Cortical and limbic brain areas are regarded as centres for learning. However, how thalamic sensory relays participate in plasticity upon associative learning, yet support stable long-term sensory coding remains unknown. Using a miniature microscope imaging approach, we monitor the activity of populations of auditory thalamus (medial geniculate body) neurons in freely moving mice upon fear conditioning. We find that single cells exhibit mixed selectivity and heterogeneous plasticity patterns to auditory and aversive stimuli upon learning, which is conserved in amygdala-projecting medial geniculate body neurons. Activity in auditory thalamus to amygdala-projecting neurons stabilizes single cell plasticity in the total medial geniculate body population and is necessary for fear memory consolidation. In contrast to individual cells, population level encoding of auditory stimuli remained stable across days. Our data identifies auditory thalamus as a site for complex neuronal plasticity in fear learning upstream of the amygdala that is in an ideal position to drive plasticity in cortical and limbic brain areas. These findings suggest that medial geniculate body's role goes beyond a sole relay function by balancing experience-dependent, diverse single cell plasticity with consistent ensemble level representations of the sensory environment to support stable auditory perception with minimal affective bias.
Associative learning depends on the reliable integration of sensory stimuli from the environment and their association with specific aversive or appetitive outcomes to shape future behaviours. Many cortical and limbic brain areas have been identified as centres for associative learning. However, how thalamic sensory relays like the auditory thalamus (medial geniculate body, MGB), which provide direct sensory input to these areas, participate in plasticity upon associative learning, yet ensure stable long-term sensory coding remains unknown. Auditory fear conditioning, a well-studied classical conditioning paradigm, identified the amygdala as a core brain area for associative learning of stimulus-predicted (conditioned stimulus, CS, e.g., tone) aversive outcomes (unconditioned stimulus, US, e.g., mild foot shock)\(^1\)–\(^4\). At its input site, amygdala response plasticity is thought to be driven by synaptic potentiation in basolateral amygdala (BLA) neurons\(^5\)–\(^6\). However, early work demonstrated that the higher order MGB, a major auditory input hub to the amygdala\(^7\), is a site of CS-US integration and plasticity upon fear learning\(^8\)–\(^15\). Enhanced responses to conditioned stimuli and increased synaptic drive from presynaptic MGB neurons to the BLA might act as an additional plasticity mechanism for associative fear learning. Nevertheless, the role of MGB in neuronal response plasticity upon fear learning has been controversially discussed\(^1\)\(^6\)–\(^17\) and recent physiological studies of fear conditioning mostly omitted this site of sensory integration and response potentiation upstream of the amygdala and auditory cortex. It is currently unknown if individual MGB neurons exhibit complex response dynamics upon adaptive associative and defensive behaviours and how this potential heterogeneity is balanced with reliable representations of sensory inputs from the environment. Furthermore, we are currently lacking a concept of the ensemble level activity and dynamics in this widely projecting thalamic auditory relay site, which is crucial to delineate the distributed population code underlying associative learning and adaptive defensive behaviours\(^18\)–\(^19\).

Here we used a combination of deep brain Ca\(^{2+}\)-imaging, miniature microscopy and fear conditioning in freely moving mice to reveal the response dynamics and plasticity of large populations of auditory thalamus neurons\(^20\)–\(^22\). We find that individual auditory thalamus neurons exhibit mixed selectivity of CS and US responses with highly diverse plasticity patterns during associative learning, while the ensemble representation of auditory stimuli remains stable along learning. These findings suggest that auditory thalamus plays a role beyond a classic relay function by balancing experience-dependent plasticity with stable ensemble level representations of the sensory environment to support stable auditory perception with minimal affective bias.

Next, we used a classic four-day fear conditioning and fear extinction paradigm\(^28\) (Fig. 2a, b), in which mice learn to associate a mild foot-shock unconditioned stimulus (US) with a predictive conditioned stimulus (CS+, 6 or 12 kHz pure tones, 200 ms pips, 27 pips per CS). After fear conditioning, mice exhibited enhanced freezing to the CS+ (61 ± 6%) when compared to a neutral CS− (42 ± 8%, \(N = 15\), \(p < 0.01\), Mann–Whitney test, Fig. 2b, Supplementary Fig. 2a), which extinguished upon repetitive CS+ presentation (Friedman test, \(p < 0.001\), followed by Dunn’s multiple comparisons test, Extinction 1 early vs. Extinction 1 late \(p < 0.01\), Extinction 1 early vs. Extinction 2 late \(p > 0.05\), Extinction 2 early vs. Extinction 2 late \(p < 0.005\)). During fear conditioning, the total neuronal population (Fig. 2c) as well as individual MGB neurons (Fig. 2d, e) were strongly responsive to both the CS+ and the US. The proportion of US responsive neurons (75 ± 5%) was significantly higher than the proportion of CS+ (27 ± 3%) and CS− neurons (20 ± 2%, \(N = 9\) mice, see “Methods” for classification of responsive neurons), while similar proportions of neurons were responsive to the CS+ and the CS− (Fig. 2f; Supplementary Fig. 2b, c, Friedman test, \(p < 0.01\) followed by Dunn’s multiple comparisons test CS+ vs. CS− \(p > 0.05\), CS+ vs. US \(p < 0.05\), CS− vs. US \(p < 0.001\)). Furthermore, we found mixed selectivity in subpopulations of neurons that were responsive to combinations of tones and foot shocks, yet they were not enriched beyond chance level in the total population (Fig. 2g). These multisensory neurons were spatially intermingled in MGB and not locally clustered (Fig. 2h–j).

To test if US responses are solely driven by movement of the animal or self-induced sounds, e.g., escape runs or low frequency harmonic vocalizations during the aversive foot shock, we correlated the activity of individual MGB neurons with movement speed or the occurrence of sounds in the context. First, a large proportion of MGB neurons exhibited an apparent correlation between movement speed and Ca\(^{2+}\)-activity during the US. However, this is most likely due to the simultaneous occurrence and conflation of the 2 s aversive foot shock and the behavioural output (escape), given that the activity in the large majority of MGB neurons was not motion or speed correlated during the habituation period (Supplementary Fig. 3). In addition, US responses in MGB cannot be solely explained by the auditory environment, i.e., movement sounds or low frequency harmonic vocalization\(^29\)–\(^30\) of the animal during the aversive foot shock, given that US and CS+ responses were typically substantially larger in sound correlated neurons than responses to self-evoked sounds of the animal (Fig. 2k; Supplementary Fig. 4). This indicates that MGB US responses are most likely driven by direct somatosensory input, pain signals or aversive state switches.

In summary, our data demonstrates that auditory thalamus neurons are strongly responsive to both pure auditory tones as well as aversive stimuli. This integration of CS and US inputs underlines that MGB neurons are ideal candidates for sensory plasticity upon associative learning\(^9\)–\(^17\).

Neural response dynamics of MGB neurons upon fear conditioning. MGB neurons, particularly in the medial subdivision, have been shown to potentiate auditory CS responses upon fear learning\(^17\). However, their response diversity and dynamics on the population level upon associative learning remain unknown. To understand the learning-related dynamics of MGB neurons, we followed the activity of large populations of individual MGB neurons across the 4-day fear conditioning paradigm. Using a cluster analysis approach, we classified CS+ responsive neurons according to their response dynamics before and after fear conditioning and fear extinction (Fig. 3a, b). On the habituation day
and on the two extinction days, we identified eight subgroups of CS+ responsive neurons. 7 ± 1% of cells show stable CS+ responses across days. The remainder could be separated in the following subgroups of plastic neurons: neurons that abolish their complete (21 ± 3%, CS down cells) or onset (9 ± 2%, on-down cells) CS+ response after fear conditioning, neurons with enhanced CS+ responses when the animal is in a high fear state (23 ± 5%, fear cells), neurons that are inhibited when the animal is in a high fear state (4 ± 1%, fear-down cells) as well as neurons that enhance or decrease their response when the animal extinguished the fear behaviour (14 ± 3%, extinction-up cells; 8 ± 2%, extinction-down cells). In addition, we identified cells that had stable, enhanced CS+ responses after fear learning (14 ± 4%, persistent cells, Fig. 3c). Similar subgroups were found for CS− responsive neurons. However, in contrast to the US-paired CS+, the group of CS− stable cells was most prominent across days (Supplementary Fig. 5a–c). In addition, we found similar proportions of CS+ (Supplementary Fig. 2b) and CS− responsive (Supplementary Fig. 2c) neurons during the habituation, fear conditioning and extinction days. However, the proportion of neurons that were plastic and changed their CS responses across days was significantly higher in the CS+ group (93 ± 1%) compared to the CS− group (60 ± 7%, 2-way ANOVA, p < 0.0001, followed by Tukey post hoc test, p < 0.05), while the proportion of stable neurons was higher in the CS− (40 ± 7%) compared to the CS+ group (7 ± 1%, 2-way ANOVA, p < 0.0001, followed by Tukey post hoc test, p < 0.05, Fig. 3d), indicating that neural response plasticity is learning-specific and more prominent for the paired conditioned stimulus than the control stimulus. To rule out that CS+ plasticity is not just a result of a general drift in tone responsiveness across days, we performed an unpaired conditioning paradigm where the foot shock is not temporally contingent with the tone CS+, such that the mice experience similar sensory stimuli to the fear conditioned animals but do not associate the CS+ with the US and fail to learn to freeze to the CS+ (Supplementary Fig. 6a, b). Unpaired conditioning did not affect the proportions of tone and foot shock responsive neurons in MGB (Supplementary Fig. 6c–h). However, compared to fear conditioned animals, the majority of neurons exhibited stable across-day CS+ tone responses in the unpaired condition (61 ± 9%), while the proportion of plastic neurons was significantly decreased (39 ± 9%, N = 5 mice, Fig. 3e). This data indicates that
MGB single cell tone responses are by-and-large stable across days, while CS+ response dynamics of individual neurons after fear conditioning are due to associative learning-induced plasticity and not just a general drift of tone-responsiveness in MGB across days. Notably, for the minority of MGB neurons that exhibited CS+ plasticity, the relative proportion of fear cells is significantly decreased (Supplementary Fig. 6i) when compared to fear conditioned animals, indicating that the emergence of this population of neurons with potentiated CS+ responses is specific to auditory fear learning.

All-in-all, this data reveals a broad response diversity of MGB neurons upon fear learning that extends previous observations of fear conditioning potentiated neurons15,16,31. The plastic CS+ subgroups are similar to previously described functional groups in the amygdala21,22,28,32. While fear and extinction neurons are the most prominent groups, they appear alongside other distinct subgroups, indicating that diverse CS+ response plasticity occurs not only in the amygdala, but also upstream in auditory thalamus.

MGB neurons are diversely tuned to auditory frequencies9 and individual neurons were reported to change their frequency tuning upon associative learning11,12,31. To estimate changes in auditory frequency tuning in large populations of individual MGB neurons before and after fear conditioning, we presented 200 ms pips of at least eleven different pure tone frequencies (1–40 kHz) at 65–85 dB to freely moving mice while simultaneously imaging MGB Ca2+ activity (Fig. 3f). We found that the mean pip response of the MGB population across all frequencies was nearly doubled after fear conditioning (Fig. 3g). Furthermore, the proportion of tone responsive neurons was increased post-conditioning (Fig. 3h, before: 22 ± 7%, after: 37 ± 6%, Wilcoxon signed-rank test, p < 0.0002, Ext.2e vs Ext.2 l). Friedman test, p < 0.001), followed by Dunn’s multiple comparisons test: CS+ vs. US, p = 0.029; CS− vs. US, p = 0.0005 (N = 9 mice). Boxplots represent median, 2nd, 3rd quartile, minimum and maximum. Cross indicates mean. Proportion of mixed selectivity CS± and US coding neurons. Red line indicates chance overlap level (N = 9 mice). Boxplots represent median, 2nd, 3rd quartile, minimum and maximum. Cross indicates mean. Example spatial map of unisensory and multisensory mixed selectivity CS and US coding neurons in MGB. i Relationship between within response group and across response group pairwise spatial distance between neurons (N = 855 cells, N = 9 mice). j Cumulative distribution function of pairwise distances between all, US-responsive, CS+ and CS− responsive neurons (N = 855 cells, N = 9 mice). k Mean Ca2+ activity (± s.e.m) of sound-correlated neurons during shock evoked sound events e.g., mouse escape sounds and low frequency harmonic vocalizations (LFH, orange), the first CS+ pip (blue) and the US (green) from N = 550 CS+/US or N = 4956 sound event trials from 110 cells out of N = 3 mice. *, **, *** indicate p values smaller than 0.05, 0.01 and 0.001, respectively.

**Fig. 2 Mixed selectivity tone CS+ and shock US coding of MGB neurons upon fear conditioning.** a Details of the 4-day fear conditioning paradigm. b Conditioned stimulus (CS) CS+ and CS− freezing (mean ± s.e.m.) during the habituation, fear conditioning as well as extinction days (Ext. 1, Ext. 2. e and l indicate early and late phases of extinction, i.e., the first four or last four CS+ of the session. Friedman test, p < 0.001, followed by Dunn’s multiple comparisons test, Ext.1e vs Ext.1 l p = 0.0069, Ext.1e vs Ext.2 l p = 0.0002, Ext.2e vs Ext.2 l p = 0.0281, N = 15 mice). Boxplots represent median, 2nd, 3rd quartile, minimum and maximum. Cross indicates mean. c Population response of one example animal to the CS+ and US (unconditioned stimulus, mean ± s.e.m.). Blue dots indicate CS+ tone pips. Green bar indicates shock US. Example cell response to the CS+ (d) and US (e). Mean ± s.e.m. of five trials. Dots indicate CS+ tone pips. Inset represents average response to single pips. f Proportion of CS+, CS− and US responsive neurons. Friedman test, p < 0.001, followed by Dunn’s multiple comparisons test: CS+ vs. US, p = 0.029; CS− vs. US, p = 0.0005 (N = 9 mice). Boxplots represent median, 2nd, 3rd quartile, minimum and maximum. Cross indicates mean. g Proportion of mixed selectivity CS± and US coding neurons. Red line indicates chance overlap level (N = 9 mice). Boxplots represent median, 2nd, 3rd quartile, minimum and maximum. Cross indicates mean. h Example spatial map of unisensory and multisensory mixed selectivity CS and US coding neurons in MGB. i Relationship between within response group and across response group pairwise spatial distance between neurons (N = 855 cells, N = 9 mice). j Cumulative distribution function of pairwise distances between all, US-responsive, CS+ and CS− responsive neurons (N = 855 cells, N = 9 mice). k Mean Ca2+ activity (± s.e.m) of sound-correlated neurons during shock evoked sound events e.g., mouse escape sounds and low frequency harmonic vocalizations (LFH, orange), the first CS+ pip (blue) and the US (green) from N = 550 CS+/US or N = 4956 sound event trials from 110 cells out of N = 3 mice. *, **, *** indicate p values smaller than 0.05, 0.01 and 0.001, respectively.
Fig. 3 Single cell response plasticity in MGB upon fear learning. a Heat map of single cell CS+ responses on the habituation, extinction 1 and extinction 2 days. Cells were clustered into groups depending on their CS+ response pattern (N = 386 cells, N = 9 mice). b Average traces ± s.e.m of neuronal clusters in (a). c Proportion of individual plasticity groups within CS+ responsive cells/animal (Kruskal-Wallis test, p < 0.05, followed by Dunn’s multiple comparisons test; Stable vs cs down, p = 0.013, cs down vs fear down, p = 0.0003, cs down vs extinction down, p = 0.035, fear vs fear down p = 0.0041; N = 9 mice). Boxplots represent median, 2nd, 3rd quartile, minimum and maximum. Cross indicates mean. d Proportion of CS+ and CS− stable and plastic neurons (2-way ANOVA followed by Sidak multiple comparisons test, p < 0.001; stable CS− vs. stable CS+, p = 0.002; stable CS− vs. plastic CS+, p < 0.001; stable CS+ vs. plastic CS−, p < 0.001; stable CS+ vs. plastic CS+, p < 0.001; plastic CS− vs. plastic CS+, p = 0.002; N = 9 mice, data presented as mean values ± s.e.m.). Circles represent individual animals. e Proportion of CS+ stable and plastic neurons in a pseudoconditioning paradigm compared to fear conditioned animals (2-way ANOVA followed by Sidak multiple comparisons test, p < 0.001; stable vs plastic, unpaired vs plastic, p = 0.019; stable vs. plastic paired, p < 0.001; stable vs. plastic, unpaired vs plastic, p < 0.001; plastic vs. plastic paired, p < 0.001; Unpaired group N = 5 mice, Paired group N = 9 mice, data presented as mean values ± s.e.m.). Circles represent individual animals. f Experimental paradigm. Auditory tuning was tested before and after fear conditioning. g Population response to individual 200 ms pips before (black) and after (orange) fear conditioning (mean ± s.e.m). * Significance after (orange) fear conditioning. h Heat maps of single cell US responses to the five US stimulations during the fear conditioning day (N = 634 cells, N = 9 mice). i Proportion of tone-responsive cells before and after fear conditioning (Two-tailed, Wilcoxon signed-rank test, p > 0.05). j Proportion of US responsive cells/animal (Kruskal-Wallis test, p = 0.0015; n = 284 neurons, 7 mice). Horizontal lines represent median. k Population statistics for BF tuning towards the CS+ (two-tailed Wilcoxon signed-rank test, p = 0.001, n = 284 neurons, 7 mice). Horizontal lines represent median. l Heat maps of single cell US responses to the five US stimulations during the fear conditioning day (N = 634 cells, N = 9 mice). m Average response ± s.e.m. of plastic subtypes of US-responsive MGB neurons (N = 634 cells, N = 9 mice, see also Supplementary Fig. 3). n Proportion of individual plasticity groups within US responsive cells/animal (Kruskal-Wallis test, p < 0.001; followed by Dunn’s multiple comparisons test; Stable vs down, p = 0.043; down vs off-up, p = 0.0006; down vs off-down, p < 0.001; down vs inh, p = 0.005; off-down vs inh type 1, p = 0.018; N = 9 mice). Boxplots represent median, 2nd, 3rd quartile, minimum and maximum. Cross indicates mean. o Proportion of US (N = 5 mice, p < 0.05, Wilcoxon signed-rank test) and CS plastic cells (N = 5 mice, p < 0.05, two-tailed Wilcoxon signed-rank test) in the ventral (MGBv) and medial (MGBm) subdivisions of MGB. Insert: Schematic of GRIN lens location above the different MGB subdivisions. *, **, *** indicate P values smaller than 0.05, 0.01 and 0.001, respectively.
MGB preserves a broad tuning range for reliable sensory representation, despite a stronger representation of the CS+.

In addition to plasticity of CS tone representations, we found that US responsive neurons can be subdivided into stable (8 ± 1%) and plastic cells (92 ± 1%) during fear conditioning. The majority of US responsive cells was plastic and exhibited dynamic intra-session representations of the US (Mann–Whitney test, p < 0.001). Using a similar cluster analysis approach, we identified neurons that demonstrated intra-session potentiation or depression and subtypes of inhibited as well as off-responsive neurons that potentially signal relief from the shock (Fig. 3i–n). However, despite its prominent diversity, the US response type was not predictive of CS plasticity in MGB neurons. US response and plasticity type did not overlap with CS response and plasticity type above chance levels (Supplementary Fig. 7), indicating that US inputs per se do not drive plasticity in MGB neurons. Nevertheless, adaptive US responses in MGB could act as an upstream teaching signal in addition to local circuit mechanisms39, which direct plasticity in downstream areas like the amygdala.

MGB is subdivided into a first order, auditory cortex-projecting nucleus (MGBv) as well as higher order nuclei (MGBd, MGBm), which send axons to cortical and limbic brain areas, e.g., the amygdala (Doron and LeDoux, 1999). To test if MGB CS and US plasticity types are different between first order and higher order nuclei, we subdivided the cells depending on their location in the GRIN lens field of view between MGBv and MGBm after anatomical verification of the lens front location for all mice where MGBv and MGBm were simultaneously imaged (N = 5 mice, Supplementary Fig. 8a, b). Similar to the total population of MGB neurons, large fractions of MGBv and MGBm neurons exhibited plasticity to the US or CS+ (Fig. 3o). Nevertheless, plastic cells were not significantly different between either subdivision (Fig. 3o) and the diversity of plasticity subtypes was similar in the first order (MGBv) vs. higher order (MGBm) area of auditory thalamus (Supplementary Fig. 8c–e).

Overall, we found that responses of individual MGB neurons to the CS and US are plastic upon fear conditioning. In addition to previously reported potentiated auditory neurons, we find highly diverse subtypes of CS or US plastic neurons that go beyond FC-driven response potentiation and are distributed similarly across both first order and higher order MGB subdivisions. US response subtypes were not predictive of CS plasticity, nor were the proportions of subgroups or their enrichment predictive of behavioural outcomes on an animal-by-animal basis (Supplementary Table 1), indicating that MGB neurons might play diverse roles in guiding memory formation during associative learning.

Activity in the MGB → BLA pathway is necessary for fear memory consolidation and stabilizes MGB plasticity. To test if activity in MGB → BLA projection neurons is necessary for fear learning8,41–44 and MGB plasticity, we specifically expressed the inhibitory opsin ArchT in MGB → BLA projection neurons (Fig. 5a, b, Supplementary Fig. 10a-e). Inhibition of MGB → BLA projectors during CS-US pairing on the conditioning day (Fig. 5c) had no effect on fear acquisition (Fig. 5d, mean freezing during the last two CS+ presentations, GFP: 65 ± 4%, N = 13 mice; MGB → BLA ArchT: 60 ± 6%, N = 9 mice; p > 0.05, Mann–Whitney test, Supplementary Fig. 10f). However, freezing levels were significantly reduced during the fear test 24 h later (Fig. 5e, mean freezing during the first four CS+, GFP: 48 ± 6%, N = 13 mice; MGB → BLA ArchT: 22 ± 5%, N = 9 mice; p < 0.01, Mann–Whitney test), indicating that activity in MGB → BLA projectors is necessary for the consolidation of fear memories. In addition, and similar to amygdala-projecting MGB neurons (Fig. 5a–e), we found that inhibition of neuronal activity in the total MGB population (Supplementary Fig. 10f–i) or in the first order ventral nucleus of MGB (Supplementary Fig. 10j) had no effect on fear acquisition during the conditioning session but suppressed fear memory consolidation.

To test how activity in MGB → BLA neurons during fear learning affects across-day plasticity of the total MGB population, we used an all-optical miniaturized microscope imaging approach (Fig. 5f). We specifically expressed ArchT or tdTomato in MGB → BLA neurons (Fig. 5g) and suppressed their activity during the CS-US pairings on the fear conditioning day (Fig. 5c)
while simultaneously imaging the activity of the total MGB population (CaMKII-driven GCaMP6f). Given that MGB → BLA neurons represent only a fraction of the total MGB neuronal population (ca. 11 ± 2% in total MGB, N = 4 mice, see also Fig. 4c), inhibiting MGB → BLA neurons during fear conditioning did not affect the mean CS+ and US response of the total imaged MGB population (Fig. 5h and Supplementary Fig. 11a–e). However, when comparing the CS+ plasticity across fear learning conditions, inhibiting MGB → BLA neurons did affect the mean CS+ and US response of the total imaged MGB population (Fig. 5h and Supplementary Fig. 11a–e).
we find that inhibition of MGB → BLA neurons during fear conditioning enhances the proportion of plastic neurons after fear consolidation (Fig. 5i, stable neurons: tdTom = 7 ± 2%; plastic: tdTom = 73 ± 6%, ArchT = 93 ± 2%; 2-way ANOVA, F; 1, 9) = 10.09, p < 0.05, Sidak’s multiple comparisons test, p = 0.0104 each). Boxplots represent median, 2nd, 3rd quartile, minimum and maximum. Cross indicates mean. *Freezing of GFP and ArchT-expressing animals upon fear recall during early extinction 1 (Ext. 1, mean freezing during the first four CS+, GFP: N = 13 mice, MGB → BLA ArchT: N = 9 mice, p > 0.05, two-tailed Mann–Whitney test). Boxplots represent median, 2nd, 3rd quartile, minimum and maximum. Cross indicates mean. *, ** indicate p values smaller than 0.05 and 0.01, respectively.

Population coding and representation of the conditioned stimulus across days. Next, we tested if the CS+ and CS− can be decoded from Ca2+ activity based on MGB population activity. First, we trained a three-way quadratic decoder to distinguish between CS+, CS− and baseline activity within the same session (see “Methods”). To balance for different cell population sizes between animals, we randomly sub-selected 40 cells for each animal and averaged decoder accuracy across 50 independent runs. Furthermore, to account for different numbers of CS+ and CS− presentations, we only decoded the first four CS+ and CS− presentations. Within each individual session, the decoders achieved high classification accuracy (Fig. 6a, >80% compared to 33% chance level) indicating a distinct representation of the individual CSs by the MGB population. Surprisingly, decoding accuracy was higher in the population of MGB → BLA projectors compared to the total MGB population, except for the fear conditioning day. To test if CS tones can be accurately detected across days from MGB population activity, we next trained sets of two-way decoders to distinguish between baseline and CS+ or CS− responses for each experimental day and tested the trained decoders across days (Fig. 6b). Strikingly, we found that decoder accuracy is robust across days reaching decoding levels of ca. 70% or higher, for both the CS+ and the US (Fig. 6d). This is in contrast to amygdala population coding, where decoding levels for the CS+ drop to chance levels after fear conditioning 21, indicating that MGB population representations of CS tones are stable despite associative learning. Furthermore, we found a drop in CS+ encoding in MGB → BLA projectors during the fear conditioning day, which recovered afterwards (Fig. 6c, see also Fig. 6a), indicating temporary changes in CS+ encoding during associative learning.

Finally, we compared the population vector distance (PVD) between the evoked population responses to the CS+ and CS− and the evoked population responses to the US (Fig. 6d-g). During fear conditioning, we found a decrease of the PVD for the CS+ and CS− compared to the US (Fig. 6d, see also Fig. 6a), indicating different changes in PVD at the end of the session indicating different population dynamics for this subgroup of...
MGB neurons during associative learning (Friedman test, \( p < 0.01 \), 1st and 3rd versus 5th pairing; Dunn-Sidak multiple comparisons test, \( p < 0.05 \)). Importantly, no changes were found between the evoked responses to the CS− and US during conditioning. In contrast to previous observations in the amygdala21, the PVD between the CS+ and the US changes were not preserved post conditioning on the extinction days and the population representation recovered to pre-conditioning levels similar to the observations using two-way or three-way decoders (Fig. 6a, c). The same was observed for the across-day population vector distance of the CS+ to itself (Supplementary Fig. 12). However, note that we found a significant difference between the CS− to US PVD of the total MGB population between the conditioning day and the second extinction day (FC versus Ext. 2; Friedman test, \( p < 0.05 \), Dunn-Sidak multiple comparisons test, \( p < 0.05 \)). Nevertheless, we did not find a change in the CS− to US PVD between the habituation, fear conditioning and early extinction days indicating that the CS− representation is stable during high fear states in relation to the habituation day and shows no significant drift intraday during fear conditioning. Furthermore, the CS− drifts further away from the US in comparison to the habituation day which might be reflective of an enhanced safety signal after extinction.

In addition to the general lack of consolidation of population level changes across fear learning, the strength of PVD-changes between the CS+ and US were not predictive of learned freezing behaviour on an animal-by-animal basis (Supplementary Fig. 13).

Finally, using an all-optical approach to inhibit MGB → BLA projectors during fear learning (see Fig. 5), we find that the suppression of activity in MGB → BLA leads to a reduced shift in the population vector distance between the CS+ and the US on the fear conditioning day (Fig. 6h, 5th pairing, relative change control: \(-30 \pm 13\%\), ArchT: \(-2 \pm 3\%\), 2-way ANOVA, \( p < 0.05 \), Sidak multiple comparisons test, \( p < 0.05 \)), which might be crucial for downstream population plasticity in BLA. However, suppression of MGB → BLA activity did not affect the post-conditioning CS-US PVD-change reset (Supplementary Fig. 11f–h).

Taken together, this data indicates that high dimensional representations of CS+ tones are stably encoded in MGB populations across associative fear learning, despite plastic changes in single cell response patterns. In contrast to the basolateral amygdala21, MGB population representations of sensory stimuli only transiently change during associative fear learning and reset overnight, which might be crucial for unbiased representations of stimuli from the environment.

**Discussion**

By imaging large populations of MGB neurons, we find that auditory thalamus is a site of diverse neuronal plasticity during associative fear learning on the level of single cells as well as the total MGB population. However, changes in MGB population level coding are only transient and do not consolidate overnight, which might be instructive for plastic changes in downstream
structures during learning, yet allows for long-term stability of sensory coding across days.

On the level of individual MGB neurons, we observed associative learning-induced CS+ response potentiation that resembles classic studies which demonstrated that auditory thalamus exhibits enhanced responses to aversive conditioned tones.4–16,45. However, recording simultaneously from large populations of individual neurons in MGB during fear conditioning, we find diverse plasticity patterns (at least 7) that are similar or go beyond previously reported plasticity types in cortical or limbic areas.51,52,58,59 downstream of MGB as well as calretinin-positive subpopulations of lateral thalamus neurons.44 Changes in CS responsiveness of individual MGB neurons upon fear learning and extinction were bidirectional, i.e., potentiated or depressed, and depended on the behavioural state of the animal. For example, we find different functional types that are enhanced or depressed particularly in high fear or extinction states. This extends the notion of unidirectional response potentiation in MGB upon associative learning and demonstrates that auditory thalamus neurons exhibit heterogeneous, adaptive signalling of threat-predicting auditory stimuli of the environment. Plasticity in MGB is specific to auditory fear conditioning and not just a general drift of single cell responses across days. However, by-and-large, the proportion of individual plasticity subtypes was not correlated with the strength of post-conditioning freezing or discrimination levels57 of the animals (Supplementary Table 1, Supplementary Fig. 13b), suggesting that individual functional neuronal types in MGB are per se not predictive of an animal’s behavioural output. Nevertheless, the heterogeneity of the neuronal response plasticity might be necessary to enhance the computational capacity and memory specificity of MGB.48–51

Furthermore, precise behavioural outputs might be orchestrated by heterogeneous and complex activity in MGB in coordination with a distributed downstream network. For example, MGB neurons project onto amygdala principal neurons and several amygdala interneuron subclasses,33,44,52–55 suggesting that the diversity of MGB plasticity is tuned to the precise MGB → BLA connectivity. This in turn would be crucial for the specific activation of distinct amygdala circuits and amygdala memory engrams to recruit input specific behavioural outputs.44,56–58

Besides auditory stimuli, MGB neurons signal the aversive foot shock (US) during fear conditioning.9–15. Indeed, we found that the proportion of foot shock encoding neurons exceeds the number of tone CS+ encoding neurons in auditory thalamus of freely moving animals. The large proportion of US encoding neurons in MGB and the strength of the US signal on the population level could not be explained by movement of the animal or self-vocalization-induced activation of MGB.39,60

Aversive US responses are considered to be more prominent in higher order auditory thalamus (MGBm).9 Our imaging sites covered both first order (MGBv) and higher-order (MGBm) MGB, and US encoding neurons were equally present in both sites. This demonstrates that multisensory encoding is not an exclusive feature of higher-order areas of MGB in freely moving animals, but can also occur in the first order ventral subdivision that projects to auditory cortex, suggesting that auditory thalamus conveys aversive US information to a broad range of cortical and limbic downstream areas during associative fear learning. Strikingly, US responses were heterogeneous across the population of MGB neurons during fear conditioning. Besides stable US responders, we identified several plasticity types of US responsive neurons, including short term facilitating, depressing or off-responsive neurons. This functional diversity of US neurons indicates that first and higher order auditory thalamus can signal distinct types of instructive information, for example adaptive teaching signals for associative fear learning as well as relief or safety signals upon termination of the US. Future studies need to address if and how these non-uniform adaptive MGB US signals are relayed to specific circuits elements in downstream areas like the amygdala or auditory cortex.53–57,61–67

CS and US coding neurons are spatially intermingled in auditory thalamus and a large fraction of MGB neurons exhibit mixed selectivity for both, the CS tone and US foot shock. The convergence of diverse CS and US responses in individual MGB neurons renders auditory thalamus an ideal site for neuronal plasticity in associative learning,34,35,37,44,85,86 which is supported by the finding of large numbers of different subgroups of plastic neurons upon fear conditioning. Nevertheless, similar to observations downstream in the basolateral amygdala,21,22, the convergence of CS and US responses in MGB neurons was not predictive of the response plasticity of a given neuron. Instead, MGB neurons exhibit manifold functional classes and outcomes of CS/US conversion upon learning (e.g., CS/US responsive cells can become potentiated fear cells or CS down cells), suggesting that heterogeneous fear conditioning-induced auditory response plasticity in MGB is most likely governed by multiple cellular or circuit mechanisms of neuronal plasticity. Indeed, we find subsets of neurons in all groups of CS plastic neurons that were not US responsive during FC (see Supplementary Fig. 7). Converging subthreshold CS and US inputs in dendrites, which cannot be detected by extracellular measurement of somatic neuronal activity during fear conditioning, yet might induce local dendritic plasticity mechanisms68–71, could be a potential source of CS response adaptation. Alternatively, subsets of MGB neurons might not require converging CS and US input15 to drive functional plasticity upon associative learning, arguing for additional plasticity mechanisms that go beyond classical Hebbian plasticity and coincidence detection on a millisecond timescale,72, and might additionally involve slower, neuromodulatory mechanisms73–75 and the amygdala circuits21,76 or consolidation during sleep.14

Furthermore, brain-wide distributed interacting circuit mechanisms could play a role in the formation of single cell plasticity upon associative fear learning, not only in MGB but across multiple fear-related brain areas16,28,77–82. The detailed computations within this distributed network19 and the role of auditory thalamus are poorly understood. Plasticity and adaptive changes in MGB depend on uni- or multi-synapse feedback circuits from distinct brain areas including the amygdala,16,83 and cortex.84 Nevertheless, our data supports the notion that learning-induced modifications of neuronal activity in MGB could drive plastic neuronal responses in downstream areas.17 This suggests that at least a part of the heterogeneous response plasticity in amygdala or cortex during associative fear learning could be inherited in a feedforward fashion from adaptive changes in the thalamic relay independent of and in addition to local synaptic and circuit mechanisms33,61,63

Both, first order auditory cortex-projecting MGBv as well as higher order areas of MGB (including calretinin-positive lateral thalamus → BLA neurons)44 are necessary for memory consolidation and we found similar proportions of CS and US plastic neurons in both subregions of MGB. Auditory cortex is involved in the formation of fear memories to complex sound stimuli and plasticity in auditory cortex-projecting MGBv neurons might play an instructive role. Higher order areas of MGB are more broadly-projecting areas of auditory thalamus and target among others auditory cortex, striatum and amygdala.34,35,37,44,45,85,86

Given the amygdala’s prominent role in associative fear learning and our finding that activity in the MGB → BLA pathways is crucial for fear memory consolidation (note that we did not find a recently observed effect of MGB → BLA calretinin-positive neurons on fear memory acquisition on the conditioning day44, which might due to differences in the strength of the conditioning protocols), we hypothesized that CS and US plastic neurons are
Specifically enriched in MGB → BLA projection neurons when compared to the total population including less plastic MGBv neurons. Using a retrograde viral approach, we specifically imaged BLA-projecting MGB neurons to distinguish these cells from the general population. Surprisingly, we found that the proportion of plastic neurons was not enhanced in amygdala-projecting neurons and was similar to the proportion of plastic neurons in the total MGB population. This lack of enrichment of neurons with dedicated functions in associative learning suggests that the MGB → BLA pathway is most likely not a labelled line, but that MGB potentially propagates experience-dependent changes of neuronal activity in associative fear learning to a wider brain network, including auditory cortex and striatum. This is reminiscent of recent findings showing that heterogeneous behaviour-related neural activity of projection neurons of a given brain area is broadcast simultaneously and in parallel to different downstream targets irrespective of the output pathway. Interestingly, this broadcasting might be achieved by the same subsets of individual MGB neurons given that higher order MGB → BLA neurons also project to auditory cortex (Supplementary Fig. 14). Finally, perturbing activity in the MGB → BLA feedforward pathway during fear conditioning led to enhanced, aberrant across-day single cell plasticity patterns in the total MGB population after fear conditioning. MGB “over-plasticity” could be a compensatory mechanism due to the lack of amygdala activity and polysynaptic amygdala feedback to MGB, e.g., via the reticular nucleus of the thalamus, which might be crucial to fine-tune single cell plasticity in MGB, further supporting the notion that distributed activity across brain areas and networks is necessary to stabilize neuronal plasticity and facilitate precise memory formation as well as behavioural output. Work including simultaneous multi-site recordings, targeted activity-dependent neural manipulations and computational neuroscience tools will be required to delineate how this heterogeneous, widely distributed population code is established, subsequently interpreted by different downstream regions and related to complex behavioural output.

Locally, on the level of the auditory thalamus, CS responses could be decoded reliably from the population level responses of the total MGB ensemble. Within a given day of the fear conditioning paradigm, we could train decoders that accurately classify individual cells during fear conditioning, which can be stable over weeks. This data is supported by the observation that the MGB population vector difference between the CS+ and US decreases during fear conditioning, yet recovers to baseline levels on the next day after the conditioning session. Thus, MGB exhibits stable tone representations on the population level across associative fear learning. This suggests that MGB ensembles exhibit stable population level tone representation across days, despite the plasticity of CS responses of individual cells during fear conditioning, which can be stable over weeks. This data is supported by the observation that the MGB population vector difference between the CS+ and US decreases during fear conditioning, yet recovers to baseline levels on the next day after the conditioning session. Thus, MGB exhibits stable tone representations on the population level across associative fear learning. This suggests that MGB ensembles exhibit stable population level tone representation across days, despite the plasticity of CS responses of individual cells during fear conditioning, which can be stable over weeks. This is in stark contrast to fear-biased population level changes in sensory representation in the amygdala that are further stabilized and consolidated after learning, and prevent the decoding of tone responses across fear conditioning. While the population code of the amygdala stabilizes “fear hijacking” of the sensory representation, MGB exhibits transient changes in population level encoding, which provides a clean slate for future perception that is unaffected by a valence bias. Nevertheless, the transient population level changes during fear conditioning in MGB, which are dependent on MGB → BLA projection neuron activity, might be crucial to guide long term population level changes in the amygdala or other downstream areas upon associative learning.

Taken together, our data indicates that auditory thalamus is ideally positioned to exhibit a complex role in guiding neuronal plasticity and valence assignment during associative learning that goes beyond the classical role of auditory processing and response potentiation during conditioning and potentially extrapolates to a broad set of behavioural functions. Delineating the neural circuit mechanisms that underlie these highly dynamic representations of uni- and multisensory stimuli in MGB and their experience-dependent plasticity will open new avenues to understand the role of early, pre-cortical sensory relays like auditory thalamus in the formation of sensory percepts and memories that mediate complex behaviours.

Methods

Animals: In total, 8 to 11-week-old C57BL/6JR mice were used throughout the study. Behavioural experiments were performed with male mice. All experiments were done in accordance with institutional guidelines (University of Basel, Tierschutz) and were approved by the Cantonal Veterinary Office of Basel-Stadt, Switzerland. Animals were housed on a 12-h light / dark cycle at an ambient mean temperature and humidity of 22°C and 55%, respectively. Food and water were provided ad libitum.

Surgery, virus injection and GRIN lens as well as optical fibre implantation. Mice were injected with the help of a stereotaxic apparatus (Kopf Instruments) in the medial geniculate body (for imaging experiments 500 nl, AAV2/5.CaMKII.GCaMP6f. WPRE.SV4. 200, Penn Vector Core, for optogenetic experiments 500 nl AAV2/5.CAG. flex.Arch.TGFp, UNC Vector Core, AAV2/5.CaMKII.EGFP.WPRE.HGFp(A), VVF Zurich, AAV2/5.CaMKII.eArchT3.0.2.A.EGFP.WPRE, VVF Zurich or AAV2/2.Syn. dlox.GFP.dlox.WPRE, VVF Zurich, for all-optical experiments 500 nl, AAV2/2.CAG. flex.Arch.Id.Tomo, UNC Vector Core or AAV2/5.CAG.dlox.Id.Tomo.dlox.PO. WPRE, VVF Zurich, for imaging experiments 500 nl, AAV2/2.Syn. flex.aXon.GCaMP6f.94, Addgene: coordinates: AP: −3.28, ML: −1.9, DV: −3.1 mm), or basolateral amygdala shunting AAV2/9.CaMKII.GCaMP6f.37.50, Penn Vector Core; rAAV2-retro. EF1a.GCaMP6f.WPRE, Georg Keller, FMI Vector Core, for optogenetic experiments 300 nl, rAAV2-retro.hSyn.mCherry.WPRE.HGFp(A), VVF Zurich; or 50 nl CTB 555, Invitrogen, for optogenetic experiments 300 nl AAV2-retro.hSyn1.mCherry. mCherry.ire.c.WPRE.HGFp(A), VVF Zurich, for all-optical experiments 300 nl AAV2-retro.CaMKII.eCre.WPRE.eGFP(A), VVF Zurich, for fluorescence imaging experiments, one week after virus injection, a gradient refractive index (GRIN) lens (0.5 or 0.6 mm diameter, Incöpsix) was implanted during a second surgery (anaesthesia and anaesthesia see above). A 0.8 mm diameter craniotomy was drilled above the MGB and a small track was cut with a 0.7 mm sterile needle. The GRIN lens was then slowly advanced into the brain (coordinates: AP: −3.28, ML: −1.9, DV: −3.0 mm), fixed to the skull with light curable glue (Loctite 4305, Henkel) and the skull was sealed with Scotchbond (3 M). The other end of the Grin lens was sealed on a piece of tubing (Coulbourn, Noldus). The wound and skull were fixed and sealed in a similar manner to GRIN lens implantations. Animals were provided with analgesia (buprenorphine, ropivacaine) and their well-being was monitored throughout the entire experimental period.

Behavioural paradigms and analysis. Behavioural experiments were performed during the animal’s light period. A four-day auditory fear conditioning paradigm was performed in a habituation / test context (days 1, 3, 4) and a fear conditioning context (day 2). Mice were presented with 5 intermingled CS+ and CS− during each session 1,5 Hz (6 kHz and 12 kHz, counterbalanced) in a round plexiglass box. CSs were composed of 27 tone pips (200 ms, 75 dB) presented at a rate of 1.1 Hz (Tucker-Davis Technologies, TDT 78 or RZ6). Fear conditioning was performed in a ca. 25 cm square plexiglass box and a shock grid floor (Coboulm, Noldus). The CS+ (6 kHz and 12 kHz, counterbalanced) was terminated by a 2 s 0.65 mA foot shock. During the last tone pip, the light was turned on (day 1 and day 4, habituation context), 4 CS− and 12 CS+ were presented. For optogenetic experiments, animals were habituated to the optical fibre attachment procedure for

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3 days before the start of the fear conditioning paradigm. On the fear conditioning day, optical fibres were attached to the optical fibre implant via a ceramic mating sleeve (Thorlabs). MGB ArchT-expressing neurons were continuously inhibited during the five CS-US pairings (starting 2 s before CS onset until 2 s after US offset) with a 565 nm LEDs (M565D2, Thorlabs). Optical stimulation was controlled with a custom-built stimulation setup consisting of an Arduino board (Arduino Uno REV3, Arduino) and LED drivers (LED121, Thorlabs). The light intensity measured at the optical fibre tip was 19 mW, which had a typical attenuation of 30%. Optogenetic experiments were performed and analyzed in a blinded fashion. Behavioural experiments were performed and analysed using Cineplex 3.4.1 (Plexon Inc) or Ethovision 14 (Noldus). Behavioral tracking based on the position of the centre of mass of the mouse was performed using infrared CS +/− US responses were recorded with a PCB Precision Condenser Microphone (Model 377C01) microphone and a RZ6 Auditory Processor (Tucker-Davis Technologies) 50 cm above the floor. The fear conditioning context at 195 kHz simultaneous to miniature microimage during the fear conditioning session. Sound waves were high-pass filtered at 1 kHz and spectrograms were computed using short-time Fourier transforms (spectrogram function, Signal Processing Toolbox, MATLAB, Mathworks). To detect sound-level correlated neuronal activity, the cross-correlation coefficient between the squared acoustic signal binning in 50 ms and the corresponding Ca2+ signal of individual neurons was computed with a maximum lag of 500 ms. Cells were classified as sound-squared if they exceeded a maximal cross-correlation coefficient of 0.2. Acoustic event onsets were detected based on the peak of the differentiated squared and binned sound wave. Acoustic events were not distinguished between animal movement-related sounds and vocalizations of the animal.

**Miniature microscope imaging.** The miniature microscope (nVista2.0 or nVoke, Incyscop). The stage was head-fixed at the head bar on a flying saucer style running wheel. Mice were initially habituated to this procedure. MGB Ca2+ activity was imaged throughout the four-day fear conditioning and three-day paradigm. On day one, the animals were exposed to the same presentation of pure tone pips as on day 1. MGB neural responses within one CS (or the baseline period), such that each training set were trained using a tenfold cross-validation procedure. Decoder accuracy was calculated as the mean of the diagonal of the confusion matrix. Classifiers were trained for each individual animal and are presented as mean decoding accuracy across animals. To balance for unequal cell numbers between the different animals, we randomly selected 40 neurons from each animal and calculated the mean accuracy from 50 independent runs.

**Image analysis.** Raw image data was analysed previously described21,22. Briefly, movies from all behavioural sessions were spatially down sampled (2x), bandpass filtered (Fourier transform) and normalized by the filtered image (ImageJ). The movies from all days were concatenated into a single time and motion corrected using Turboreg (min. three rounds)26. Only movies that motion-corrected successfully across days with final spatial displacements of < 2 μm were used for Ca2+ trace extraction. Principal component analysis (PCA)-based detection of individual neurons of interest (ROIs) was performed on down sampled (5 Hz) A/F/F movies. ROIs were truncated at 50% peak intensity and limited to a size of 30 pixels (ca. 60 μm). ROIs were initially oversized (300 ICs) and then overlaid with the maximum intensity projection of the 4-day movie. ROIs that did not match individual neurons of interest were removed. We typically retained 97 ICs per animal or CaMKII-GCaMP6f, N = 19 mice, 69 ± 9 ICs per animal for rAAV2-retro.EF1α.GCaMP6f, N = 6 mice, 90 ± 2 ICAs per animal for tdTomato nVoke experiments, N = 5 mice and 94 ± 2 ICAs per animal for ArchT nVoke experiments, N = 6 mice. These ICAs were then applied to the 20 Hz motion corrected raw fluorescence movie to extract single cell GaMP6f traces for further processing. The detected ICs from the concatenation method overlap > 99% with a similar individual day detection and post-hoc alignment method and was thus deemed more suitable and efficient (Supplementary Fig. 15).

**Ca2+ data analysis.** All analysis was based on linearly detrended and z-scored Ca2+ traces of individual neurons. Ca2+ traces were baseline to the time periods preceding CS or US onset. To identify CS− and US-responsive neurons and their plastiCity types across days, 30 s CS and 2.8 s US responses were analysed using a combined statistical and supervised cluster analysis approach as previously described22. Auditory tuning curves were measured using the mean Ca2+ response during the 250 ms time window after pip onset. Cells were classified as tone-responsive to individual frequency pips if their mean response exceeded 0.5 zfs for at least two of the frequencies tested. The best frequency (BF) of a neuron was defined as the frequency that produced the maximal Ca2+ response averaged across trials. The difference in BF to the CS− for comparison across fear conditioning is calculated on an animal-by-animal basis in absolute values as ABF = [BF+ − BF−].

Baseline responses were sampled from the 30 s periods preceding the CS+ and CS−. Classifiers were trained on the mean response of five consecutive pip presentations within one CS (or the baseline period), such that each training set contained 20 input variables per condition (i.e., 40 for two-way decoders and 60 for three-way decoders). The mean response was calculated based on a 300 ms time window after pip onset and classifiers were trained using a tenfold cross-validation procedure. Decoder accuracy was calculated as the mean diagonal of the confusion matrix. Classifiers were trained for each individual animal and are presented as mean decoding accuracy across animals. To balance for unequal cell numbers between the different animals, we randomly selected 40 neurons from each animal and calculated the mean accuracy from 50 independent runs.

**Histology.** After completion of the behavioural experiment, mice were transcardially perfused with ca. 5 ml phosphate buffered saline (PBS, Thermofisher) fol- lowed by 40 ml 4% paraformaldehyde (PFA) in PBS (pH = 7.4). Brains were removed and stored overnight in 4% PFA. Of 150 μm coronal slices were prepared using a vibrate (Campden Instruments) and immunostained for calretinin using the following solutions and protocol: carrier solution: 1% normal horse serum (NHS, Vector Laboratories) with 0.5% Triton (ThermoFisher) in phosphate buffered saline (PBS, Thermofisher), blocking solution: 10% NHS with 0.5% Triton in PBS. After several rounds of PBS washes, slices were blocked for two hours at room temperature and incubated in primary antibody to carrier solution (goat anti-calretinin, 1:1000, Swant; rabbit anti-NeuN, 1:3000, Abcam; rabbit anti-GABA, 1:500, SigmaAldrich) overnight at 4°C. Slices were washed again in PBS and incubated for 2 h at room temperature in secondary antibody in carrier solution (donkey anti-goat 647, 1:1000, Thermofisher; donkey anti-rabbit 405, 1:1000, Abcam; donkey anti-rabbit 555, 1:1000, Thermofisher). After four final washes, slices were mounted on slides and cover slipped using 22 × 50 mm, 0.16–0.18 mm thick cover glasses (FisherScientific). Images were acquired with a LSM710 confocal microscope (Zeiss) and stitched with Zen 2.1 (black, Zeiss). Confocal images were post processed with ImageJ (Version: 2.0). Cells were manually counted using the cell counter plugin (https://imagej.nih.gov/ij/plugins/cell-counter.html) for ImageJ.

**Statistical analysis.** Statistical analysis was performed using Matlab (Mathworks) and Prism 8 (Graphpad). Unless otherwise indicated, normal distribution of the data was not assumed and non-parametric tests were performed. Values are pre- sented as mean ± SEM unless stated otherwise. Box and whisker plots indicate median, interquartile range as well as the minimum to maximum value of the distribution. Statistical tests are mentioned in the text or figure legends. * , ** , *** indicate p values smaller than 0.05, 0.01 and 0.001, respectively.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** The source data underlying the main figures and Supplementary information are available as Source Data file.

**Code availability** Custom code used for analysis in this manuscript is available upon request.

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