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Abstract

For neuronal circuits in the brain to mature, necessary synapses must be maintained and redundant synapses eliminated through experience-dependent mechanisms. However, the functional differentiation of these synapse types during the refinement process remains elusive. Here, we addressed this issue by distinct labeling and direct recordings of presynaptic terminals fated for survival and for elimination in the somatosensory thalamus. At surviving terminals, the number of total releasable vesicles was first enlarged, and then calcium channels and fast-releasing synaptic vesicles were tightly coupled in an experience-dependent manner. By contrast, transmitter release mechanisms did not mature at terminals fated for elimination, irrespective of sensory experience. Nonetheless, terminals fated for survival and for elimination both exhibited developmental shortening of action potential waveforms that was experience independent. Thus, we dissected experience-dependent and -independent developmental maturation processes of surviving and eliminated presynaptic terminals during neuronal circuit refinement.

Significance statement

During neuronal circuit development, there is a surplus of synapses that undergo selective strengthening or elimination via experience-dependent mechanisms. However, the functional differentiation of presynaptic terminals during the refinement process remains elusive. To address this issue, we performed direct electrophysiological recordings selectively from presynaptic terminals fated for survival or elimination in the somatosensory thalamus during neural circuit refinement. With development, the transmitter release mechanisms matured in an experience-dependent manner at surviving presynaptic terminals but not at those that were later eliminated. However, the maturation of action potential waveforms was indistinguishable between surviving and eliminated terminals. Thus, we clarified a distinct functional development of presynaptic terminals fated for survival and for elimination during neuronal circuit refinement.

Introduction

The developmental maturation of neural circuits involves the initial formation of a surplus of synapses, followed by selective survival of strengthened synapses and elimination of others. This selective pruning occurs throughout the brain and is considered a fundamental event for the maturation of neuronal networks. Extensive studies have been performed to understand the underlying mechanisms (1, 2), and several model systems in the developing brain have been proposed (3-6). Such studies have shown that this refinement process is remarkably sensitive to sensory experience during postnatal development (5, 7), indicating that the strengthening of winner synapses and elimination of loser synapses occurs in an experience-dependent manner. However, it is not known if the transmitter release mechanism, crucial for synaptic transmission, develops differently at presynaptic terminals that are fated for survival and at those fated for elimination. Furthermore, how sensory experience affects presynaptic functional development is not clear because direct observations are lacking.

To examine transmitter release kinetics, the most direct methods are capacitance measurements of presynaptic terminals or paired recordings from a presynaptic terminal and postsynaptic cell (8, 9). However, these require patch-clamping directly on presynaptic terminals, which is often not possible because of their small size at most central nervous system synapses. These methods have been applied to large presynaptic structures (10-13), but presynaptic functional changes before circuit maturation have only be explored at the calyx of Held presynaptic terminal (14-20), and the transmitter release properties from terminals fated for elimination remain mostly unknown.

To address these issues, we focused on the rodent whisker sensory pathway, in which sensory fiber synapses onto excitatory thalamocortical neurons of the somatosensory

thalamus have been used to study experience-dependent circuit development. In rodents, whisker-mediated sensory information is transmitted via glutamatergic afferents (lemniscal fibers) from the V2 region of the principal trigeminal nucleus (PrV2) to the somatosensory thalamus (the ventral posteromedial nucleus [VPM]). It has been shown that VPM neurons also receive ectopic innervation from fibers originating from non-PrV2 regions, but these synapses are eliminated during circuit maturation (7). The elimination of these ectopic connections is prevented by whisker deprivation (WD), indicating that circuit refinement in the VPM is experience dependent (7). Therefore, transmitter release mechanisms of terminals fated for survival or elimination during development can be examined independently by recording from PrV2-originating lemniscal fiber terminals (whisker-LFTs) or non-PrV2-origin lemniscal fiber terminals (ectopic-LFTs), respectively. Furthermore, experience-dependent modulations of transmitter release mechanisms can be easily examined by employing WD (7).

Here, we used genetic and viral approaches to fluorescently label whisker- and ectopic-LFTs. The labeling enabled us to perform direct patch-clamp recordings from small LFTs and measure capacitance. By also using paired recordings from an LFT and a target VPM neuron, we were able to extensively examine the kinetics of transmitter release at terminals fated for survival or for elimination. Our data show that the transmitter release kinetics at whisker-LFTs exhibited developmental and sensory experience-dependent maturation, whereas ectopic-LFTs remained immature and insensitive to sensory experience. Thus, we clarified the distinct functional developments between “surviving” and “to-be-pruned” presynaptic terminals during neural circuit refinement.

Results

Distinct and direct measurements of transmitter release kinetics from whisker- and ectopic-LFTs

To distinguish whisker- and ectopic-LFTs, we generated Krox20-Ai34D mice, in which whisker-LFTs were identified with tdTomato-tagged synaptophysin (21, 22). Krox20-Ai34D mice were generated by crossing a Krox20-Cre knock-in line and a Cre-dependent synaptophysin-tdTomato reporter line (Ai34D). Krox20, a transcription factor in the developing hindbrain, has a specific expression pattern in rhombomere 3, which corresponds to PrV2 (7). Therefore, by crossing Krox20-Cre and Ai34D lines, whisker-LFTs (i.e., PrV2-originating LFTs) were labeled with tdTomato fluorescence (Fig. 1*A*). The ectopic-LFTs (i.e., non-PrV2-originating LFTs) were selectively labeled by introducing "Cre-Off" AAV (AAV9-Ef1a-DO-ChETA-EYFP [23]) into the Krox20-Ai34D mouse brainstem principal trigeminal nucleus (Pr5), including non-whisker regions (24) (Fig. 1*A*). The Cre-off vector expresses the encoded protein (i.e., ChETA-EYFP) unless Cre-mediated recombination reverses the orientation of the transgene (23). Thus, whisker-LFTs were labeled with tdTomato, and ectopic-LFTs were labeled with EYFP (Fig. 1*B*). The fluorescent labeling enabled us not only to distinguish between whisker- and ectopic-LFTs but also to perform direct presynaptic patch-clamp recordings (Fig. 1*C*). In a 200–300 μm parasagittal thalamic acute slice, labeled LFTs were clearly fluorescent and visible for direct patch-clamp recordings (Fig. 1*C*).

Kinetics of transmitter release were examined by capacitance measurement (25) or by deconvolution of the excitatory postsynaptic current (EPSC) response (26, 27). In a capacitance measurement configuration, depolarizations of presynaptic LFTs induced clear calcium currents and capacitance jumps (Fig. 1*D*). The amplitudes of capacitance

123 jumps reflect the total increase of membrane surface caused by depolarization-induced
124 exocytosis of synaptic vesicles. Accordingly, the capacitance jumps were blocked by 500
125 nM tetanus toxin infusion, which blocks synaptic vesicle fusion (Fig. S1*A, B*). Because
126 membrane capacitance cannot be measured during depolarization, we assessed the time
127 course of exocytosis from postsynaptic EPSCs in addition to examine the time course of
128 exocytosis directly. Under the paired-recording configuration, VPM neuron EPSCs were
129 induced by presynaptic LFT depolarizations (Fig. 1*E, F*). From the recorded EPSCs, the
130 time course of exocytosis was calculated by deconvolution with asynchronous miniature
131 EPSCs (mEPSCs; postsynaptic responses to single synaptic vesicle release events, Fig.
132 S2*A*-*D*), which provided the vesicle release rate. This method was based on the
133 assumption that EPSCs are a linear summation of mEPSCs (27). Because the frequency
134 of the asynchronous mEPSCs was nearly 10-fold higher than that before the stimulus
135 (mean ± SEM: 3.2 ± 1.1 Hz vs. 31.4 ± 5 Hz, $p < 0.01$, see Fig. S2*A*, S3*A* for example
136 traces), we surmised that the asynchronous mEPSCs mostly originated from the recorded
137 LFTs. Integrating this release rate provides the cumulative number of vesicles released at
138 a given time after the stimulus (Fig. 1*F*) (26, 27). The capacitance jumps and the number
139 of released vesicles calculated from EPSC deconvolutions scaled linearly for
140 depolarizations of 2–100 ms (Fig. 1*G, H*; Fig. S3*A, B*).

141 By comparing the capacitance jumps and EPSC deconvolutions, we calculated the
142 connection ratio between single LFTs and target VPM neurons. Whereas capacitance
143 measurements sample the total release from the entire terminal, EPSC deconvolution
144 samples only a fraction of total release from the terminal that is directed toward the single
145 recorded VPM neuron. Therefore, quantitative comparison of the number of released
146 vesicles with both techniques results in an estimate of the number of postsynaptic VPM

147 neurons per LFT for each recording pair. Assuming a single vesicle capacitance of 0.1 fF
148 (which may vary from 0.07 to 0.1 fF depending on vesicle diameter [11, 28, 29]), the
149 comparison revealed an average of 1.13 VPM neurons per LFT, indicating that a single
150 LFT targeted a single VPM neuron in most cases.

151 These results validated estimations of transmitter release both by capacitance
152 measurements and by EPSC deconvolutions under our experimental conditions.
153 Therefore, the ability to make direct measurements of transmitter release from single
154 LFTs enabled us to examine functional differences between whisker- and ectopic-LFTs.

155

156 **Developmental and experience-dependent changes of transmitter release kinetics**
157 **occur at whisker-LFTs but not at ectopic-LFTs**

158 The lemniscal fibers show unique developmental innervations, and three
159 developmental phases have been identified (Fig. 2*A*) (7). In rodents, the 1st week after
160 birth is a "synapse formation" phase, when a VPM neuron is weakly innervated by several
161 lemniscal fibers. The 2nd week is a "functional differentiation" phase, when connections
162 from several fibers are first strengthened in parallel, and then the number of innervated
163 fibers is reduced thereafter. Individual VPM neurons receive intermingled innervations
164 from whisker [(Krox20(+)]) and ectopic [Krox20(−)]) fibers until the end of the 2nd week.
165 The 3rd week is a "mature" phase, when a VPM neuron is strongly innervated by a single
166 whisker [Krox20(+)]) fiber, and ectopic [Krox20(−)] fibers are mostly eliminated. With
167 WD (whisker sensory deprivation from postnatal day 12 [P12]–13 to the recording day
168 after P16), multiple fiber innervations of a single VPM neuron and ectopic fiber terminals
169 remain in the "mature" phase (Fig. 2*A*) (7, 30). Therefore, the whisker sensory inputs
170 during the functional differentiation phase drive the selective strengthening of whisker

171 [Krox20(+)] fibers and elimination of ectopic [Krox20(−)]) fibers.

172 Pathway-specific developmental and experience-dependent changes of the transmitter
173 release kinetics were examined by capacitance measurements (Fig. 2*B, C*). To examine
174 the characteristics of the readily releasable pool (RRP) of synaptic vesicles, capacitance
175 jumps were recorded in response to various durations of depolarization. We defined the
176 size of the RRP as the maximum amount of capacitance jump evoked by depolarizations,
177 which is also termed RRP_{depol} or total releasable pool (31). The capacitance jumps
178 correlated positively with basal capacitances (e.g., size), but the basal capacitances varied
179 up to ~7-fold (0.55 to 4.1 pF; Fig. S4*A*). This variability compromised statistical
180 comparisons between different experimental conditions. Therefore, we normalized the
181 capacitance jumps to the basal capacitances. At whisker-LFTs, capacitance jumps at P4–6
182 were very small, with maximum capacitance jumps (reflecting the total RRP size)
183 estimated to be ~4 fF/pF (Fig. 2*B, D, E*). This is consistent with previous studies
184 demonstrating that VPM neuron EPSCs induced by lemniscal fiber stimulations were
185 weak before P6 (4, 7). The total RRP sizes at whisker-LFTs enlarged thereafter and, at
186 P8–14, were already comparable to those of "mature" P16–25 terminals (Fig. 2*B, D, E*).
187 The total RRP size per 1 pF was ~30 fF (Fig. 2*D, E*). Considering the capacitance jump
188 per single vesicle fusion of 0.1 fF (28), this corresponds to a total RRP size of ~300
189 vesicles per 1 pF. On the other hand, capacitance jumps of P8–14 ectopic-LFTs were very
190 small, similar to P4–6 whisker-LFTs (Fig. 2*C, F, G*). The results indicate that the
191 transmitter release machinery did not develop further at ectopic-LFTs.

192 Between P8–14 (differentiation phase) and P16–25 (mature phase), the transmitter
193 release kinetics of ectopic-LFTs remained essentially the same, but that of whisker-LFTs
194 further matured. The capacitance jumps after shorter (10 ms or less) depolarizations were

195 significantly larger at P16–25 than under other conditions, indicating a development of
196 the fast component of the RRP, which was released with a few-millisecond time constant
197 (Fig. 2*B, D, E*) (28). At P16–25 whisker-LFTs, the release time course was fitted by a
198 double exponential, with τ = 1.2 and 54 ms, unlike at P4–6 or at P8–14, when the release
199 time courses were fitted by single exponentials, with τ = 28.8 ms and 44.9 ms,
200 respectively (Fig. 2*D*). On the other hand, the enlargement of the capacitance jumps was
201 not significant when the capacitance jumps were not normalized to the basal capacitances
202 (Fig. S4*B*). The enlargement of the fast component of transmitter release was verified via
203 paired recordings (see Fig. 4). The development of the fast component of the RRP was
204 accompanied by enhanced vesicle recycling (Fig. S5*A, B*). The release time courses of
205 the mature whisker-LFTs were comparable to those of other glutamatergic presynaptic
206 terminals (11, 13, 32).
207 The developmental changes of the presynaptic transmitter release properties were
208 inconsistent with a previous report showing that the paired-pulse ratios (PPRs) of EPSCs
209 evoked by external single-fiber stimuli did not change from P7 to P24 (4). However, we
210 found that the PPR underwent clear developmental changes (Fig. S5*C-E*) by investigating
211 PPRs with various interstimulus intervals under our experimental conditions. When we
212 blocked AMPA receptor desensitization of AMPA receptors by applying cyclothiazide and
213 emphasized paired-pulse depletion by raising the external Ca^{2+} concentration from 1.5
214 mM (4) to 3 mM, we detected a significant difference in PPRs at the different
215 developmental phases (Fig. S5*C-E*). The strong effect of desensitization on AMPA EPSC
216 responses and the resultant changes of PPRs, especially at shorter inter stimulus intervals,
217 were similar to what has been reported for synapses in the lateral geniculate nucleus
218 synapse (33). Altogether, the results suggest that the RRP for the fast component is

developmentally enlarged and accompanied by enhanced vesicle recycling.

Development of the fast component of transmitter release at whisker-LFTs was prevented by WD, indicating that sensory experience is necessary for this process (Fig. 2*B, D, E*). On the other hand, capacitance jumps for ectopic-LFTs were not affected by WD (Fig. 2*C, F, G*), strongly suggesting that these synapses were not strengthened via inhibition of sensory experience dependent activity at whisker-LFTs. The results indicate that the enlargement of the fast component of transmitter release at whisker-LFTs is whisker-mediated sensory experience dependent, whereas the development of the transmitter release machinery at ectopic-LFTs is insensitive to sensory experience.

Developmental changes of action potential waveforms are not pathway specific

Are the pathway- and experience-dependent modifications specific to the transmitter release kinetics or do they affect other presynaptic properties as well? To answer this question, we examined presynaptic terminal sizes, calcium currents, and action potential (AP) waveforms. We focused on these parameters because they are closely related to transmitter release kinetics. Terminal size correlates with synaptic strength in some synapses (7, 34), and calcium currents and AP waveforms strongly affect transmitter release, as they are directly related to the kinetics of the calcium influx that triggers exocytosis.

Terminal sizes were evaluated by basal membrane capacitance, and calcium currents (assessed as calcium current density) were evoked by applying depolarization to 0 mV. The basal membrane capacitance and calcium current density of whisker-LFTs increased from P4–6 to P8–14 (mean ± SEM: basal capacitance, from 1.59 ± 1.1 pF to 2.28 ± 1.4 pF; calcium current density, from 10.2 ± 1.2 pA/pF to 23.3 ± 2.4 pA/pF, respectively), but

243 those of ectopic-LFTs showed no developmental changes (Fig. 3*A-D*). Basal capacitance
244 and calcium current density of ectopic-LFTs were similar to those of P4–6 whisker-LFTs
245 throughout the development phases (Fig. 3*B, D*). The calcium current amplitudes were
246 consistent with the current densities (Fig. S6*A, B*). These results suggest that at ectopic-
247 LFTs, not only the transmitter release kinetics but also terminal sizes and calcium currents
248 remain immature during development. The results also suggest that the enlargement of
249 the fast component of RRP between P8–14 and P16–25 was not caused by a change of
250 calcium influx kinetics, as current density as well as the rise time of calcium currents
251 remained unchanged (mean ± SEM: 0.79 ± 0.13 ms and 0.65 ± 0.12 ms at P8–14 and
252 P16–25, respectively, $p = 0.44$).
253 APs were evoked with 1 ms current injections under current clamp mode. AP
254 waveforms at other synapses become shorter with development (14, 16, 35). LFTs are no
255 exception, as they also exhibited developmental shortening. In contrast to the terminal
256 sizes and calcium currents, the time course of developmental shortening of AP waveforms
257 was not different between whisker- and ectopic-LFTs (Fig. 3*E-G*). With development, the
258 full width at half maximum (FWHM) of APs gradually became shorter, reaching ~0.2 ms
259 at maturation (Fig. 3*G*). On the other hand, peak AP amplitudes increased at P8–14, unlike
260 peak AP amplitudes at the calyx of Held, which remain constant throughout development
261 (14, 15). This suggests a delayed upregulation of K^+ conductance compared with that of
262 Na^+ conductance. The developmental change in peak AP amplitudes occurred in both
263 whisker- and ectopic-LFTs (Fig. S6*C-E*). To our surprise, WD manipulation did not affect
264 the terminal size (Fig. 3*A*), calcium current (Fig. 3*C*), or AP waveform (Fig. 3*E, G*; Fig.
265 S6*C, E*), suggesting that these properties are experience independent during development.
266

Fast component of transmitter release is advantageous for AP-evoked transmitter release

The results so far indicate that the prominent developmental change of whisker-LFTs between P8–14 and P16–25 is the enhancement of the fast component of transmitter release. To examine this directly and with sub-millisecond time resolution, we performed EPSC deconvolution analysis against EPSCs evoked by whisker-LFT depolarization under a paired-recording configuration. The calculated release time courses were fitted by double exponentials, which correspond to the fast and slow components of transmitter release, clearly revealing the enhancement of the fast component of transmitter release at whisker-LFTs at P16–25 compared with that at P8–14 (mean fraction ± SEM: 0.16 ± 0.03 vs. 0.07 ± 0.02, respectively, $p = 0.02$; Fig. 4*A, B*). This enhancement was abolished under the WD condition (mean fraction ± SEM: [P16–25] 0.16 ± 0.03 vs. 0.06 ± 0.01 for WD, $p = 0.01$; Fig. 4*A, B*), consistent with the results of capacitance jumps normalized to basal capacitance (Fig. 2*B, D, E*). The time courses of the fast (several milliseconds) and slow (tens of milliseconds) components of transmitter release were similar at P8–14, P16–25, and P16–25 with WD (Fig. S7*A, B*). Because the fast component of transmitter release is thought to be responsible for the synchronized transmitter release induced by APs (28, 36), we examined the number of vesicles released in response to AP-like stimulation (1 ms depolarization to +40 mV). The AP-like stimulation evoked the release of more vesicles at P16–25 than at P8–14 or under the WD condition (Fig. 4*C, D*). Furthermore, we calculated the release probability evoked by the AP-like stimulation (P_{rAP}) by comparing the AP-like stimulation-evoked release number against the fast component size or the total RRP size. P_{rAP} per the fast component at P16–25 was somewhat larger than at P8–14 and under the WD condition, but these had large amounts of variability

caused by the small sizes of the fast component (Fig. S8*A*). However, P_{rAP} per RRP was significantly larger at P16–25 than at P8–14 or under WD conditions (Fig. S8*B*). Thus, a larger fraction of the RRP was triggered for exocytosis by an AP-like stimulation at P16–25 than at P8–14 or under WD conditions, most likely a result of the larger fast component. These results strongly suggest that the larger fast component is advantageous for AP-evoked transmitter release *in vivo*.

Fast component of transmitter release is mediated by vesicles tightly coupled to calcium channels

In general, transmitter release from the presynaptic terminal is triggered by calcium influx through voltage-gated calcium channels (VGCCs). Thus, the development of the fast component of transmitter release may reflect enhanced coupling of VGCCs to fast-releasing vesicles (18). The coupling distance between VGCCs and releasable vesicles can be examined by investigating the sensitivity to a calcium chelator, e.g., EGTA, as a large amount of intracellular EGTA predominantly blocks exocytosis of loosely coupled releasable vesicles (9, 37). Therefore, the spatial coupling properties of VGCCs and releasable vesicles can be examined by measuring the capacitance jump blockade induced by EGTA dialysis.

We increased the intracellular concentration of EGTA from 0.1 mM to 5 mM and examined the time course of transmitter release under each condition by capacitance measurements. The larger amount of EGTA strongly reduced maximum capacitance jumps of whisker-LFTs at P8–14, at P16–25, and under WD conditions (Fig. 5*A, B*), but the amount of blockade against shorter depolarizations was significantly smaller at P16–25 whisker-LFTs (Fig. 5*A*-*C*). The results indicate that the enlargement of the fast

315 component of transmitter release was caused by tighter coupling of VGCCs to fast-
316 releasing vesicles. This process occurred between P8–14 and P16–25 (i.e., between
317 functional differentiation and mature phases) in an experience-dependent manner.
318

Discussion

In this study, we directly examine the transmitter release kinetics from presynaptic terminals fated for survival or for elimination during development in the mammalian central nervous system. The unique features of LFT-VPM neuron synapses provide (i) direct accessibility of presynaptic terminals with the patch-clamp technique, (ii) clear distinction of terminals that will survive and those to be eliminated during development, and (iii) controllability of pathway-specific whisker sensory-mediated inputs for surviving presynaptic terminals. We show that transmitter release mechanisms develop at these so-called whisker-LFTs but not at ectopic-LFTs that are fated for elimination. Deprivation of sensory experience prevents the maturation of transmitter release machinery at whisker-LFTs but does not promote the development of the transmitter release machinery at ectopic-LFTs. In contrast to the transmitter release kinetics, the developmental shortening of AP waveforms is comparable between whisker- and ectopic-LFTs and shows no experience dependence. Taken together, we propose that sensory experience affects synapse development independently at whisker- and ectopic-LFTs of the somatosensory thalamus. At whisker-LFTs, sensory experience drives the development of specific elements of transmitter release machinery. By contrast, sensory experience does not affect functional differentiation at ectopic-LFTs but rather promotes structurally pruning (7).

A large pool of releasable vesicles and fast component at whisker-LFTs

Our results indicate the presence of a very large pool of releasable vesicles at whisker-LFTs. The RRP per pF (300 vesicles) of the mature whisker-LFTs is within the range of previously reported sizes at other large presynaptic terminals, such as calyx of Held

terminals (35 to 400 vesicles/pF [15, 38-40]), mossy fiber boutons of hippocampus (1,000 vesicles/pF [11]), and mossy fiber boutons of the cerebellum (250 to 500 vesicles/pF [12, 31]). Given that our recordings are biased to large and isolated terminals and that the basal membrane capacitances (~2 pF) are similar to those of hippocampal mossy fiber boutons (41), our data represent LFTs of large size (~4 μm diameter [42, 43]). The number of active zones within an average-sized LFT with a 2 μm diameter is estimated to be three in mature mice (42). Thus, the number of active zones within a single LFT in our recordings is estimated to be ~10. The fraction of the fast component of transmitter release calculated from EPSC deconvolution was ~0.15 (Fig. 4), which corresponds to 4.5 fF/pF assuming a total RRP size of 30 fF/pF (Fig. 2). Again, by scaling this value for a basal membrane capacitance of 2 pF, the size of the fast component is ~9 fF, or ~90 vesicles per terminal. Thus, each active zone contains ~9 fast-releasing vesicles. The number is higher than reported for large presynaptic terminals (11, 13) but matches well with the number of docked vesicles within a single active zone at a small conventional-sized excitatory glutamatergic synapse (44, 45).

The total RRP size of mature whisker-LFTs was 60 fF (30 fF/pf × 2 pF), or ~600 vesicles, which translates to ~60 vesicles per active zone. The number is much higher than that for other depressive large presynaptic terminals, such as the calyx of Held (3–6 [46]) or cerebellar mossy fiber boutons (3 [12]), and is even higher than that for facilitative hippocampal mossy fiber boutons (30 [11]). The large RRP and depressive property of the LFTs suggests a slow but massive amount of replenishment. We note that the total RRP size estimated from the capacitance jump evoked by a long depolarization in this study may include replenished vesicles during the stimulus; thus the number could be an overestimation.

Developmental maturation of release machinery at whisker-LFTs

Synapses change their morphological and functional properties during their development from immature to mature states, and newly formed immature synapses often possess functional properties distinct from those of mature synapses (47). Unlike postsynaptic developmental maturation that has been relatively well documented, the small sizes of presynaptic terminals at most synapses in the central nervous system have prevented direct measurements of their developmental maturation. The giant terminal of the calyx of Held synapse is an exception, and the developmental changes of their presynaptic properties have been explored extensively (14-20). Those studies indicated that the calyx of Held terminal becomes functionally mature just before the onset of hearing (~P14 in rodents); thus the transmitter release mechanisms mature independently of sound experience (48). Studies of lemniscal fiber-VPM neuron synapses have indicated that the synaptic wiring matures at around P16 (4, 7), and our study found that the release machinery of whisker-LFTs matures at around the same age. However, the whisker sensory pathway is already active well before this timepoint, as a passive whisker deflection is thought to be crucial for suckling at a few days after birth (49), and active whisking begins at P14 (50). The different timings of synaptic maturation compared with sensory onset are of interest. Because the enlargement of the fast component of transmitter release occurs just after active whisking begins, and WD prevents this enlargement, active whisking may be crucial for functional maturation. Despite our finding that the maturation of the presynaptic release properties and synaptic wiring are highly correlated, causation cannot currently be established and remains to be elucidated. It is interesting to note that the development of the transmitter release mechanism is

391 pathway and experience dependent, whereas developmental shortening of AP waveforms
392 is pathway and experience independent. The results suggest that the construction of
393 release units by active zone scaffolds (e.g., neurexins, RIMs, and Munc13 [51, 52]), but
394 not AP waveform regulating ion channels (e.g., N^+ and K^+ channels [35, 53]), is pathway
395 and experience dependent, and that VGCCs are recruited to these release units for tight
396 couplings between VGCCs and release sites (54, 55). Alternatively, it is also possible that
397 the extreme changes in the calcium sensitivity of the vesicles for exocytosis may underlie
398 the enlargement of the fast component of the RRP.
399
400 **Developmental changes of release machinery at ectopic-LFTs**
401 The elimination of early-formed unnecessary synapses is crucial for the formation of
402 mature functional neural circuits. However, presynaptic properties of the to-be-pruned
403 terminals remain unknown, as recordings from presynaptic terminals that are known to
404 be eliminated after maturation were impossible. In this study, we selectively recorded
405 presynaptic terminals fated for elimination by taking advantage of genetic and viral
406 labeling. Our data clearly indicate that the RRPs of these terminals remain small and never
407 enlarge (Fig. 2). The calcium currents and the basal membrane capacitances (i.e., terminal
408 sizes) also remain small, which suggests that the release properties of ectopic-LFTs
409 remain immature throughout development. Given that WD (i.e., inhibition of whisker-
410 LFTs) does not promote the strengthening of ectopic-LFTs, it is suggested that sensory
411 experience-dependent functional developments of “winner” fibers and “loser” fibers do
412 not interfere with each other. On the other hand, the elimination of loser fiber synapses is
413 prevented by WD (7), indicating that the structural pruning is an experience-dependent
414 process.

In contrast to the RRP size, the AP waveform at ectopic-LFTs becomes shorter with development similarly to whisker-LFTs. The shorter presynaptic APs are usually associated with smaller EPSCs because of the decreased release probability as a result of shorter calcium influx (18, 56). Therefore, our results suggest that the synaptic transmission from ectopic-LFTs *in vivo* becomes gradually unreliable with development as a result of the progressive shortening of APs without RRP enlargement, unlike at whisker-LFTs, where RRP enlargement balances the decrease of release probability caused by AP shortenings, as reported at calyx of Held synapses (57). Given the previous findings that a VPM neuron is innervated by a single whisker fiber after P16 (7), AP-evoked transmitter release from ectopic-LFTs is not capable of evoking a postsynaptic response and/or the corresponding postsynaptic structure is lost.

At the climbing fiber-to-Purkinje cell synapse, the sizes of postsynaptic densities formed by weak (putative "loser") fibers are indistinguishable from those formed by strong (putative "winner") fibers in the functional differentiation phase (58). On the other hand, at the immature calyx of Held synapse, the synaptic contact area of the large (putative "winner") terminals is clearly larger than that of the small (putative "loser") terminals (59). Our results indicate that at LFT-VPM neuron synapses, transmitter release kinetics are clearly distinguishable between whisker- and ectopic-LFTs in the functional differentiation phase, and WD does not affect the transmitter release from ectopic-LFTs (Fig. 2). This is consistent with our previous morphological finding that ectopic-LFTs are smaller than whisker-LFTs, and WD did not promote enlargement of the ectopic-LFTs (7). It will be interesting to examine the morphological difference between whisker- and ectopic-LFTs in more detail (e.g., distribution of synaptic vesicles) in the future.

It should be noted that technical limitations biased our recordings to large terminals;

thus small terminals were essentially ignored in this study. We normalized the responses to the basal capacitance to mitigate this, but we cannot rule out the possibility that small responses of ectopic-LFTs, nevertheless, might be overestimated, and that the difference between whisker- and ectopic-LFTs may be even larger. We should also mention the lack of Krox20 at ectopic-LFTs. Because Krox20 is a transcription factor with many targets, many parameters other than pool maturation could be different between Krox20(+) whisker-LFTs and Krox20(−) ectopic-LFTs.

Comparison with remodeling of other neuronal circuits

Developmental synapse refinement has been extensively studied in several regions in the brain (3-6), particularly, cerebellar climbing fiber-to-Purkinje cell synapses and retinogeniculate synapses in the lateral geniculate nucleus. In these areas, the presynaptic functional differences between strong and weak fibers (60, 61), or undeveloped and developed fibers (33), have been proposed based on recordings of postsynaptic responses. The studies suggest that at strong or developed presynaptic terminals, the number of released vesicles/AP is increased, and the AP-induced transmitter release is more synchronized. Our results directly demonstrate that the development of the fast component of transmitter release is the underlying mechanism.

In the lateral geniculate nucleus, changes to the terminal (or bouton) clustering (but not axon retraction) contribute to synapse refinement during the functional differentiation phase. We previously showed that a proportion of ectopic-LFTs (~13% of the total) structurally remain in the VPM after single-fiber innervation is established (i.e., after synaptic connections mediated by ectopic-LFTs are eliminated [7]). In this study, we confirmed this finding, with ectopic-LFTs remaining at P16–25, indicating that fibers

463 with inputs that are fated for elimination remain in the VPM. Together with our previous
464 finding that the number of input fibers can increase after circuit maturation via peripheral
465 afferent nerve cutting (7), it is likely that synapse elimination precedes axon retraction in
466 the VPM as well as in the lateral geniculate nucleus. It is interesting to note that the
467 number of presynaptic terminals formed by the afferent fiber with the strongest input in
468 a mature circuit (~60) is similar between retinogeniculate synapses and LFT-VPM neuron
469 synapses (62, 63). It is not clear whether similar changes to terminal (or bouton) clustering
470 occur and contribute to climbing fiber-to-Purkinje cell synapse refinement in the
471 developing cerebellum.
472 To gain a general view of neuronal circuit formation, it is crucial to investigate the
473 similarities and differences between the different models of developmental synapse
474 refinement. Our findings shed new light on this and on the synapse elimination theory,
475 which may help to reveal a common principle of neuronal circuit formation.
476

Methods

Experimental animals

All experiments were approved by the Animal Care and Use Committee of the Tokyo Women's Medical University, and were performed according to the institutional guidelines concerning the care and handling of experimental animals. Krox20-Cre mice (JAX#025744 [21]) were backcrossed to C57BL/6 mice for more than three generations before use. Cre-dependent synaptophysin-tdTomato reporter mice (Ai34D) [B6;129S-Gt(ROSA)26Sortm34.1(CAG-Syp/tdTomato)Hze/J; JAX#012570] (22) were obtained from the Jackson Laboratory (Bar Harbor, ME). To visualize PrV2-originating lemniscal fibers, male Krox20-Cre heterozygotes and female Ai34D homozygotes or heterozygotes were crossed, and Cre^+ pups (Krox20-Ai34D mice) were used. Krox20-Ai34D pups were detected by tdTomato signals in vibrissal follicles expressed beginning at late pregnancy. Male and female mice (P4–25) were used. All efforts were taken to minimize animal numbers.

Preparation of acute thalamic slices

Mice were deeply anesthetized with isoflurane (Abbott) and decapitated, and brains were removed in accordance with the guidelines of the Physiological Society of Japan. Parasagittal thalamic slices 200–300 μm thick were obtained using a Leica VT1200S microslicer (Leica Microsystems) in ice-cold slice medium containing the following (in mM): 234 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 10 $MgCl_2$, 0.5 $CaCl_2$, 25 $NaHCO_3$, 0.5 myoinositol, and 11 glucose equilibrated with 95% O_2 and 5% CO_2. Slices were then incubated at 32°C for >0.5 h in an artificial cerebrospinal fluid (ACSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 26 $NaHCO_3$,

20 glucose (pH 7.4, gassed with 95% O_2 and 5% CO_2). Slices were visualized with IR-DIC on an upright microscope (BX-51, Olympus) in a recording chamber filled with ACSF. A slice in a recording chamber was perfused with 30–32°C ACSF at a rate of 2.5–3.0 ml/min. PrV2-originating LFTs of Krox20-Ai34D mice were identified by 565 nm LED (Thorlabs) excitation. Non-PrV2-originating LFTs of AAV9-Ef1a-DO-ChETA-EYFP-WPRE-pA-injected Krox20-Ai34D mice were identified by 470 nm LED (Thorlabs) excitation. Both IR-DIC and the fluorescence of tdTomato or EYFP were imaged with an sCMOS camera (Zyla; Andor), controlled by SOLIS software (Andor). During terminal recordings with capacitance measurements, 1 μM TTX and 10 mM TEA-Cl were added to block Na^+ and K^+ channels. For paired recordings, 50 μM AP5 and 100 μM cyclothiazide were included to block NMDA receptors and the desensitization of AMPA receptors, respectively. For terminal recordings with AP measurements, all blockers were removed. Recordings were performed within 6 h after cutting the slices. For EPSC recordings with external stimulation, 10 μM (−)-(-)-bicuculline methochloride, 50 μM AP5, and 100 μM cyclothiazide were added to block GABA receptors, NMDA receptors, and the desensitization of AMPA receptors, respectively.

Whole-cell recordings

LFTs were whole-cell voltage clamped at −70 mV using an EPC10/2 amplifier (HEKA, Germany) controlled by PatchMaster software (HEKA). The patch pipettes were filled with intracellular solution containing the following (in mM): 135 Cs-gluconate, 20 TEA-Cl, 10 HEPES, 5 Na_2-phosphocreatine, 4 MgATP, 0.3 GTP, 5 QX-314, and 0.1 EGTA (pH 7.2). In some experiments, EGTA was raised to 5 mM. Membrane currents were low-pass filtered at 3 kHz and sampled at 20–100 kHz. Leak currents were subtracted using

525 appropriately scaled responses against small voltage steps (usually 20% of the test step)
526 to measure the calcium currents. Data were analyzed off-line with IgorPro software
527 (Wavemetrics). Membrane capacitance measurements were performed using an EPC 10/2
528 amplifier in the sine + DC configuration. A sine wave (30 mV in amplitude, 1,000 Hz in
529 frequency) was superimposed on a holding potential of −80 mV. To evoke exocytosis,
530 depolarizing pulses to 0 mV were applied. To limit the membrane capacitance (C_m)
531 change to single presynaptic terminal, recordings were made from isolated terminals with
532 few neighboring varicosities. Patch pipettes (Harvard, GC150F-10) typically had a
533 resistance of ~ 3 MΩ, and the average series resistance was ~50 MΩ, which was similar
534 to previously reported values for small presynaptic recordings (41). We stopped
535 recordings when the leak currents exceeded 100 pA at resting potential. Depolarizing
536 pulses were applied every minute to allow for complete recovery of responses from
537 depression. For AP recordings, Cs-gluconate and TEA-Cl were replaced by K-gluconate,
538 and QX-314 was omitted. APs were induced by 1 ms current injections.
539 VPM neurons were whole-cell clamped at −70 mV with a patch pipette (Harvard,
540 GC150-10) with a resistance of ~4 MΩ. Average series resistance was <15 MΩ, which
541 was compensated so that residual resistance was ~3 MΩ. For VPM neuron recordings,
542 TEA-Cl was removed, and EGTA was raised to 1 mM in the intracellular solution. To
543 evoke lemniscal fiber-mediated EPSCs, a concentric bipolar electrode (tip diameter, 25
544 μm) (IMB-16820; InterMedical) was used. The stimulus electrode was placed on the
545 medial lemniscal fiber bundle (4, 7), and a 100 μs electrical square pulse was applied. We
546 ensured that recorded EPSCs were from lemniscal fibers, not from corticothalamic fibers,
547 according to established criteria (7): depression of EPSCs in response to paired-pulse
548 stimuli and all-or-none responses with distinct thresholds in response to increasing

549 stimulus intensity. Lemniscal fiber-mediated EPSCs displayed the paired-pulse
550 depression throughout development in the absence of cyclothiazide (4).
551
552 **Whisker deprivation**
553 All unilateral mystacial vibrissae on the snout were deprived every other day from P12–
554 13 to the slice recording day, ranging from P16 to P25. Under a dissecting microscope,
555 vibrissae of isoflurane-anesthetized mice were carefully plucked out using fine tweezers
556 by applying slow and steady tension to the base of the vibrissa until the vibrissa slipped
557 out of the follicle (24).
558
559 **Viral vector construction and stereotaxic surgery**
560 To express fluorescent proteins selectively in Cre^- non-PrV2-originating lemniscal fibers,
561 we used the viral vector AAV9-Ef1a-DO-ChETA-EYFP-WPRE-pA (23). AAV9 viral
562 particles were kindly packaged and purified by the Gunma University Initiative for
563 Advanced Research Viral Vector Core. The genomic titer of the purified AAV9 viral
564 particles as determined by real-time PCR was 1.65×10^{13} vector genomes/ml. A standard
565 stereotaxic injection surgery (24) was made under isoflurane (3% for induction, 2% for
566 maintenance) anesthesia and local lidocaine (2%) analgesia. In brief, the head of a P3–7
567 mouse pup was fixed on a stereotaxic apparatus, and a dorsal midline skin incision was
568 made to align the bregma and lambda points. A small hole was made in the skull above
569 the left occipital cortex with a HP1/4 carbide bar (Midwest) attached to a hand grinder
570 (V112J; Minitor), and 1 μl AAV9 vector was injected into the left Pr5 (4.2–4.3 mm
571 posterior and 1.7–1.8 mm lateral from the bregma, 3.7–3.8 mm below the dura) through
572 a microneedle syringe (NF35BV and NANOFIL; WPI) by continuous pressure from a

573 microsyringe pump (Micro4; WPI). After injection, the needle tip was held in the Pr5 for
574 5 min. After the injection, the wound was stitched with nylon sutures, and mice were
575 returned to their mothers for recovery. Slice experiments were conducted 5–18 days after
576 injection. Because it was necessary to wait more than 5 days for sufficient expression of
577 the fluorescent protein (EYFP), the recordings from the ectopic-LFTs were performed
578 only after P8.
579
580 **Immunohistochemistry**
581 Brainstem regions of the AAV9-Ef1a-DO-ChETA-EYFP-WPRE-pA-injected Krox20-
582 Ai34D mice used to prepare the thalamic slices were fixed with 4% paraformaldehyde
583 and 0.2% picric acid in 0.1 M phosphate-buffered solution at least overnight and then
584 coronally cut into 100 μm sections using a vibratome (Leica VT1000; Leica
585 Microsystems, Germany). Sections were incubated overnight at 4°C with a rat
586 monoclonal antibody against GFP (Nacalai Tesque; 1:1,000) and a rabbit polyclonal
587 antibody against RFP (Rockland; 1:1,000) in 0.05 M phosphate-buffered saline (PBS)
588 containing 10% normal donkey serum and 0.3% Triton X-100. After washing in PBS, the
589 sections were reacted with secondary antibodies conjugated to Alexa Fluor 488 (for GFP)
590 (Abcam; 1:500) and Alexa Fluor 647 (for RFP) (Abcam; 1:500) for 2 h at room
591 temperature or overnight at 4°C. To quantify EYFP and tdTomato expression using an
592 epifluorescence upright microscope (BX-51; Olympus), sections were mounted on glass
593 slides, sealed with SlowFade Diamond antifade mountant (Thermo Fisher Scientific), and
594 coverslipped with spacers (50 μm depth × 3). Images were acquired using a 5× lens
595 objective and a sCMOS camera (Zyla; Andor), controlled by SOLIS software (Andor).
596

Analysis

Data were analyzed using IgorPro and Excel (Microsoft). Transmitter release rates were calculated by deconvolving EPSCs with the mEPSCs (26, 27). The mEPSC amplitudes were measured by detecting asynchronous release events following the stimulation. The EPSCs were assumed to be a linear summation of mEPSCs and have no residual currents due to delayed clearance of glutamate in the synaptic cleft (27). The calculated release rates were validated by comparing the cumulative release with capacitance jumps.

Quantification and statistical analysis

Means and standard errors were calculated in Igor or Excel. Data are presented as means ± SEMs. *p*-values were determined with two-tailed Student's *t*-test or ANOVA. Details, including sample sizes, can be found in figure legends.

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836 **Figure legend**

837

838 **Figure 1. Direct and selective recordings from whisker- and ectopic-LFTs enabled**
839 **analysis of pathway-specific transmitter release kinetics.**

840 (*A*) A schematic view of distinct labeling of PrV2-originating [Krox20(+), whisker] and
841 non-PrV2-originating [Krox20(–), ectopic] pathways. (*B*) Distinct labeling of Krox20-
842 Cre^{+} (i.e., PrV2 origin, whisker) and Krox20-Cre^{-} (i.e., non-PrV2 origin, ectopic)
843 pathways by injecting "Cre-off" AAV to Krox20-Ai34D mice. Scale bars: top, 20 μm;
844 bottom, 500 μm. The fluorescence signal for Pr5 in the lower-magnification image was
845 amplified by immunostaining. (*C*) An LFT imaged with DIC (left) and epifluorescence
846 (right). Scale bar = 10 μm. Bottom shows a schematic view of capacitance measurement
847 configuration. (*D*) Example traces of depolarization induced calcium current and C_m jump.
848 (*E*) A paired recording imaged with DIC (left) and epifluorescence (right). Scale bar = 10
849 μm. Bottom shows a schematic view of paired-recording configuration. (*F*) Example
850 traces of depolarization-induced EPSC from postsynaptic VPM neuron, deconvolved
851 release rate, and cumulative release. (*G*) Superimposed preterminal capacitance (C_m) and
852 EPSC deconvolution estimated cumulative release number (N_{rel}) from the same
853 recording: 2 ms (black) and 100 ms (red) depolarization-induced responses. (*H*) The
854 capacitance jumps against EPSC deconvolution-estimated number of vesicles released.
855 Dotted line indicates a 1:1 relation between LFT and VPM neuron based on single-vesicle
856 capacitance as 0.1 fF. n = 10 cells. Data for *D* to *H* were from whisker-LFTs.

857

858

859 **Figure 2. Readily releasable pool enlarges first, and the fast-releasable pool develops**
860 **thereafter at whisker-LFTs in an experience-dependent manner, but the readily**

releasable pool of ectopic-LFTs remains small.

(*A*) A schematic view of experimental conditions. Example capacitance jumps induced by 10 and 100 ms depolarizations in each condition at whisker-LFTs (*B*) and at ectopic-LFTs (*C*). (*D*) Averaged ΔC_m per pF values were plotted against pulse durations for whisker-LFTs under each condition. Enlarged traces are shown on the bottom. n = 8–14, 12–14, 8–18, and 18–21, for P4–6, P8–14, P16–25, and WD, respectively. P16–25 responses were significantly larger than those for the three other conditions at pulse durations of 2 ($p < 0.01$), 5 ($p < 0.01$),) and 10 ms ($p < 0.05$, one-way ANOVA followed by Tukey's *post hoc* test). For the pulse durations longer than 20 ms, there were no significant difference between P8–14, P16–25, and P16–25 (WD). P4–6 responses were significantly smaller than those for the three other conditions at all pulse durations ($p <$ 0.05, one-way ANOVA followed by Tukey's *post hoc* test) except for the 2 ms response against WD. (*E*) Averaged ΔCm per pF evoked by 10 ms (top) and 200 ms (bottom) depolarizing pulses. (*F*) Same as in (*D*) but for ectopic-LFTs. Enlarged traces are shown on the bottom. n = 10–11, 5–7, and 4–5 for P8–14, P16–25, and WD, respectively. There were no significant differences between the three conditions. (*G*) Same as in (*E*) but for ectopic-LFTs. *$p < 0.05$, **$p < 0.01$, one-way ANOVA followed by Tukey's *post hoc* test. Error bars show SEMs.

Figure 3. Maturation of terminal sizes and calcium currents, but not action potential waveforms, are pathway specific.

(*A*) Averaged basal capacitances under each condition at whisker-LFTs. n = 21, 21, 37, and 31 for P4–6, P8–14, P16–25, and WD, respectively. (*B*) Same as in (*A*) but at ectopic-LFTs. n = 11, 10, and10, 5 for P8–14, P16–25, and WD, respectively. (*C*) Example traces

of calcium currents (left) and averaged calcium current density (right)induced by a 10 ms depolarization under each condition at whisker-LFTs. n = 10, 34, 15, and 24 for P4–6, P8–14, P16–25, and WD, respectively. (*D*) Same as in (*C*) but at ectopic-LFTs. n = 6, 5, and 5 for P8–14, P16–25, and WD, respectively. (*E*) Example normalized APs (left) and averaged AP half width (right) under each condition at whisker-LFTs. n = 6, 28, 8, and 10 for P4–6, P8-–14, P16–25, and WD, respectively. (*F*) Same as in (*E*) but at ectopic-LFTs. n = 5 and 4 for P8–14 and P16–25, respectively. (*G*) The AP full width at half maximum (FWHM) values were plotted against postnatal days. Blue dotted lines in (*B*), (*D*), and (*F*) show values of P4–6 whisker-LFTs for comparison. *$p < 0.05$, **$p < 0.01$, one-way ANOVA followed by Tukey's *post hoc* test. Error bars show SEMs. In the calcium currents shown in (*C*) and (*D*), the capacitive artifact might have been emphasized by the leak subtraction procedure (see Methods).

Figure 4. Fast component of transmitter release is more efficiently triggered for release by AP-like stimulations at whisker-LFTs.

(A) Example cumulative release kinetics estimated from EPSC deconvolutions evoked by a 50 ms depolarization under each condition. Fast and slow components are shown superimposed as dotted lines. A schematic view of paired-recording configuration is shown on the top. (*B*) Averaged fractions of the fast component under each condition. n = 8, 8, and 7 for P8–14, P16–25, and WD, respectively. (*C*) Example release number estimated from EPSC deconvolutions evoked by an AP-like stimulus under each condition. (*D*) Averaged number of released vesicles under each condition. n = 10, 8, and 7 for P8–14, P16–25, and WD, respectively. *$p < 0.05$, one-way ANOVA followed by Tukey's *post hoc* test. Error bars show SEMs.

Figure 5. Fast component of transmitter release is mediated by vesicles tightly coupled to VGCCs at whisker-LFTs.

(A) Example capacitance jumps induced by 10 and 100 ms depolarizations under each condition with 0.1 mM or 5 mM EGTA internal solutions. A schematic view of capacitance measurement configuration is shown on the top. (*B*) Averaged ΔC_m per pF values were plotted against pulse durations under each condition with 0.1 mM or 5 mM EGTA internal solutions. Bottom shows enlarged traces of boxed regions. (*C*) Averaged fractions of ΔC_m evoked by a 10 ms depolarization in 5 mM EGTA compared with those in 0.1 mM EGTA. $n = 4$, 18, and 12 for P8–14, P16–25, and WD, respectively. $^{**}p < 0.01$, one-way ANOVA followed by Tukey's *post hoc* test. Error bars show SEMs.