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Research paper

The role of spontaneous activity in development of the endbulb of Held synapse

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Abstract

In the mouse brainstem cochlear nucleus, the auditory nerve to bushy cell synapse (endbulb of Held) is specialised for rapid, high-fidelity transmission. Development of this synapse is modulated by auditory nerve activity. Here we investigate the role of spontaneous auditory nerve activity in synaptic transmission using *deafness* (*dn*/*dn*) mutant mice that have abnormal hair cells and lack spontaneous auditory nerve activity. Evoked and miniature alpha amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor-mediated excitatory post-synaptic currents (eEPSCs, mEPSCs) were compared in *deafness* and normal mice before the age of hearing onset (post-natal day 7–11: P7–11) using variance-mean, miniature event and tetanic depression analyses. Amplitudes were significantly greater in *deafness* mice for eEPSCs (2.1-fold), mEPSCs (1.4-fold) and quantal amplitudes (1.5-fold). eEPSCs in *deafness* mice decayed more rapidly with increasing age, indicating an input-independent transition in post-synaptic AMPA receptor properties. A comparison of normal mice before and after the onset of hearing showed a change in synaptic parameters with an increase in eEPSC (1.7-fold), mEPSC (1.6-fold) and quantal amplitude (1.7-fold) after hearing onset while release probability remained constant (0.5). Overall, the results in *deafness* mice suggest that synaptic strength is altered in the absence of spontaneous auditory nerve activity.

Keywords: Endbulb of Held; Auditory; Development; AMPA; *dn*/*dn*; Spontaneous

1. Introduction

The endbulb of Held is an unusually large and powerful calyceal synapse between spiral ganglion neurons and spherical bushy cells in the auditory pathway. Spiral ganglion neurons transfer auditory input from the inner hair cells in the organ of Corti to bushy cells in the anteroventral cochlear nucleus (AVCN). Each spherical bushy cell receives input from up to four large endbulb terminals (Brawer and Morest, 1975; Ryugo and Sento, 1991). At maturity, the endbulb of Held is specialised for rapid, high-fidelity transmission via glutamatergic alpha amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor-mediated transmission. The AMPA-receptor mediated responses allow presynaptic action potentials to generate post-synaptic spikes with very few failures and at very high (up to 800 Hz) frequencies. Such rapid synaptic transmission is necessary for the precise processing of binaural cues required for sound localisation (Brenowitz and Trussell, 2001b; Gardner et al., 1999; Taschenberger et al., 2002).

Abbreviations: AMPA, alpha amino-3-hydroxy-5-methyl-4-isoxazole propionate; eEPSC, evoked excitatory postsynaptic currents; mEPSC, miniature excitatory postsynaptic currents; AVCN, anteroventral cochlear nucleus; aCSF, artificial cerebral spinal fluid; TTX, tetrodotoxin; Pr, the probability that a vesicle will be released from an active zone after the arrival of a single action potential; N, the number of release sites; Q_{av}, average quantal amplitude; RRP, ready releasable pool; P_{ves}, the probability of release for a single vesicle from the pool of readily releasable vesicles

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How does this synapse develop to produce rapid, high-fidelity properties of synaptic transmission? Is spontaneous auditory nerve activity important in development of the endbulb synapse? Previous reports show that hearing begins around postnatal day 12 (P12) (Kamiya et al., 2001; Romand, 1983, 2003) and sound-driven activity is required for development of frequency-selective tuning and organization in the auditory cortex (Rubel and Fritzsch, 2002; Zhang et al., 2001). Spontaneous nerve activity, which is present before auditory input (Jones et al., 2001; Lippe, 1994; Lu et al., 2007), regulates membrane properties and tonotopic maps (Leao et al., 2005, 2006; Walmsley et al., 2006). The *deafness* (*dn*/*dn*) mutant mouse offers an opportunity to examine the role of spontaneous auditory nerve activity in the development of synaptic transmission. The *deafness* mouse is profoundly deaf from birth and, unlike normal mice, lacks spontaneous auditory nerve activity (Durham et al., 1989; Leao et al., 2006). A mutation is present in the transmembrane cochlear-expressed gene 1 (Kurima et al., 2002) and inner hair cells fail to acquire mature potassium currents and exocytotic machinery (Marcotti et al., 2006). Hair cells with damaged stereocilia have been shown to result in a significant reduction in spontaneous nerve activity (Liberman and Dodds, 1984). The hair cells in the *deafness* mouse are initially intact with minor ultrastructural abnormalities (Pujol et al., 1983; Steel and Bock, 1980) and degenerate from postnatal days 15 to 40 (P15–40) (Faddis et al., 1998; Marcotti et al., 2006; Webster, 1992). Hence, this mouse provides an excellent model to study the absence of spontaneous activity in the auditory nerve without the complication of other abnormalities.

Here we investigate the effect of spontaneous auditory nerve input on synaptic transmission by comparing *deafness* mice to normal mice before the age of hearing onset (P7–11). We also test the effect of sound-driven activity by comparing normal mice before and after the onset of hearing (P7–11 versus P13–16). This study represents a novel comparison compared to previous reports in mice ($P < 25$ days versus $P > 40$) (Wang and Manis, 2005) and provides additional information using variance-mean, miniature event and tetanic depression analyses. Results from the *deafness* mutant mice confirm previous reports of an increase in synaptic strength in *deafness* compared to normal mice (Oleskevich et al., 2004; Oleskevich and Walmsley, 2002) and present new evidence regarding the role of spontaneous auditory activity in development of synaptic transmission at the endbulb synapse.

2. Methods

Brainstem slices were obtained from normal CBA mice before (P7–11, $n = 14$) and after ear canal opening (P12–16, $n = 19$) and from *deafness* (*dn*/*dn*) mutant mice (P7–11; $n = 9$; P12–16; $n = 13$). *Deafness* (*dn*/*dn*) mutant mice were obtained from the MRC Institute of Hearing Research, Nottingham, UK. These mice were recently crossed with CBA mice (to improve breeding), and subsequently bred to obtain homozygous *dn*/*dn* mice with a CBA background. Auditory brainstem responses cannot be recorded in mice until P12 and response onset correlates closely with opening of the ear canal (Kamiya et al., 2001). Because P12 represents a transition day in hearing onset, data from P12 mice ($n = 2$) were not included in comparisons of age-grouped data but were included in correlation analysis of postnatal day. All normal mice that were P13 or older had their ears and eyes open at the time of the experiment. Tissue was collected in accordance with the Garvan Institute of Medical Research/St. Vincent's Animal Experimentation Ethics Committee. Saggital slices of AVCN (200 μm for P7–10, 150 μm for P11–16) were cut in low calcium/high magnesium ice-cold artificial cerebral spinal fluid (aCSF) containing (in mM): 125 NaCl, 25 $NaHCO_3$, 3 KCl, 1.25 NaH_2PO_4, 5 Mg_2SO_4, 1 $CaCl_2$, 25 glucose, bubbled with carbogen (95% O_2, 5% CO_2). Slices were transferred to normal aCSF (mM): 125 NaCl, 25 $NaHCO_3$, 3 KCl, 1.25 NaH_2PO_4, 1 Mg_2SO_4, 2 $CaCl_2$, 25 glucose, bubbled with carbogen and maintained at 35 °C for 30–60 min. Thereafter, slices were kept in normal aCSF at room temperature.

Whole-cell recordings were made at room temperature from AVCN neurons visualised using differential interference contrast optics. Patch electrodes (4–7 MΩ) were filled with internal solution containing (mM): 120 CsCl, 4 NaCl, 4 $MgCl_2$, 0.001 $CaCl_2$, 10 Hepes, 2 Mg-ATP, 0.2 GTP-tris, 10 EGTA, adjusted to pH 7.3 and 295–300 mOsm with sorbitol. Series resistance, which was <10 MΩ, was compensated by 80%. A caesium chloride-based internal solution blocked potassium conductances and intracellular addition of lidocaine *N*-ethyl bromide (QX-314, 2 mM) blocked sodium channels thus minimising variance in the amplitude of the synaptic currents. All recordings were filtered at 10 kHz with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) and sampled at 20 kHz. Data acquisition and analysis were performed with AxoGraphX (AxoGraph Scientific, Australia).

Evoked excitatory post-synaptic currents (eEPSCs) were recorded in voltage clamp (−60 mV) by stimulation of auditory nerve afferents. The stimulating electrode was filled with normal aCSF and stimulation intensity was set at 1.5 times the response threshold for each cell (0.1 ms; 0.2 Hz). Thresholds ranged from 15 to 70 V for both *deafness* and normal mice. Since spherical bushy cells have one or two inputs and globular bushy cells and stellate cells can have up to 40 inputs, the evoked currents were identified as single-fibre synaptic currents by their all-or-none response to graded stimulation intensities, minimal eEPSC amplitude of greater than 1 nA and fast kinetics at a membrane potential of −60 mV (Isaacson and Walmsley, 1995; Oleskevich et al., 2000). AVCN neurons did not exhibit slow mEPSCs (at −60 mV), as recorded in some types of neurons in other regions of the cochlear nucleus (Gardner et al., 2001). The electrode was placed between 50 and 100 μm from the cell at a tissue depth of approximately 50 μm. Paired stimuli

were delivered at 10 ms intervals, and tetanic stimuli were delivered as trains of 15 stimuli at 100 Hz at 30 s intervals. AMPA eEPSCs were isolated by perfusion of bicuculline methochloride (10 μM; Tocris, Bristol, UK), (+)-2-amino-5-phosphonopentanoic acid (30 μM; Tocris) and strychnine hydrochloride (1 μM, Sigma).

Spontaneous miniature excitatory post-synaptic currents (mEPSCs) were detected using a sliding template procedure that detected spontaneous events with amplitudes 2.5–4.0 standard deviations above background noise (Clements and Bekkers, 1997). The template has a time course typical of a synaptic event and is optimally scaled to fit the trace at each position. Miniature EPSCs were recorded in the absence of tetrodotoxin (TTX) and were indistinguishable from those recorded in the presence of TTX (Isaacson and Walmsley, 1996).

Variance-mean analysis (Clements and Silver, 2000; Reid and Clements, 1999) was used to estimate pre- and post-synaptic parameters of synaptic transmission. Parameters were estimated from the parabolic relationship between the variance and mean amplitude of eEPSCs recorded under different release probability conditions. The degree of curvature is directly proportional to the release probability (the probability that a vesicle will be released from an active zone after the arrival of a single action potential, Pr), the width or spread of the variance-mean parabola relates to the number of release sites (N) and the initial slope of the parabola gives an estimate of average quantal amplitude (Q_{av}). Pr and N are parameters of presynaptic function, whereas Q_{av} is a post-synaptic parameter. Pr was modulated by increasing or decreasing the extracellular calcium concentration and extracellular magnesium concentration was adjusted accordingly to maintain a consistent divalent cation ratio (in mM Ca^{2+} 0.5:Mg^{2+} 2.5; Ca^{2+} 1:Mg^{2+} 2; Ca^{2+} 1.5:Mg^{2+} 1.5; Ca^{2+} 2.0:Mg^{2+} 1.0; Ca^{2+} 3.0:Mg^{2+} 0.0). The mean eEPSC amplitude and variance were calculated over a stable epoch of 30–150 events after wash-in of each extracellular solution. Regular presynaptic stimulation continued during wash-in. After solution exchange was complete, the eEPSC amplitude remained stable throughout the subsequent analysis epoch. The variance attributable to recording noise was estimated in the region prior to the test pulse and was subtracted from the eEPSC variance. A zero point was included in each variance-mean plot to indicate that the noise variance was subtracted. In approximately 15% of epochs, the synaptic response decreased during the recording period and the variance was calculated after subtracting a fitted regression line. This rundown adjustment was more likely to be required under conditions where Pr was high. If the decrease was >25%, the data was not used. The validity of the rundown correction procedure was previously tested (Oleskevich et al., 2000). Due to the asynchrony of release, the contribution of individual quanta to the peak amplitude of the eEPSC will be less than expected (Isaacson and Walmsley, 1995; Bellingham et al., 1998), leading to an underestimate of mean quantal size. At the endbulb-bushy cell connection, this factor may be considerable, due to the fast kinetics of the AMPA receptor channels (Isaacson and Walmsley, 1995; Bellingham et al., 1998). In order to compare the miniature EPSC amplitudes with quantal amplitude obtained from the variance-mean analysis, a correction factor has been calculated and applied to the quantal size from the variance-mean analysis, obtained directly from the peak amplitude to charge ratios of the evoked and miniature EPSCs (Isaacson and Walmsley, 1995; Bellingham et al., 1998). Variance-mean analysis was performed with AxoGraphX.

Estimates of the ready releasable pool (RRP) and the probability of release for a single vesicle from the pool of readily releasable vesicles (P_{ves}) were measured using a technique first described at a nerve-muscle synapse (Elmqvist and Quastel, 1965; Schneggenburger et al., 1999). Trains of stimuli (100 Hz) were applied and the cumulative eEPSC amplitude (NQ_{av}) was plotted versus stimulus number (data not shown). A linear fit was applied from stimulus number 10–15 and the y-intercept of the linear fit provided an estimate of NQ_{av}. Using the mean value of Q_{av} from the variance-mean analysis, the number of vesicles in the RRP was calculated from NQ_{av}/Q_{av}. P_{ves} was calculated from the ratio of the first eEPSC amplitude and the RRP.

To provide a full description of development at the endbulb synapse and analysis of the effects of deafness on synaptic transmission some previously published data (Oleskevich et al., 2004; Oleskevich and Walmsley, 2002) has been reanalysed for this study. All data from normal and *deafness* mice at P7–10 is new, as is all data on mEPSC frequency and decay at P13–16. Replication of previous experiments was avoided to comply with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2004). Statistics are quoted as mean ± standard error of the mean (SE). The F statistic was used to test for different variances between populations. If variances were similar then the Student's t-test was used, otherwise differences were tested using an unpaired t-test with Welch's correction for unequal variance. Correlation was tested using the non-parametric Spearman's Rank Correlation. All statistical tests were two-tailed and statistical analysis was performed using Prism (GraphPad, San Diego, CA).

3. Results

3.1. Synaptic strength is greater in deafness mutant mice

To investigate the role of spontaneous auditory nerve activity on development of synaptic transmission at the endbulb synapse, we examined evoked AMPA receptor-mediated currents in *deafness* and normal mice before and after the age of hearing onset (P7–11 versus P13–16) (Fig. 1a; Table 1). Before the age of hearing onset, the mean eEPSC amplitude in *deafness* mice (3.29 ± 0.65 nA) was

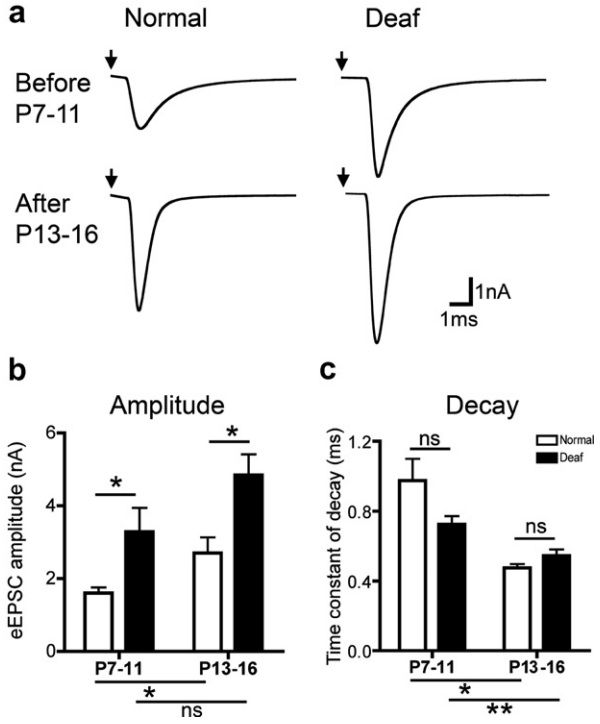

Fig. 1. Synaptic strength is greater in *deafness* mutant mice. (a) Averaged AMPA evoked EPSCs for individual cells recorded at the endbulb of Held synapse in AVCN slices in response to focal stimulation of the auditory nerve in normal and *deafness* mice before and after the onset of hearing. Arrows indicate focal stimulation of auditory nerve. (b) Summary data showing that mean eEPSC amplitude is significantly greater (2.2-fold) in *deafness* mice before the age of hearing onset suggesting that a lack of spontaneous auditory nerve activity can modulate synaptic strength. In normal mice, mean eEPSC amplitude increased after hearing onset (1.7-fold). (c) eEPSCs in *deafness* mice decayed more rapidly with increasing age (P7–11 versus P13–16), indicating an input-independent transition in post-synaptic AMPA receptor properties. Bars display mean values and SE ($^{*}p < 0.05$, $^{**}p < 0.01$).

significantly greater (2.1-fold) than the mean eEPSC amplitude in normal mice (1.60 ± 0.17 nA; $p = 0.03$; Fig. 1b). Variability of the amplitudes was significantly greater in *deafness* (range 0.99–9.70 nA; $n = 15$; $p < 0.0001$) than normal mice (range 0.64–2.6 nA; $n = 15$). Similarly, after the age of hearing onset, the mean eEPSC amplitude in *deafness* mice (4.84 ± 0.57 nA) was significantly greater (1.8-fold) than in normal mice (2.70 ± 0.46 nA; $p = 0.02$) and again the amplitudes were more variable in the *deafness* mice (range 0.81–13.00 nA; $n = 34$) than in normal mice (range from 0.56 to 7.74 nA; $n = 21$, $p < 0.01$).

To investigate the role of sound-driven auditory nerve activity on endbulb development, synaptic transmission was examined before and after hearing onset in normal mice. The mean amplitude was significantly increased after hearing onset (from 1.60 ± 0.17 nA to 2.70 ± 0.46 nA; $p = 0.025$; Fig. 1b). A correlation analysis of postnatal day and eEPSC amplitude confirmed that the eEPSC amplitudes increased between P7 and P16 ($r = 0.38$; $p = 0.02$).

Previous reports at the rat and chick endbulb synapse show that a developmental increase in eEPSC amplitude is accompanied by faster decay kinetics (Bellingham et al., 1998; Brenowitz and Trussell, 2001b). The mean time constant of decay of eEPSCs in *deafness* mice was significantly faster after ear canal opening (decrease from 0.72 ± 0.05 ms to 0.54 ± 0.04 ms; $p = 0.001$; Fig. 1c), which was supported by correlation analysis of postnatal day and time constant of decay ($r = -0.56$, $p = 0.001$). The mean time constant of decay of eEPSCs in normal mice was also significantly faster after hearing onset (decrease from 0.98 ± 0.12 ms to 0.47 ± 0.02 ms; $p = 0.003$) as confirmed by correlation analysis between time constant of decay and postnatal age ($r = -0.51$, $p = 0.006$).

In summary, the observation that the eEPSC amplitude was greater in *deafness* versus normal mice before the age of hearing onset suggests that a lack of spontaneous auditory nerve activity, which is the predominant difference between the *deafness* and normal animals, modulates synaptic strength at the endbulb synapse. This increase in eEPSC amplitude in *deafness* versus normal mice is consistent with previous observations made during or immedi-

Table 1
Synaptic properties at the endbulb of Held

	Before age of hearing onset P7–11		After age of hearing onset P13–16	
	Deafness	Normal	Deafness	Normal
eEPSC amplitude (nA)	3.29 ± 0.65 (15)	1.60 ± 0.17 (15)*	4.84 ± 0.57 (34)	2.70 ± 0.46 (21)$^{\#,*}$
eEPSC decay (ms)	0.72 ± 0.05 (15)	0.98 ± 0.12 (15)	0.54 ± 0.04 (34)$^{\#\#}$	0.47 ± 0.02 (21)$^{\#}$
mEPSC amplitude (pA)	85 ± 7 (12)	62 ± 7 (15)*	99 ± 7 (22)	102 ± 17 (20)$^{\#}$
mEPSC decay (ms)	0.40 ± 0.05 (12)	0.34 ± 0.03 (15)	0.21 ± 0.01 (22)$^{\#\#}$	0.23 ± 0.03 (20)$^{\#}$
mEPSC frequency (Hz)	1.77 ± 0.69 (12)	2.10 ± 0.48 (15)	2.69 ± 0.54 (22)	2.67 ± 0.60 (20)
Q_{av} (pA)	88 ± 13 (5)	59 ± 6 (8)*	105 ± 13 (6)	103 ± 13 (8)$^{\#\#}$
Pr	0.64 ± 0.05 (5)	0.47 ± 0.06 (8)	0.77 ± 0.07 (6)	0.48 ± 0.10 (8)*
N (number of sites)	143 ± 32 (5)	116 ± 27 (8)	97 ± 13 (6)	91 ± 24 (8)
Tetanic Depression (%)	88 ± 3 (7)	82 ± 4 (9)	93 ± 1 (18)$^{\#\#}$	89 ± 1 (9)*
P_{ves}	0.53 ± 0.19 (7)	0.55 ± 0.16 (9)	0.61 ± 0.14 (16)	0.53 ± 0.01 (7)*
RRP	92 ± 21 (7)	52 ± 19 (9)	72 ± 12 (16)	109 ± 17 (7)

Number of cells in parentheses.
* Significant difference between *deafness* and normal ($p < 0.05$).
$^{\#}$ Significant difference between before and after age of hearing onset ($p < 0.05$).
$^{\#\#}$ Significant difference between before and after age of hearing onset ($p < 0.01$).

ately after the age of hearing onset (P11–14) (Oleskevich et al., 2004; Oleskevich and Walmsley, 2002).

3.2. Post-synaptic properties are modulated in deafness mice before the age of hearing onset

Variance-mean analyses were performed to investigate if the changes in eEPSC amplitude were due to changes in presynaptic function (release probability, *Pr*; the number of release sites, *N*) and/or post-synaptic function (the average quantal amplitude, Q_{av}). Synaptic parameters are derived from the parabolic relationship between the variance and the mean amplitude of eEPSCs recorded under different release probability conditions (Clements and Silver, 2000; Reid and Clements, 1999). Examples of eEPSCs recorded in response to varying calcium concentrations are shown in Fig. 2a and b and representative parabolas for individual cells are displayed in Fig. 2c–f. In low calcium concentrations when *Pr* is low, most sites do not release transmitter and the trial-to-trial variance of eEPSC amplitude is low. When calcium concentrations (2 mM) and *Pr* are moderate (around 0.5), the number of sites that release transmitter varies widely from trial-to-trial, and eEPSC amplitude variance is high. When calcium concentrations (3 mM) and *Pr* are high (approaching 1.0), almost all sites release transmitter after every stimulus and the eEPSC amplitude variance is again low. A plot of the mean EPSC amplitude versus variance will yield a parabola with varying degrees of curvature. The mean eEPSC amplitude in normal mice before and after the onset of hearing showed significant variability at high calcium concentrations (3 mM) and thus the parabola showed a decreased degree of curvature in comparison to the parabolas estimated for *deafness* mice before and after the onset of hearing.

The mean Q_{av} was significantly greater (1.5-fold) in *deafness* (88 ± 13 pA; $n = 5$) versus normal mice (59 ± 6 pA; $n = 8$; $p = 0.039$; Fig. 2g) before the age of hearing onset and did not change significantly during development in *deafness* mice (P7–11: 88 ± 13 pA, $n = 5$ versus P13–16: 105 ± 13 pA, $n = 6$). Before hearing onset in normal mice, mean Pr at the endbulb synapse (0.47 ± 0.06) was not significantly different to the mean Pr in *deafness* mice

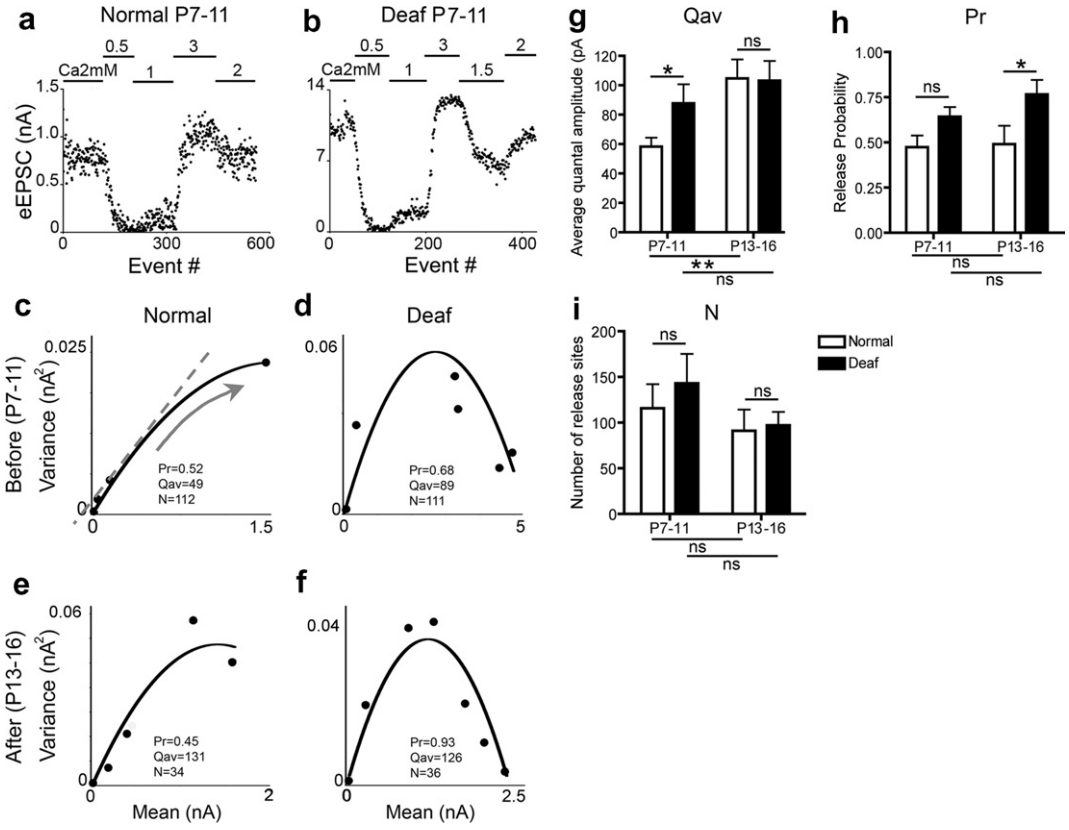

Fig. 2. Post-synaptic properties are modulated in *deafness* mice before the age of hearing onset. (a and b) Variance-mean analysis was used to measure pre- and post-synaptic parameters of endbulb transmission. Single AMPA eEPSCs recorded during superfusion with a range of extracellular Ca^{2+} concentrations from a normal mouse (a) and a *deafness* mouse (b) before the age of hearing onset. (c–f) Parabolas are fit from the relationship between the variance and mean eEPSC amplitudes recorded at different Ca^{2+} concentrations (see Section 2). The post-synaptic parameter (Q_{av}; initial slope) was greater (1.5-fold) in *deafness* mice compared to normal mice while presynaptic parameters (*Pr* and *N*; degree of curvature) were not significantly different as shown by variance-mean parabolas for individual cells. After the age of hearing onset, *Pr* was significantly greater (1.4-fold) in *deafness* mice, consistent with previous studies. In normal mice, Q_{av} increased (1.7-fold) after hearing onset. In (c), dashed grey line indicates initial slope (Q_{av}) and arrow indicates degree of curvature (*Pr* and *N*). (g–i). Summary data for Q_{av}, *Pr*, and *N* in normal and *deafness* mice before and after hearing onset. Bars display mean values and SE ($^{*}p < 0.05$, $^{**}p < 0.01$).

(0.64 ± 0.05, $p = 0.06$). After the age of hearing onset, Pr was significantly greater (1.6-fold) in *deafness* (0.77 ± 0.07) compared to normal mice (0.048 ± 0.01, $p = 0.04$; Fig. 2h) in keeping with previous studies (Oleskevich et al., 2004; Oleskevich and Walmsley, 2002). There were no significant differences between the mean N estimates before the age of hearing onset for *deafness* (143 ± 32 sites) or normal mice (116 ± 27 sites) or after the age of hearing onset between *deafness* (97 ± 13 sites) and normal mice (91 ± 24 sites; $p = 0.81$; Fig. 2i).

In normal mice, mean Q_{av} increased significantly by 1.7-fold after hearing onset (from 59 ± 6 pA to 103 ± 13 pA; $p = 0.004$) consistent with a strong positive correlation between Q_{av} and postnatal age ($r = 0.76$, $p = 0.002$). The onset of hearing did not affect Pr (P7–11: 0.47 ± 0.06 versus P13–16: 0.48 ± 0.10) or N (P7–11: 116 ± 27 sites versus P13–16: 91 ± 24 sites).

In summary, post-synaptic parameters (Q_{av}) may underlie the increase in eEPSC amplitude in *deafness* versus normal mice before the age of hearing onset, while presynaptic parameters (Pr) may underlie the increase in eEPSC amplitude after the age of hearing onset. In normal mice, the increase in eEPSC amplitude observed after hearing onset was correlated with changes in post-synaptic parameters (Q_{av}) while presynaptic parameters (Pr and N) were unchanged during development.

3.3. Deafness mice show an input-independent transition in post-synaptic AMPA receptor properties

We next examined the amplitude and kinetics of spontaneous mEPSCs in *deafness* and normal mice before and after the age of hearing onset. The mEPSC elicited by the spontaneous release of a vesicle from a nerve terminal is a direct measure of the quantal current and can be compared to the estimate of Q_{av} from variance-mean analysis. Decay kinetics of mEPSCs were measured to provide insight into developmental changes in post-synaptic receptor composition. Fig. 3a displays representative mEPSCs traces and corresponding examples of averaged mEPSCs from individual neurons.

Before the age of hearing onset, the mean mEPSC amplitude was significantly greater (1.4-fold) in *deafness* mice (85 ± 7 pA, $n = 12$) versus normal mice (62 ± 7 pA, $n = 15$, $p = 0.03$, Fig. 3b), in keeping with the differences observed in Q_{av}. mEPSC decay kinetics were similar between *deafness* (0.40 ± 0.05 ms) and age-matched normal mice (0.34 ± 0.03 ms) before the age of hearing onset. After the age of hearing onset, the mean mEPSC amplitude in *deafness* mice (99 ± 7 pA, $n = 22$) was similar to normal mice (102 ± 17 pA) as was mEPSC decay kinetics in *deafness* (0.21 ± 0.01 ms) and normal mice (0.23 ± 0.03 ms; $n = 20$; Fig. 3c). Interestingly, the mean decay kinetics in

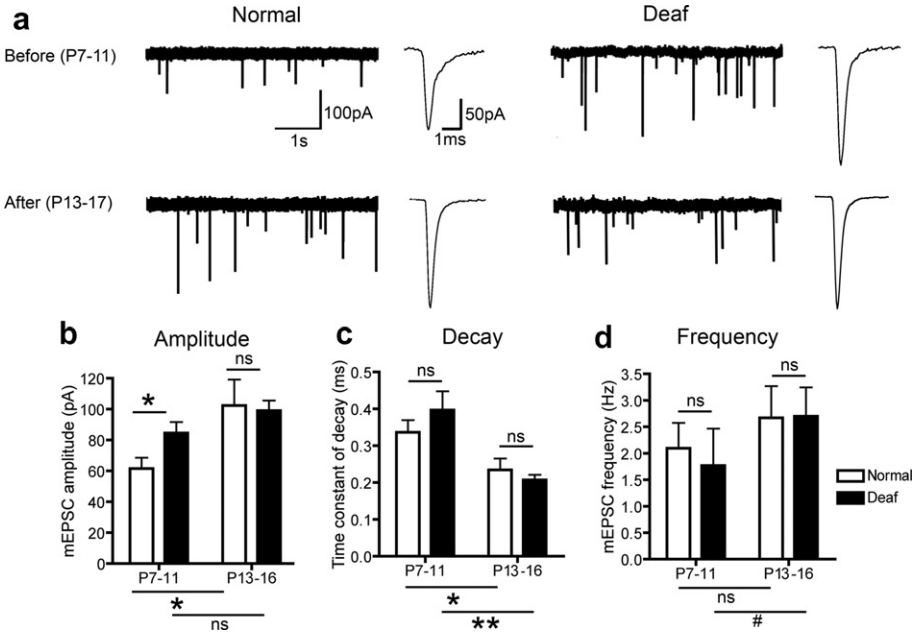

Fig. 3. Deafness mice show an input-independent transition in post-synaptic AMPA receptor properties. (a) Representative continuous recordings of mEPSCs for individual cells in normal and *deafness* mice recorded before and after the age of hearing onset. Averaged mEPSCs are displayed beside each trace. (b–d) The mean mEPSC amplitude is significantly greater in *deafness* mice before the age of hearing onset. This is consistent with the observed increase in Q_{av} and suggests that a lack of spontaneous auditory nerve activity affects synaptic strength by modulating post-synaptic parameters of endbulb transmission. In normal mice, Q_{av} and decay time constants changed after the onset of hearing suggesting that sound-driven auditory nerve activity can modulate synaptic strength via post-synaptic parameters. mEPSCs in *deafness* mice decayed more rapidly with increasing age (P7–11 versus P13–16), indicating an input-independent transition in post-synaptic AMPA receptor properties. There is a slight increase in the mEPSC frequency with increasing age in *deafness* mice but only when mEPSC frequency is correlated to postnatal day ($^{\#}p < 0.05$, Spearman's correlation). Bars display mean values and SE ($^{*}p < 0.05$, $^{**}p < 0.01$).

deafness mice were 1.9-fold faster after the age of hearing onset (0.21 ± 0.01 ms versus 0.40 ± 0.05 ms, $p = 0.003$, Fig. 3c), as confirmed by correlation analysis ($r = -0.47$, $p = 0.02$).

The frequency of mEPSCs was similar between *deafness* (1.77 ± 0.69 Hz) and normal mice before the age of hearing onset (2.10 ± 0.48 Hz) and between *deafness* (2.69 ± 0.54 Hz) and normal mice after the age of hearing onset (2.67 ± 0.60 Hz; Fig. 3d). There was a trend towards an increase in mEPSC frequency in *deafness* mice after the age of hearing onset ($p = 0.08$) as confirmed by correlation analysis between mEPSC frequency and postnatal day ($r = 0.42$, $p = 0.02$). The frequency of mEPSCs did not change significantly during development in normal mice.

At the normal endbulb synapse, the mean mEPSC amplitude significantly increased by 1.6-fold from 62 ± 7 pA to 102 ± 17 pA ($p = 0.02$) after hearing onset, consistent with a positive correlation to postnatal day ($r = 0.40$; $p = 0.04$). The mean decay time constants were significantly faster after hearing onset, decreasing from 0.34 ± 0.03 ms to 0.23 ± 0.03 ms ($p = 0.03$, Fig. 3c).

In summary, mEPSCs in *deafness* mice decayed more rapidly after the age of hearing onset (P7–11 versus P13–16), indicating an input-independent transition in post-synaptic AMPA receptor properties. Direct measurements of mEPSC amplitude corroborate estimates of Q_{av} from variance-mean analysis ($r = 0.65$, $p = 0.001$, $n = 22$) in both *deafness* and normal mice.

3.4. Tetanic depression is not affected in deafness mice before the age of hearing onset

Synaptic depression is associated with synapses that exhibit high release probability (Zucker, 1989) and has been attributed to depletion of the readily releasable pool (Wu and Borst, 1999). Previous work has shown that *deafness* mice show greater synaptic depression than normal mice at P11–14 (Oleskevich et al., 2004; Oleskevich and Walmsley, 2002). To examine the development of synaptic depression, the auditory nerve was stimulated with trains of 15 stimuli (100 Hz; Fig. 4a). Depression at the synapse was defined as the ratio of eEPSC amplitudes at the 15th response (S15) versus the first response (S1) converted into the degree of depression.

There was no difference in the mean degree of depression before the age of hearing onset in *deafness* ($88 \pm 3\%$, $n = 7$) and normal mice ($82 \pm 4\%$, $n = 9$). However, after the age of hearing onset, depression was significantly greater in *deafness* ($93 \pm 1\%$, $n = 18$) versus normal mice ($89 \pm 1\%$, $n = 9$, $p = 0.04$; Fig. 4b), consistent with previous reports and confirmed by correlation analysis between depression and postnatal age ($r = 0.41$, $p = 0.04$).

Given that tetanic depression and release probability were significantly increased in *deafness* mice compared to normal mice after the age of hearing onset, estimates of the ready releasable pool (RRP) and the probability of release of a vesicle from the pool of readily releasable vesicles (P_{ves}) were measured using a technique first described at a nerve-muscle synapse (Elmqvist and Quastel, 1965; Schneggenburger et al., 1999) (see Section 2).

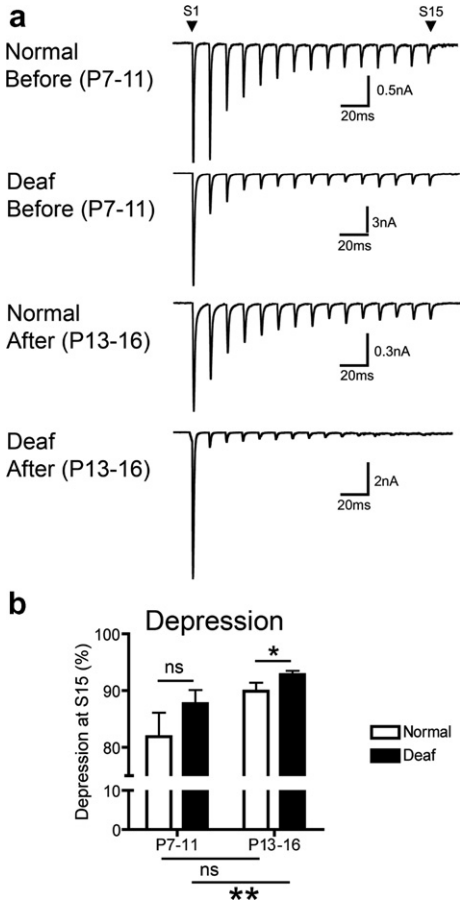

Fig. 4. Tetanic depression is increased after hearing in deafness mice. (a) Representative averaged eEPSCs show depression in response to trains of 15 stimuli at 10 ms intervals for normal and *deafness* mice recorded before and after the age of hearing onset. Tetanic depression (S15:S1) in *deafness* mice is similar to normal mice before the age of hearing onset. As tetanic depression is often correlated with presynaptic parameters, these results are consistent with the variance-mean analysis and demonstrate that a lack of spontaneous nerve activity in the *deafness* mice does not affect presynaptic parameters of endbulb transmission. (b) Bar graph summarising mean depression for normal and *deafness* mice before and after hearing onset. Mean depression is greater in *deafness* versus normal mice at P13–16 in keeping with a greater release probability in *deafness* mice at this age and consistent with previous reports (Oleskevich and Walmsley, 2002).

Before hearing onset, P_{ves} did not differ between normal (0.55 ± 0.16, $n = 9$) and *deafness* mice (0.53 ± 0.19, $n = 7$, $p = 0.81$; Fig. 5). However, after hearing onset P_{ves}, was significantly higher in *deafness* mice (0.61 ± 0.14, $n = 16$) compared to normal mice (0.53 ± 0.01, $n = 7$, $p = 0.04$). This data supports the release probability data obtained from variance-mean analysis. No differences were observed for RRP between *deafness* and normal mice before or after the age of hearing onset. RRP did not change during development in *deafness* or normal mice. For both animal and age groups, the RRP size per release site ranged between

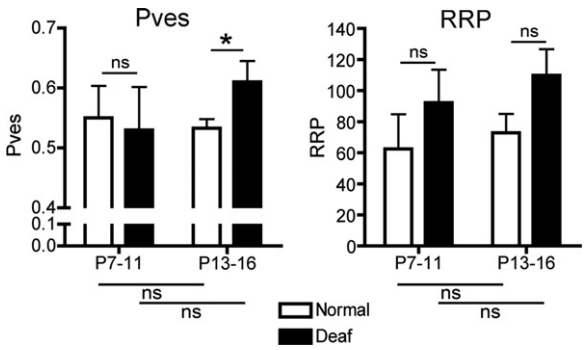

Fig. 5. Probability of release of a vesicle from the pool of readily releasable vesicles is increased after hearing in deafness mice. Summary data showing the mean probability of release of a vesicle from the pool of readily releasable vesicles (P_{ves}) and the ready releasable pool of vesicles (RRP) for normal and *deafness* mice before (P7–11) and after (P13–16) hearing onset. After hearing onset the mean P_{ves} is significantly increased in *deafness* mice compared to normal mice. Bars display mean values and SE ($^{*}p < 0.05$).

29 and 157 (Fig. 5). The paired pulse ratio (ratio of the amplitudes S2:S1) showed an inverse relationship with release probability ($r = -0.57$, $p = 0.003$). No changes in paired pulse ratio were observed between *deafness* and normal mice, or in *deafness* or normal mice with increasing age (data not shown). In summary, *deafness* mice showed a developmental increase in the amount of depression at the endbulb synapse, consistent with the increased release probability at this age.

4. Discussion

4.1. Development of synaptic strength in deafness mice

Previous studies demonstrate that synaptic transmission at the endbulb of Held is altered in *deafness* mice compared to normal mice around the onset of hearing (P11–14) (Oleskevich et al., 2002, 2004). Here we examined whether the *deafness* mutation exerted an effect before the onset of sound-driven activity. Therefore mice were separated into groups before (P7–11) and after hearing onset (P13–16). Although the endbulbs of Held continue to develop morphologically over this 10-day period (Limb and Ryugo, 2000) we observed no significant changes in any measured synaptic properties within the P7–11 age group or within the P13–16 age group.

Here we have shown that differences also exist between normal and *deafness* mice early in development, well before the age at which sound-driven activity could influence synaptic development. In young *deafness* mice, the amplitude of eEPSCs was more than twice as large as in normal mice before the age of hearing onset. The increase was primarily post-synaptic as mEPSCs and Q_{av} were increased, while Pr and N were unchanged as indicated by variance-mean analysis. Other indicators of presynaptic function (tetanic depression, paired pulse ratio, RRP, P_{ves}) were also unaffected compared to age-matched normal mice. The differences in synaptic strength previously observed at the endbulb synapse in *deafness* mice before the age at which hearing begins may not necessarily be due to a lack of sound-driven input.

How might the *deafness* mutation alter post-synaptic function? One of the characteristics of this model of congenital deafness is the absence of spontaneous auditory nerve activity (Durham et al., 1989; Leao et al., 2005) and it is established that the absence of activity *per se* can alter synaptic development in the auditory system (Walmsley et al., 2006). Up regulation of quantal amplitude in response to decreased or absent activity is well described for AMPA receptors *in vitro* following activity block with tetrodotoxin and receptor block with specific antagonists (Lissin et al., 1998; Maffei et al., 2004; O'Brien et al., 1998; Turrigiano et al., 1998; Wierenga et al., 2005). A similar effect has also been demonstrated *in vivo* where visual deprivation increases quantal amplitude in visual cortex (Desai et al., 2002; Maffei et al., 2004). An increase of quantal amplitude in response to decreased activity is well described for AMPA receptors *in vitro*. Long-term changes in input activity alter synaptic transmission by a mechanism known as homeostatic plasticity (Burrone and Murthy, 2003; Turrigiano and Nelson, 2004; Walmsley et al., 2006). Homeostatic plasticity offers a hypothesis to explain how a lack of spontaneous auditory nerve activity can alter synaptic transmission in *deafness* mice. Future studies could test this hypothesis by examining synaptic transmission in normal mice reared with sensorineural hearing loss designed to disrupt sound-driven but not spontaneous auditory nerve activity (see Tucci et al., 1987).

Consistent with previous reports (Oleskevich et al., 2004; Oleskevich and Walmsley, 2002), release probability was increased in *deafness* mice compared to normal mice at P13–16. These observations were supported by tetanic depression data, which showed greater depression in *deafness* mice at P13–16. Release probability depends in part on the concentration of calcium ions that flux into the presynaptic nerve terminal after the arrival of an action potential. An increase in calcium influx increases release probability at auditory synapses (Habets and Borst, 2006) and, at the neuromuscular junction, activity block shifts the sensitivity to calcium such that release probability is increased (Wang et al., 2004). It remains to be determined why release probability and tetanic depression were unchanged in *deafness* compared to normal mice before the onset of hearing. It should be noted that there was a trend towards a difference in these parameters before hearing ($p = 0.06$), but the considerable variability in synaptic responses from younger animals compared to later in development (Lu et al., 2007; Wu and Oertel, 1987) precluded these differences from reaching significance.

Faster decay kinetics for eEPSCs and mEPSCs were observed in *deafness* mice with increasing age. As changes in decay kinetics are linked to changes in receptor subunit composition (Taschenberger and von Gersdorff, 2000), the

data suggests an input-independent transition in AMPA receptor properties. Similar changes in decay kinetics were observed for normal mice (see below). It would be interesting to investigate the number and composition of AMPA receptors in normal and *deafness* mice to further elucidate what role auditory nerve activity plays in post-synaptic development at the endbulb synapse.

4.2. Development of synaptic strength in normal mice

Several developmental changes were observed in AMPA-mediated synaptic transmission at the endbulb of Held synapse in normal mouse AVCN. The developmental changes, including an increase in eEPSC and mEPSC amplitude and corresponding faster decay kinetics were similar to previous studies in the rat (Bellingham et al., 1998) and the chick (Brenowitz and Trussell, 2001b). In the rat, an increase in quantal size and content was reported to underly an increase synaptic strength (Bellingham et al., 1998). Although these developmental changes were not observed in mEPSCs in young (<P25) versus old mice (>P40) (Wang and Manis, 2005), the correlation between an increase in mEPSC amplitude and decrease in decay kinetics has been previously reported in the mouse (Wang and Manis, 2005) and the chick (Brenowitz and Trussell, 2001b). Additional information was provided by the use of variance-mean analysis to differentiate between presynaptic (Pr and N) and post-synaptic (Q_{av}) changes during development. Over the developmental period examined, the average quantal amplitude increased, whereas release probability and the number of release sites were unchanged. The increase in the average quantal amplitude was consistent with an increase in miniature spontaneous EPSC amplitude (a direct measure of quantal amplitude). Thus, post-synaptic changes in quantal size and response kinetics can fully account for the observed developmental changes in evoked EPSCs.

Previous studies in the chick show that release probability is decreased and the pool of readily releasable vesicles is increased during development (Brenowitz and Trussell, 2001b). We observed that release probability was initially low and remained unchanged after hearing onset. This was supported by a lack of change in tetanic depression, which normally varies with release probability (Brenowitz and Trussell, 2001a; Brenowitz et al., 1998; Oleskevich et al., 2000). It was also consistent with a lack of change in RRP size which varies with tetanic depression (Brenowitz and Trussell, 2001a; Brenowitz et al., 1998; Oleskevich et al., 2002). The number of release sites remained unchanged between P7 and P16, which is in keeping with the morphological maturation of the endbulb in the cat (Ryugo et al., 2006) and mouse (Limb and Ryugo, 2000) where the endbulb becomes more extensively branched and complex with age. Most of the anatomical changes in the mouse take place after postnatal week four and do not reach adult-like complexity until the ninth postnatal week.

In summary, data from normal mice suggest that during the postnatal period spanning the onset of sound-driven auditory nerve input, the endbulb of Held undergoes mainly post-synaptic developmental changes that lead to larger and faster evoked and quantal currents. One possible post-synaptic mechanism for the emergence of larger, faster currents is a change in AMPA receptor number and/or kinetics. The switch from NMDA to AMPA-receptor mediated transmission at auditory synapses is well documented, as is the switch in AMPA receptor subunits to flop isoforms and the decrease in the GluR2 subunit (Gardner et al., 1999; Gardner et al., 2001; Isaacson and Walmsley, 1996; Lawrence and Trussell, 2000; Youssoufian et al., 2005). The switch in subunits may account for the change in kinetics but it remains to be determined whether AMPA receptor number changes over this developmental period at the mouse endbulb. Some of the changes in synaptic transmission occurring after the onset of hearing were also observed in *deafness* mice, suggesting an input-independent mechanism of action. Summary data from *deafness* mice show that the increase in synaptic strength in *deafness* versus normal mice was apparent before the age of hearing onset. This suggests that a lack of spontaneous activity in the auditory nerve rather than a lack of sound-driven activity plays an important role in the development of synaptic transmission at the endbulb synapse in *deafness* mice.

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