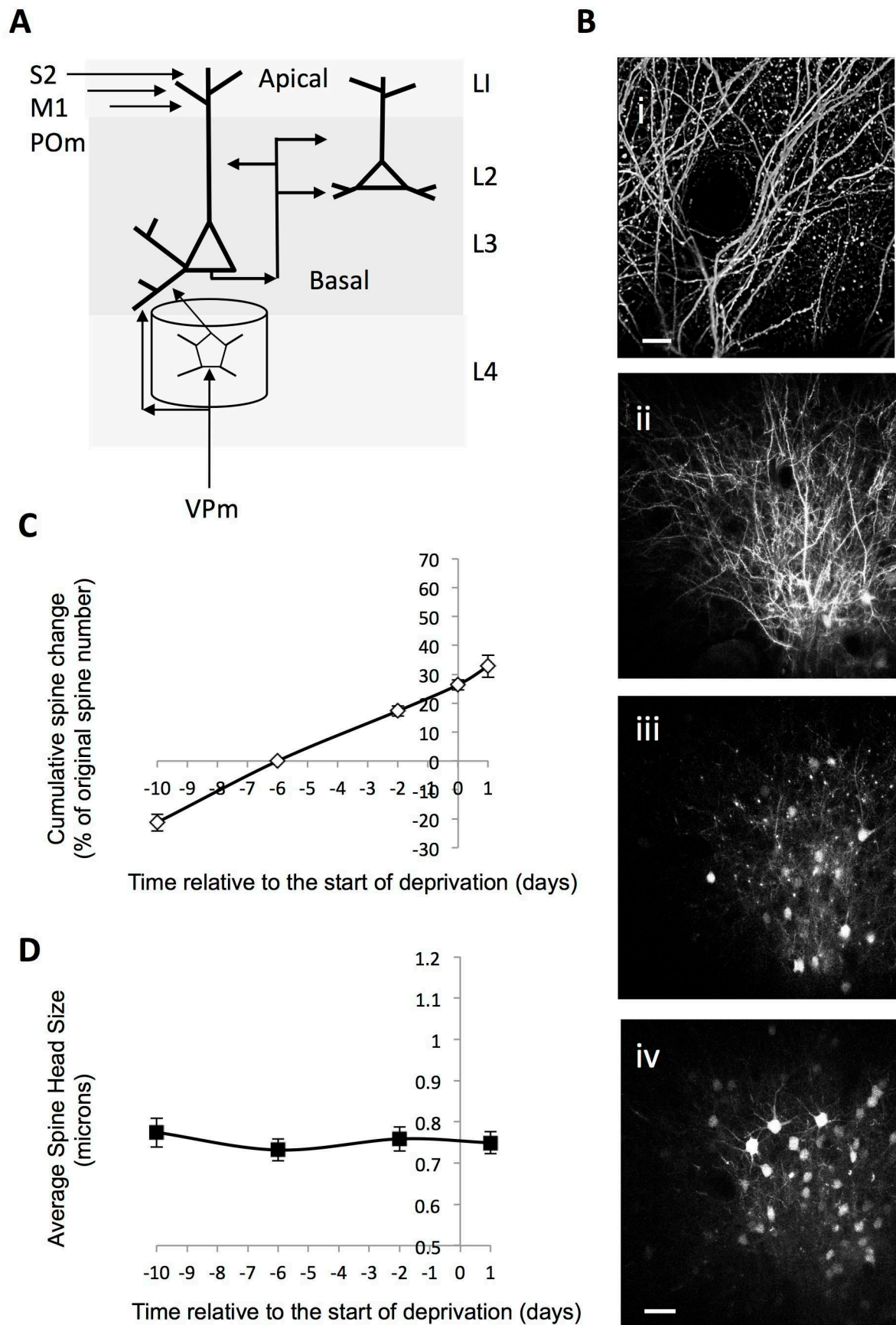


Figure 4





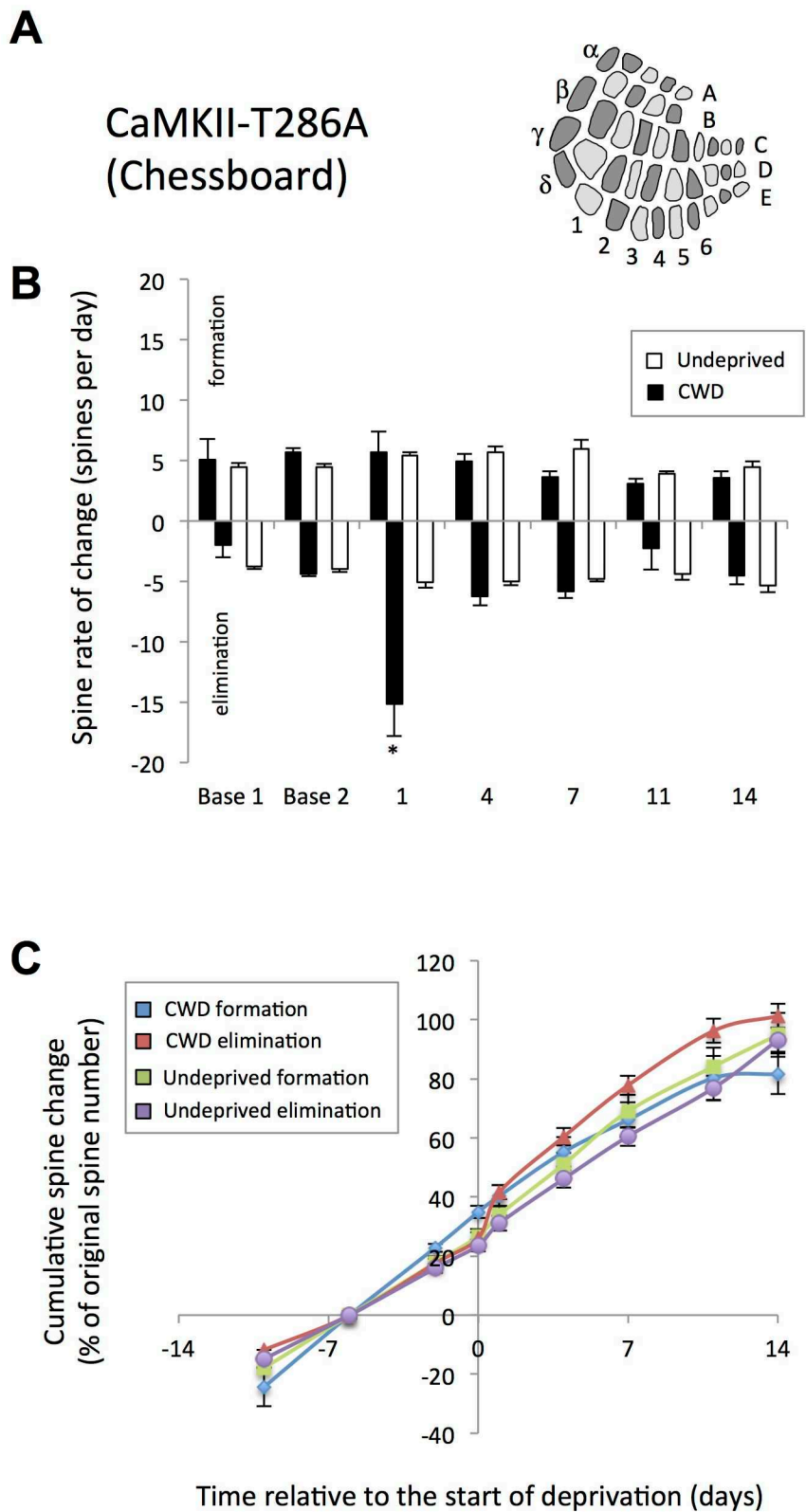
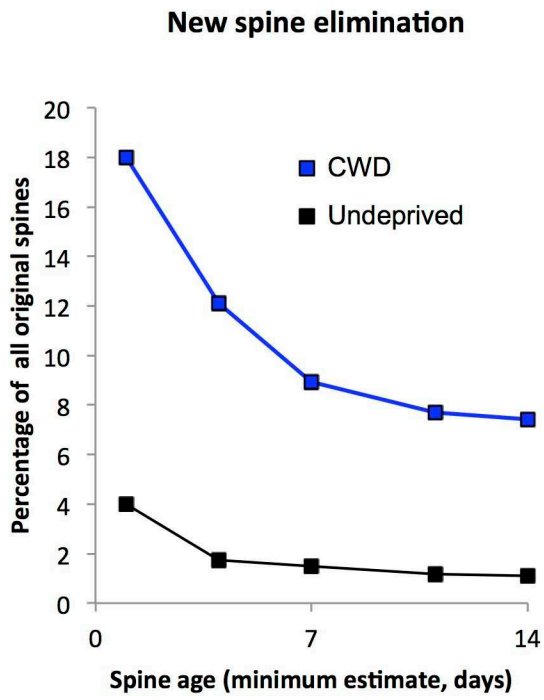
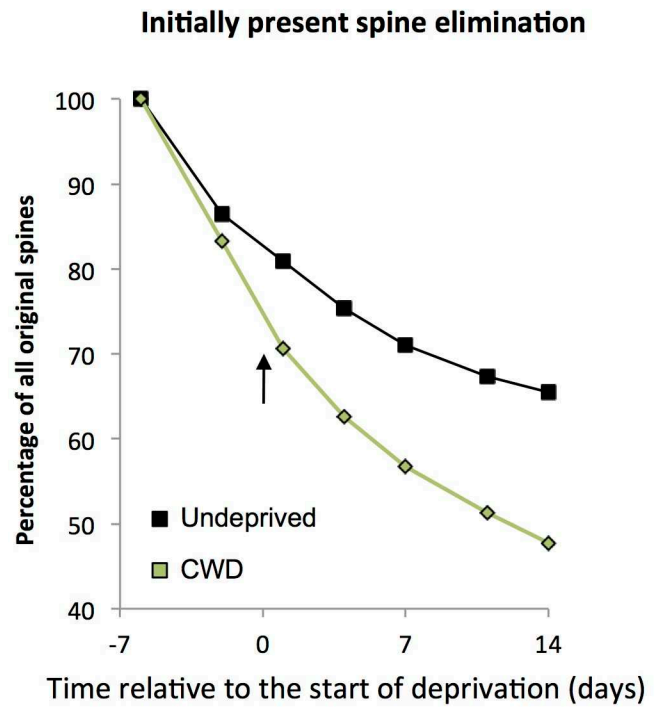


Figure 7

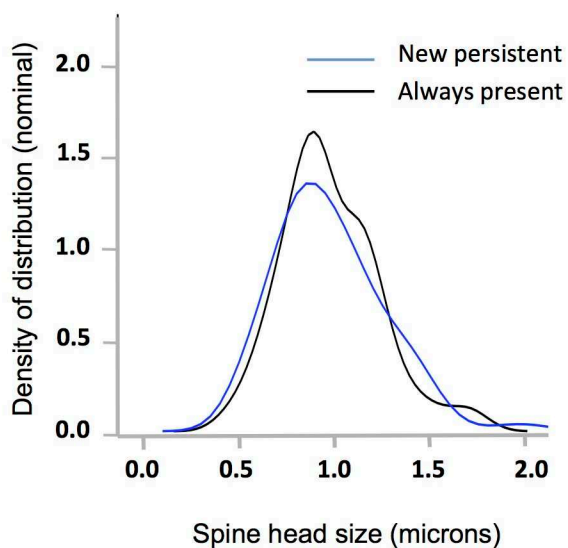
A



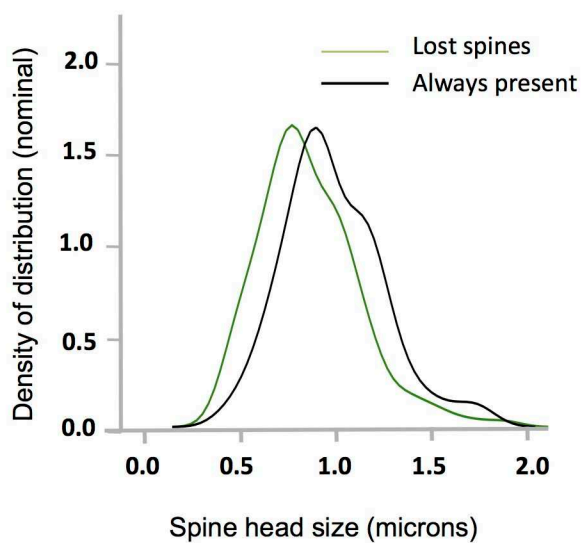
B



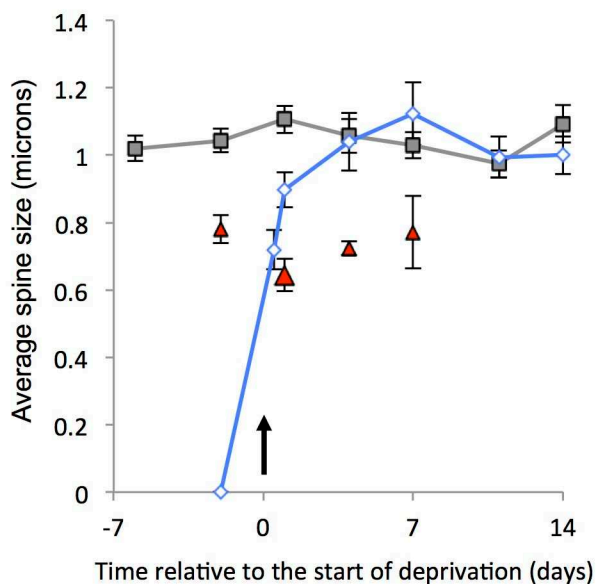
A Spine head size distribution: New persistent and always present spines



B Spine head size distribution: lost and always present spines



C Time course of spine head size changes



D Relationship between spine head size and spine persistence

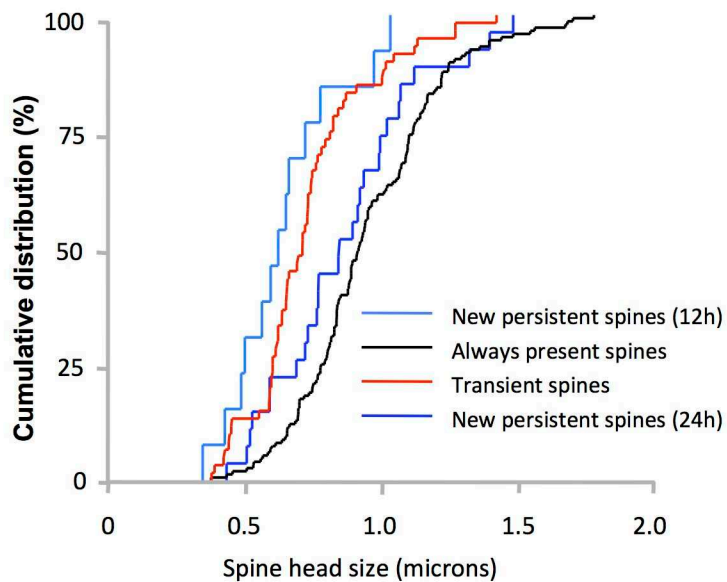


Figure 9

