

{<br>( 31 32 occur due to loss of ribbon synapses.<br>33<br>34 Introduction<br>15 In the nervous system, synapses transmit signals between neurons, and proper synapse

22 occur and to loss of the stringing properties<br>33 occur due to loss of the ribbon<br>35 occur due to ribbon system, synapses trans<br>36 occur assembly is critical for the function of 34<br>35<br>36<br>37 34 Introduction<br>35 In the nervou<br>36 assembly is cross<br>37 in synapse as:<br>38 hair cells, the In the nervous system, synapses transmit signals between neurons, and proper synapse<br>36 assembly is critical for the function of neural circuits. While many players have been identified<br>37 in synapse assembly in the centra 37 in synapse assembly in the central nervous system, relatively less is known about this process in<br>38 hair cells, the sensory receptors of the inner ear. Importantly, both noise-induced and age-<br>39 related hearing loss i 38 hair cells, the sensory receptors of the inner ear. Importantly, both noise-induced and age-<br>39 related hearing loss in humans can result from auditory synaptopathy, or synapse loss, even in<br>30 the absence of hair-cell 42 understand how to re-form these synapses and treat auditory synaptopathy. 39 the absence of hair-cell death (Liberman, 2017; Wu et al., 2020; Wu et al., 2021). By expanding<br>31 our current knowledge of the players involved in synapse assembly in hair cells, we can better<br>32 understand how to re-f the absence of hair-cell death (Liberman, 2017; Wu et al., 2020; Wu et al., 2021). By expanding<br>
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understand how to re-form the

and the players in the player of the player of the players and treat auditory synaptopathy.<br>
43 are sent to the brain. In response to sensory stimuli, apical structures called mechanosensory<br>
45 hair bundles are deflected, 43 Hair cells function to convert auditory, vestibular and lateral-line stimular are sent to the brain. In response to sensory stimuli, apical structures called n<br>44 hair bundles are deflected, opening mechanoelectrical tr 45 hair bundles are deflected, opening mechanoelectrical transduction (MET) channels and<br>46 depolarizing the cell. Hair-cell depolarization opens voltage-gated calcium channels (Ca<sub>V</sub>1.3) at<br>47 the presvnapse, resulting i 45 hair bundles are deflected, opening mechanoelectrical transduction (MET) channels and<br>46 depolarizing the cell. Hair-cell depolarization opens voltage-gated calcium channels (Ca<sub>V</sub>1.3) a<br>47 the presynapse, resulting in depolarizing the cell. Hair-cell depolarization opens voltage-gated calcium channels (Ca<sub>V</sub>1<br>the presynapse, resulting in calcium influx that triggers the release of the neurotransmitt<br>glutamate onto afferent neurons (rev the presynapse, resulting in calcium influx that triggers the release of the neurotransmitter<br>48 glutamate onto afferent neurons (reviewed in: (Fettiplace, 2017)). In order to properly encode<br>49 sensory stimuli, hair cells 48 glutamate onto afferent neurons (reviewed in: (Fettiplace, 2017)). In order to properly enco<br>49 sensory stimuli, hair cells use a specialized ribbon synapse for speed and precision (Khimich<br>40 al., 2005; Moser et al., 2 Examples 19<br>
49 sensory stimuli, hair cells use a specialized ribbon synapse for speed and precision (Khimich et<br>
50 al., 2005; Moser et al., 2006). A ribbon synapse is defined by a presynaptic density called a<br>
51 ribbon, al., 2005; Moser et al., 2006). A ribbon synapse is defined by a presynaptic density called a<br>51 sibbon, which is composed primarily of the protein Ribeye (a splice variant of CTBP2) (Schmitz<br>52 et al., 2000) (Fig 1D). Rib 51 bbon, which is composed primarily of the protein Ribeye (a splice variant of CTBP2) (Schm<br>52 et al., 2000) (Fig 1D). Ribbons are thought to be important to recruit and tether synaptic<br>53 vesicles at the presynaptic acti ribbon, which is composed primarily of the protein Ribeye (a splice variant of CTBP2) (Schmitz<br>
et al., 2000) (Fig 1D). Ribbons are thought to be important to recruit and tether synaptic<br>
vesicles at the presynaptic active

55 this critical sensory circuit remain largely undefined. Cell adhesion molecules (CAMS) are<br>56 powerful modulators of synapse formation. CAMs act to bridge pre- and post-synaptic do 54 version and presymant active zone.<br>55 this critical sensory circuit remain largel<br>56 powerful modulators of synapse format<br>57 to initiate, maintain and specify synapse 56 powerful modulators of synapse formation. CAMs act to bridge pre- and post-synaptic domains<br>57 to initiate, maintain and specify synapses (reviewed in: (Südhof, 2021)). Work in mouse<br>58 auditory inner hair cells (IHCs) 56 powerful modulators of synapse formation. CAMs act to bridge pre- and post-synaptic do<br>57 to initiate, maintain and specify synapses (reviewed in: (Südhof, 2021)). Work in mouse<br>58 auditory inner hair cells (IHCs) has i 57 to initiate, maintain and specify synapses (reviewed in: (Südhof, 2021)). Work in mouse<br>auditory inner hair cells (IHCs) has implicated the CAM Neuroplastin (Np55/Np65) and the<br>neuronal CAM (NrCAM) in the development of 58 auditory inner hair cells (IHCs) has implicated the CAM Neuroplastin (Np55/Np65) and the<br>59 neuronal CAM (NrCAM) in the development of IHC ribbon synapses (Carrott et al., 2016; Ha<br>2 59 neuronal CAM (NrCAM) in the development of IHC ribbon synapses (Carrott et al., 2016; Hair cells (NPS) and the CAM  $\frac{2}{3}$  $\frac{1}{2}$ 

()<br>()<br>() 60 et al., 2018: Newton et al., 2022). More recent work in mouse found that a well-studied family 61 of postsynaptic CAMs, neuroligins (Nlgns), are important for ribbon-synapse assembly (Ramirez<br>et al., 2022). This work found that mice lacking NLGN1/3 have fewer ribbon synapses in IHCs<br>and impaired hearing. Postsynapti et al., 2022). This work found that mice lacking NLGN1/3 have fewer ribbon synapses in IHCs<br>and impaired hearing. Postsynaptic Nlgns classically bind to a family of presynaptic CAMs, called<br>neurexins (Nrxns) (Südhof, 2021) and impaired hearing. Postsynaptic Nlgns classically bind to a family of presynaptic CAMs, cal<br>neurexins (Nrxns) (Südhof, 2021). While prior work points to the importance of NLGN1/3 in<br>synapse assembly in hair cells, wheth main. pair and impair and impair of the main of the main of the matter of NLGN1/3 in<br>neurexins (Nrxns) (Südhof, 2021). While prior work points to the importance of NLGN1/3 in<br>synapse assembly in hair cells, whether these n synapse assembly in hair cells, whether these neuroligins pair with a presynaptic neurexin in<br>
the auditory system is not known (Ramirez et al., 2022).<br>
To study the function of neurexins in hair-cell synapse assembly, we

68 model system. Numerous studies have shown that zebrafish is a relevant model for studying<br>69 the genetics of hair cells, as many of the core molecules required at hair-cell synapses (ex: For the authory system is not known (Mammed et al., 2022).<br>
66 To study the function of neurexins in hair-cell syn<br>
68 model system. Numerous studies have shown that zebra<br>
69 the genetics of hair cells, as many of the cor 68 model system. Numerous studies have shown that zebrafish is a relevant model for studying<br>69 the genetics of hair cells, as many of the core molecules required at hair-cell synapses (ex:<br>70 Ca<sub>v</sub>1.3 and Ribeye) are con 69 the genetics of hair cells, as many of the core molecules required at hair-cell synapses (ex:<br>70 Ca<sub>V</sub>1.3 and Ribeye) are conserved between zebrafish and mammals (Brandt et al., 2003; Jean<br>71 al., 2018; Lv et al., 2016  $6941.3$  and Ribeye) are conserved between zebrafish and mammals (Brandt et al., 2003; Je<br>al., 2018; Lv et al., 2016; Sidi et al., 2004). In zebrafish, hair cells are present in the inner ea<br>in the lateral-line system (Fi 71 al., 2018; Lv et al., 2016; Sidi et al., 2004). In zebrafish, hair cells are present in the inner ear and<br>72 in the lateral-line system (Fig 1A-C). These sensory systems are required for hearing and<br>73 balance or the de 121 an., 2019, 2019, 2019, 2019, 2019, 2019, 2019, 2019, 2019, 2019, 2019, 2019, 2019, 2019, 2019, 2019, 2019<br>121 in the lateral-line system (Fig 1A-C). These sensory systems are required for hearing and<br>121 balance or the in the lateral-line system (Fig 1A-C). These sensory systems are required for hearing and<br>
balance or the detection of local fluid flow, respectively (Gompel et al., 2001; Haddon and<br>
Lewis, 1996). Hair cells in the zebraf 14 Lewis, 1996). Hair cells in the zebrafish inner ear are innervated by neurons in the statoaco<br>175 banglion (SAG), while hair cells in the lateral-line system are innervated by neurons in the<br>176 anterior or posterior la 2008). The samplion (SAG), while hair cells in the lateral-line system are innervated by neurons in the<br>
26 anterior or posterior lateral-line ganglia (aLLg and pLLg) (Zecca et al., 2015).<br>
27 Our study identifies Nrxn3 as

76 anterior or posterior lateral-line ganglia (aLLg and pLLg) (Zecca et al., 2015).<br>77 Cur study identifies Nrxn3 as a presynaptic CAM required for hair-cell synapse asse<br>78 in both zebrafish and mice. In zebrafish we find Our study identifies Nrxn3 as a presynaptic CAM required for hair-cel<br>in both zebrafish and mice. In zebrafish we find that nrxn3 mutants form ~60<br>synapses in the lateral line and ~30-45% fewer ribbon synapses in the inner The study of the synapses in the lateral line and ~30-45% fewer ribbon synapses in the inner ear. We also show<br>That Nrxn3 function is conserved in mammals; 38 In both zebrafish and mice. In zebrafish we find that *mans* indicates form ~60% fewer ribbon<br>30 In synapses in the lateral line and ~30-45% fewer ribbon synapses in the inner ear. We also shot<br>30 In that Nrxn3 functio 79 19 19 that *Nrxn3* function is conserved in mammals; mice lacking NRXN3 in auditory IHCs form ~20-<br>25% fewer ribbon synapses compared to controls. In response to stimuli, we find that both pre<br>22 and post-synaptic calci 81 25% fewer ribbon synapses compared to controls. In response to stimuli, we find that both pr<br>82 and post-synaptic calcium responses in lateral-line hair cells are dramatically reduced in *nrxn3*<br>83 mutants. Surprisingly 81 25 25 Diversity our work admonstrates that nixils plays a crucial and conserved fold in holdin-synapse 83 mutants. Surprisingly, despite fewer synapses and reduced synaptic responses, we observe no<br>84 detectable deficit in the acoustic startle response, a hair-cell mediated behavior in zebrafish.<br>85 Overall, our work demons detectable deficit in the acoustic startle response, a hair-cell mediated behavior in zebrafish.<br>
85 Overall, our work demonstrates that Nrxn3 plays a crucial and conserved role in ribbon-synaps<br>
86 assembly in mice and ze Example 12 detectable definite definite transferred persons a crucial and conserved role in ribbon-synap assembly in mice and zebrafish. This knowledge will inform future research aimed to rebuild synapses and restore hear assembly in mice and zebrafish. This knowledge will inform future research aimed to rebuild<br>87 Synapses and restore hearing after auditory synaptopathy.<br>88 synapses and restore hearing after auditory synaptopathy.<br>88<br>3  $88$ <br>3

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| 89 Results<br>90 Loss of I<br>91 Neurexi<br>92 (Südhof<br>93 shown t 91 Neurexins are classic presynaptic CAMs required for synapse assembly in many c<br>92 (Südhof, 2017). Single cell RNA sequencing (scRNAseq) studies in mice and zebra<br>93 shown that *nrxn3* mRNA is enriched in hair cells, mak 91 Neurexins are classic presynaptic CAMs required for synapse assembly in many contexts<br>92 (Südhof, 2017). Single cell RNA sequencing (scRNAseq) studies in mice and zebrafish have<br>93 shown that  $nrxn3$  mRNA is enriched in

94 – assembly of ribbon synapses in hair cells (Cai et al., 2015; Elkon et al., 2015; Kolla et al., 2020;<br>95 – Lush et al., 2019; Shi et al., 2023). To determine if Nrxn3 is required for hair-cell synapse<br>96 – assembly. we 93 shown that *mxh*3 mRNA is emittied in hair cells, making it a viable candidate to drive the<br>94 assembly of ribbon synapses in hair cells (Cai et al., 2015; Elkon et al., 2015; Kolla et al., 20<br>95 Lush et al., 2019; Shi

95 Lush et al., 2019; Shi et al., 2023). To determine if Nrxn3 is required for hair-cell synapse<br>96 assembly, we used the genetically tractable zebrafish model (Fig 1A-D). In zebrafish, there are<br>97 orthologues of mammali 96 assembly, we used the genetically tractable zebrafish model (Fig 1A-D). In zebrafish, ther<br>97 orthologues of mammalian *Nrxn3*, called *nrxn3a* and *nrxn3b* (Fig 1E). Like mammalian *Nr*<br>98 both *nrxn3a* and *nrxn3b* 97 orthologues of mammalian *Nrxn3*, called *nrxn3a* and *nrxn3b* (Fig 1E). Like mammalian *Nrxn3*,<br>98 both *nrxn3a* and *nrxn3b* loci are predicted to produce two main isoforms, a long α form, and a<br>99 shorter β form (F

97 orthologues of mammalian *NYXH3*, called *HYXH3D* and *HYXH3D* (Fig 1E). Like mammalian *NYXH3*,<br>98 both *nrxn3a* and *nrxn3b* loci are predicted to produce two main isoforms, a long α form, and a<br>69 shorter β form (F 99 both *in xiisti* and *in xiisti* loci are predicted to produce two main isoforms, a long α form, and a<br>99 shorter β form (Fig 1E) (Gomez et al., 2021). For our analysis we examined the best-<br>01 forms of *nrxn3a* and

103 We next leveraged zebrafish genetics to test whether α-Nrxn3a or α-Nrxn3b is required 100 characterized α form of *mxn3b* and *mxn3b*. We first used RNA FISH, and verified that the α<br>101 forms of *nrxn3a* and *nrxn3b* mRNA are present in hair cells of the zebrafish lateral line (Fig 1<br>102 and inner ear (F 101 Forms of *mxn3b* and *mxn3b* mRNA are present in hair cens of the zebrafish lateral line (Fig 1F-H)<br>102 and inner ear (Fig S1A-B) (Choi et al., 2018).<br>103 We next leveraged zebrafish genetics to test whether α-Nrxn3a 103 We next leveraged zebrafish genetics<br>104 for the organization of ribbon synapses at 5<br>105 majority of zebrafish hair cells are mature, a<br>106 systems are functional (Kimmel et al., 1974; 104 for the organization of ribbon synapses at 5 days post fertilization (dpf). At this age, the<br>105 majority of zebrafish hair cells are mature, and both the lateral-line and inner-ear sensory<br>106 systems are functional majority of zebrafish hair cells are mature, and both the lateral-line and inner-ear senso<br>106 systems are functional (Kimmel et al., 1974; Suli et al., 2012). We obtained existing *nrxn*.<br>107 nrxn3b ENU zebrafish mutants 106 systems are functional (Kimmel et al., 1974; Suli et al., 2012). We obtained existing *nrxn3a*<br>107 *nrxn3b* ENU zebrafish mutants from the Zebrafish International Resource Center<br>108 (Kettleborough et al., 2013). Both door systems are functional (Kimmel et al., 1974, 301 et al., 2012). We obtained existing *in xifat* and<br>107 *nrxn3b* ENU zebrafish mutants from the Zebrafish International Resource Center<br>108 (Kettleborough et al., 2013) 108 (Kettleborough et al., 2013). Both alleles have early stop codons that are predicte<br>109 specifically disrupt the long  $\alpha$  form of each orthologue (Fig 1E). For simplicity, we<br>110 and Nrxn3b from here on to refer to t 109 specifically disrupt the long  $\alpha$  form of each orthologue (Fig 1E). For simplicity, we use l<br>110 and Nrxn3b from here on to refer to the long  $\alpha$  form of each Nrxn3 orthologue, unless<br>111 otherwise specified.<br>112 We 110 and Nrxn3b from here on to refer to the long α form of each Nrxn3 orthologue, unless<br>111 otherwise specified.<br>112 We first assessed the organization of ribbon synapses in lateral-line hair cells in our<br>113 *nrxn3* mu

111 otherwise specified.<br>112 be first assessed the organization of ribbon synapses in lateral-line hair cells in<br>113 nrxn3 mutants in mature hair cells at 5 dpf. For this assessment, we used<br>114 immunohistochemistry to vi 112 We first asses<br>113 *nrxn3* mutants in ma<br>114 immunohistochemist<br>115 (Fig 1C). After immun nth 113 Arthur Antistandary Correct as the original of the original of the original of the original of the organization of the immunohistochemistry to visualize presynapses (pan-CTBP) and postsynapses (pan-MAGUK (Fig 1C). 113 *mans* mutants in mature han cens at 5 upr. For this assessment, we used<br>114 immunohistochemistry to visualize presynapses (pan-CTBP) and postsyna<br>115 (Fig 1C). After immunostaining, we quantified the number of complet 115 (Fig 1C). After immunostaining, we quantified the number of complete synapses (paired CTBF<br>116 MAGUK puncta), unpaired presynapses (lone CTBP puncta) and unpaired postsynapses (lone<br>117 MAGUK puncta). Using this approa

116 MAGUK puncta), unpaired presynapses (lone CTBP puncta) and unpaired postsynapses (lone<br>117 MAGUK puncta). Using this approach, we found that there was a slight yet significant reduction<br>4

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i<br>;<br>; 118 in the number of complete ribbon synapses per hair cell in  $nrxn3a$  or  $nrxn3b$  single mutants<br>
119 compared to controls (Fig S3A-B; 18.5% reduction in  $nrxn3a$  mutants and 24% reduction in<br>
120  $nrxn3b$  mutants). In  $nrxn$ 119 compared to controls (Fig 33A-B; 18.5% reduction in *nrxn3d* mutants and 24% reduction in<br>120 *nrxn3b* mutants). In *nrxn3b* mutants, but not in *nrxn3a* mutants, we observed significantly in<br>121 unpaired presynapses c 120 *mando* mutants). In *mando* mutants, but not in *mando* mutants, we observed significantly more<br>121 unpaired presynapses compared to controls (Fig S3C). In contrast, we did not observe a<br>122 difference in the number o difference in the number of unpaired postsynapses in either *nrxn3a* or *nrxn3b* mutants (Fig<br>
123 S3D). Despite no change in unpaired postsynapses, both *nrxn3a* and *nrxn3b* single mutants<br>
124 exhibited a significant re 122 difference in the number of unpaired postsynapses in either *inxh3d* of *inxh3b* mutants (Fig<br>
123 S3D). Despite no change in unpaired postsynapses, both *nrxn3a* and *nrxn3b* single mutants<br>
124 exhibited a significan 123 S3D). Despite no change in unpaired postsynapses, both *inxrition* and *inxrition* single mutants<br>124 exhibited a significant reduction in the total number of postsynapses (paired and unpaired)<br>125 compared to controls 125 compared to controls (Fig S3D). Overall, this analysis indicates that loss of either Nrxn3a or<br>126 Nrxn3b results in fewer complete synapses in mature lateral-line hair cells. In addition, this<br>127 assessment suggests 126 Nrxn3b results in fewer complete synapses in mature lateral-line hair cells. In addition, this<br>127 assessment suggests that zebrafish hair cells may rely on both Nrxn3a and Nrxn3b for prope<br>128 pre- and post-synaptic p assessment suggests that zebrafish hair cells may rely on both Nrxn3a and Nrxn3b for prope<br>128 pre- and post-synaptic pairing and synapse organization.<br>129 As each single mutant exhibited modest defects in ribbon-synapse o

128 pre- and post-synaptic pairing and synapse organization.<br>
129 as each single mutant exhibited modest defects in ribbon-synapse organization, we<br>
130 tested whether Nrxn3a and Nrxn3b have overlapping contributions by ex 129 As each single mutant exhibited modest defects in<br>130 tested whether Nrxn3a and Nrxn3b have overlapping con<br>131 organization of synapses in lateral-line hair cells of nrxn3d<br>132 2A-F). Importantly, nrxn3a; nrxn3b neuro 129 129 129 As each single mutatic modes in the correlation of synapsis 130 131 12<br>130 129 as each single mutant exhibited modest defects of the contributions by examining the<br>131 122 124-F). Importantly, *nrxn3a; nrxn3b* organization of synapses in lateral-line hair cells of *nrxn3a; nrxn3b* double mutants at<br>132 12A-F). Importantly, *nrxn3a; nrxn3b* neuromasts were grossly normal, and the number<br>133 cells per neuromast was unchanged (Fig 2A-F). Importantly, *nrxn3a; nrxn3b* neuromasts were grossly normal, and the number of hair<br>132 cells per neuromast was unchanged (Fig 2G). However, we observed a ~60% reduction in the<br>134 number of complete ribbon synaps 132 2A-F). Importantly, *ITARISB* neuromasts were grossly normal, and the number of hair<br>133 cells per neuromast was unchanged (Fig 2G). However, we observed a ~60% reduction in the<br>134 number of complete ribbon synapses 133 cells per neuromast was unchanged (Fig 2G). However, we observed a ~60% reduction in the<br>
134 number of complete ribbon synapses per hair cell in  $nrxn3a$ ;  $nrxn3b$  mutants compared to<br>
135 controls (Fig 2A-F, H; synaps 134 Indition of complete ribbon synapses per hair cell in  $m$ xn3d,  $m$ xn3d mutants compared to<br>135 controls (Fig 2A-F, H; synapses per hair cell, control: 3.19 ± 0.09, nrxn3a;nrxn3b: 1.3 ± 0.09,<br>136 0.0001). We also obser 135 controls (Fig 2A-F,H; synapses per hanceli, control: 3.13 ± 0.09, mxh3a;mxh3b: 1.3 ± 0.09, p <<br>136 0.0001). We also observed a dramatic increase in the number of unpaired pre- and post-<br>137 synapses per cell in *nrxn3a* 140 cell (Fig 2J). In contrast, we found that the total number of presynapses per cell was unchanged 137 synapses per cell in *mansu, mansb* mutants compared to control (Fig 2F3). This result is<br>138 especially striking for postsynapses, as we observed an increase in unpaired postsynapse<br>139 despite a significant decrease especially striking for postsynapses, as we observed an increase in unpaired postsynapses<br>
139 despite a significant decrease in the total number (paired and unpaired) of postsynapses per<br>
140 cell (Fig 2J). In contrast, w 140 cell (Fig 2J). In contrast, we found that the total number of presynapses per cell was unchang<br>141 (Fig 2I). Overall, this indicates that in mature hair cells, loss of Nrxn3 results in a dramatic<br>142 decrease in ribbon 141 (Fig 2I). Overall, this indicates that in mature hair cells, loss of Nrxn3 results in a dramatic<br>142 decrease in ribbon-synapse numbers and a disruption in pre- and post-synaptic pairing.<br>143 Within lateral-line neurom

142 decrease in ribbon-synapse numbers and a disruption in pre- and post-synaptic pairing.<br>143 Within lateral-line neuromasts, there are hair cells with two different orientations<br>144 distinct populations detect fluid flow 142 distinct populations detect fluid flow in two opposing directions (for example, in the prince distinct populations detect fluid flow in two opposing directions (for example, in the prince neuromasts of the pLL, flow fr distinct populations detect fluid flow in two opposing directions (for example, in the primary<br>145 meuromasts of the pLL, flow from anterior to posterior (A to P) and flow from posterior to<br>146 anterior (P to A); see Fig S 145 neuromasts of the pLL, flow from anterior to posterior (A to P) and flow from posterior to<br>146 anterior (P to A); see Fig S4A). Because neurexins are also implicated in synapse selectivity<br>5 145 neuromasts of the pLL, flow from anterior to posterior (A to P) and flow from posterior to<br>146 anterior (P to A); see Fig S4A). Because neurexins are also implicated in synapse selectivity<br>5  $146$  anterior (P to  $\eta$ ); see Fig. S4A). Because neurexins are also implicated in synapse selectivity,

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| 147 (Gomez et al., 2021, Sudhof, 2017), we assessed whether the synaptic defects in *mxh5d*, *mxh5b*<br>148 mutants were present in hair cells of a specific orientation. We found that synapse loss was the<br>149 same across the 148 mutants were present in hair cells of a specific orientation. We found that synapse loss was the<br>
149 same across the two populations of hair cells in  $nrxn3a$ ;  $nrxn3b$  mutants (Fig S4B). These results<br>
150 show that N 149 same across the two populations of han cells in *manou, manos matants* (Fig 34B). These results<br>150 show that Nrxn3 is critical for ribbon-synapse organization in hair cells of both orientations<br>151 within the lateral

153 inner ear. We examined hair cells in the zebrafish utricle (balance organ) and the medial crista<br>154 (one of three organs detecting angular acceleration) (example images, Fig S5A-D). Within both 152 We also extend<br>153 inner ear. We examine<br>154 (one of three organs d<br>155 inner-ear epithelia, we 152 We also extended our synapse analysis of *inxh3d*, *inxh3b* mutants to han cells in the<br>153 inner ear. We examined hair cells in the zebrafish utricle (balance organ) and the medial crist<br>154 (one of three organs detec 154 (one of three organs detecting angular acceleration) (example images, Fig S5A-D). Within both<br>155 inner-ear epithelia, we found that there were significantly fewer complete synapses per hair<br>156 cell and significantly 157 compared to controls (Fig S6). Overall, our analyses demonstrate that in zebrafish, both *nrxn3a*<br>158 and *nrxn3b* are expressed in hair cells. In addition, Nrxn3a and Nrxn3b are essential for pre- and cell and significantly more unpaired presynapses and postsynapses in *nrxn3a; nrxn3b* mutants<br>157 compared to controls (Fig S6). Overall, our analyses demonstrate that in zebrafish, both *nrxn3a*<br>158 and *nrxn3b* are expre 156 cen and significantly more differed presynapses and postsynapses in mariod, mariod indicates<br>157 compared to controls (Fig S6). Overall, our analyses demonstrate that in zebrafish, both *nrxn3c*<br>158 and *nrxn3b* are ex 157 compared to controls (Fig So). Overall, our analyses demonstrate that in zebrafish, both malibum<br>158 and *nrxn3b* are expressed in hair cells. In addition, Nrxn3a and Nrxn3b are essential for pre- and<br>160 inner ear.<br>16 158 and *mxn3b* are expressed in hancens. In addition, Nrxn3a and Nrxn3b are essential for pre- and<br>159 post-synaptic pairing and ribbon-synapse organization in mature hair cells of the lateral line and<br>161 **Nrxn3** is requ 160 inner ear.<br>161<br>162 **Nrxn3 is required early in zebrafish hair-cell synapse assembly**<br>163 Our results show that loss of Nrxn3 can dramatically impact the pairing of synaptic components

161<br>162 **Nrxn3 is re**<br>163 Our result:<br>164 in hair cell 163<br>164<br>165 162 Nrxn3 is required early in zebrafish hair-cell synapse assembly<br>163 Our results show that loss of Nrxn3 can dramatically impact the<br>164 in hair cells (Figs 2, S5, S6). For these initial analyses, we examine<br>165 stage w in hair cells (Figs 2, S5, S6). For these initial analyses, we examined zebrafish hair cells at 5 dpf, a<br>165 stage where the majority of hair cells are mature. Because hair cells are already mature at this<br>166 stage, it is 165 stage where the majority of hair cells are mature. Because hair cells are already mature at this<br>166 stage, it is difficult to interpret whether Nrxn3 is required early to assemble synapses, or later in<br>167 development 166 stage, it is difficult to interpret whether Nrxn3 is required early to assemble synapses, or later if development to maintain or refine synapse numbers. Therefore, we examined hair cells in zebrafish at 3 dpf, a stage 167 development to maintain or refine synapse numbers. Therefore, we examined hair cells in<br>168 zebrafish at 3 dpf, a stage when hair cells are still developing, and sensory systems are not yet<br>169 functional. Using immuno 20168 zebrafish at 3 dpf, a stage when hair cells are still developing, and sensory systems are not yet<br>169 functional.<br>170 Using immunostaining, we quantified the number of complete synapses and unpaired<br>171 pre- and post

169 functional.<br>169 functional.<br>170 Using immunostaining, we quantified the number of complete synapses and unpaired<br>171 pre- and post-synapses in developing hair cells at 3 dpf in nrxn3a; nrxn3b mutants (example<br>172 image 170 Usin<br>171 pre- and po<br>172 images, Fig<br>173 significant, 171 pre- and post-synapses in developing hair cells at 3 dpf in nrxn3a; nrxn3b mutants (example<br>172 images, Fig 3A-F). We found that developing hair cells in nrxn3a; nrxn3b mutants showed a<br>173 ignificant, ~35% reduction i 171 pre- and post-synapses in developing handens at 3 dpf in *mxn3d*, *mxn3b* mutants (example<br>172 images, Fig 3A-F). We found that developing hair cells in *nrxn3a; nrxn3b* mutants showed a<br>173 ignificant, ~35% reduction 172 images, Fig 3A-F). We found that developing han tens in *mxh3d*, *mxh3b* mutants showed a<br>173 ignificant, ~35% reduction in the number of complete ribbon synapses per hair cell compai<br>174 to controls (Fig 3G-H). Althou 173 significant, ~35% reduction in the number of complete ribbon synapses per hair cell compared<br>174 to controls (Fig 3G-H). Although significant, this reduction in complete synapses is less dramatic<br>175 than the ~60% red 175 than the ~60% reduction observed in mature hair cells (Fig 2). In developing hair cells, we also<br>6  $175$  than the cells (Fig 2). In developing hair cells, we also than  $\frac{1}{2}$ .

י<br>ו<br>נ observed a dramatic increase in the number of unpaired presynapses (Fig 3I) and a more<br>
177 modest increase in unpaired postsynapses (Fig 3J). In contrast to mature hair cells at 5 dpf, in<br>
178 developing hair cells we did 176 178 developing hair cells we did not observe a reduction in the total number of postsynapses per<br>179 hair cell (Fig 3J). More complete synapses and a normal number of postsynapses at 3 dpf<br>180 suggests that there may be sy the complete synapses and a normal number of postsynapses at 3 dpf<br>180 suggests that there may be synapses that are able to initially pair, but later fall apart, in nrxn3<br>181 nrxn3b mutants. Overall, this analysis indicate 180 suggests that there may be synapses that are able to initially pair, but later fall apart, in n.<br>
181 nrxn3b mutants. Overall, this analysis indicates that Nrxn3 plays an early role in synapse<br>
182 assembly but may als 181 *nrxn3b* mutants. Overall, this analysis indicates that Nrxn3 plays an early role in synapse<br>182 assembly but may also play a later role in synapse maintenance in lateral-line hair cells.<br>183 Nrxn3 alters pre- and post 181 *mando mutants.* Overall, this analysis indicates that Nrand plays an early role in synapse<br>182 assembly but may also play a later role in synapse maintenance in lateral-line hair cells.<br>183 **Nrxn3 alters pre- and pos** 

183<br>183 **Arxn3 alters pre- and post-synapse size and Ca<sub>V</sub>1.3 channel distribution in zebrafish**<br>185 Studies in the central nervous system have found that loss of neurexins can alter the<br>186 morphology and clustering of p 183<br>184<br>185<br>186<br>187 184 IST NRXIIS alters pre- and post-synapse size and Cay1.3 channel distribution in zebrafish<br>185 Studies in the central nervous system have found that loss of neurexins can alter the<br>186 Inorphology and clustering of pre-186 morphology and clustering of pre- and post-synaptic components. For example, previous in neurons has shown that loss of neurexins can alter presynapse morphology and size<br>188 impact the clustering of presynaptic calciu 187 in neurons has shown that loss of neurexins can alter presynapse morphology and size, and<br>188 impact the clustering of presynaptic calcium channels (Brockhaus et al., 2018; Luo et al., 2020;<br>189 Uemura et al., 2022). 187 in neurons has shown that loss of neurexins can alter presynapse morphology and size, and<br>
188 impact the clustering of presynaptic calcium channels (Brockhaus et al., 2018; Luo et al., 2020;<br>
189 Uemura et al., 2022) 189 Uemura et al., 2022). Therefore, we next examined the morphology of synaptic components,<br>190 including presynapses, postsynapses and Ca<sub>V</sub>1.3 channels, in *nrxn3a; nrxn3b* mutants at 5 dpf.<br>191 We examined maximum int 190 including presynapses, postsynapses and Ca<sub>V</sub>1.3 channels, in *nrxn3a; nrxn3b* mutants at 5 dpf.<br>191 We examined maximum intensity projections and quantified the 2D area and average intensit<br>192 of these synaptic comp 190 Including presynapses, postsynapses and cav1.3 channels, in *mxh3d*, *in xh3b* mutants at 3 dpf.<br>191 We examined maximum intensity projections and quantified the 2D area and average intensity<br>192 of these synaptic comp 192 of these synaptic components. For our analysis, we examined the size of paired and unpaired<br>193 synaptic components separately. We predicted that if Nrxn3 is important for synaptic<br>194 organization, loss of Nrxn3 might 192 of these synaptic components separately. We predicted that if Nrxn3 is important for synaptic<br>194 organization, loss of Nrxn3 might specifically impact the architecture of paired synapses.<br>195 We first examined how los

organization, loss of Nrxn3 might specifically impact the architecture of paired synaps<br>195 We first examined how loss of Nrxn3 impacts the average area (size) of presyn<br>196 puncta (CTBP). We found that the average size of 195 We first examined how loss of Nrxn3 impacts the average area (size) of presynapt<br>196 puncta (CTBP). We found that the average size of paired presynapses (paired CTBP-MAGI<br>197 puncta) was significantly increased in *nrx* puncta (CTBP). We found that the average size of paired presynapses (paired CTBP-MAGUK<br>197 puncta) was significantly increased in *nrxn3a; nrxn3b* mutants compared to controls (Fig 4A<br>198 We then examined the size of the u puncta (CTBP). We found that the average size of paired presynapses (paired CTBP-MAGUK<br>
puncta) was significantly increased in *nrxn3a; nrxn3b* mutants compared to controls (Fig 4A).<br>
We then examined the size of the unpai 197 puncta) was significantly increased in *mansu, mansu mutantis* compared to controls (Fig 4A).<br>198 We then examined the size of the unpaired presynapses in *nrxn3a; nrxn3b* mutants, and foun<br>199 they were a similar size 198 We then examined the size of the unpaired presynapses in *mxh5d*, *mxh5b* mutants, and found<br>199 they were a similar size compared to controls (Fig 4B). We also examined how loss of Nrxn3<br>200 impacts that size of posts 200 impacts that size of postsynaptic puncta (MAGUK). We found that the average size of paired<br>201 postsynapses (paired CTBP-MAGUK puncta) were significantly larger in *nrxn3a; nrxn3b* mutar<br>202 while unpaired postsynapses postsynapses (paired CTBP-MAGUK puncta) were significantly larger in *nrxn3a; nrxn3b* mutants,<br>202 while unpaired postsynapses were a similar size compared to controls (Fig 4C,D). Altogether,<br>203 this analysis indicates th 201 postsynapses (paired CTBP-MAGOK puncta) were significantly larger in *mxh5d*, *mxh5b*-mutants,<br>202 while unpaired postsynapses were a similar size compared to controls (Fig 4C,D). Altogether,<br>203 this analysis indicate 202 while unpaired posts jumpers were a similar size complete to control (Fig 1C) experiences,<br>202 this analysis indicates that in addition to a decrease in the number of complete synapses,<br>204 nrxn3a; nrxn3b mutants form 204 *nrxn3a; nrxn3b* mutants form larger pre- and post-synapses compared to controls. This su<br>
204 **number of complete synapses** of controls. This sure of complete synapses of complete synapses compared to controls. This 204 nrxn3a; nrxn3b mutants form larger pre- and post-synapses compared to controls. This suggests

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205 that synapses do not properly coalesce in *mxn5d*, *mxn5b* double mutants, even when properly<br>206 paired.<br>207 Previous work has shown that in hair cells, presynaptic Ca<sub>V</sub>1.3 channel distribution is<br>208 shaped by pres 207<br>207<br>208 shaped<br>209 area an<br>210 and dist 207 Previous work has shown that in hair cells, presynaptic Ca<sub>V</sub>1.3 channel distribution is<br>
208 shaped by presynapse size (Sheets et al., 2017). In our present study, we found that presynapse<br>
209 area and number was al 209 area and number was altered in  $nrxn3a$ ;  $nrxn3b$  mutants. Therefore, we examined the number<br>210 and distribution of Ca<sub>V</sub>1.3 channel puncta in  $nrxn3a$ ;  $nrxn3b$  using an immunostain to label<br>211 presynapses (CTBP) and C 209 area and number was artered in *mxnod*, *mxnob* matalitis. Therefore, we examined the number<br>210 and distribution of Ca<sub>V</sub>1.3 channel puncta in *nrxn3a; nrxn3b* using an immunostain to label<br>211 presynapses (CTBP) and 210 and distribution of Cay1.3 channel puncta in *mxh3d*, *mxh3b* using an immultiostant to label<br>211 presynapses (CTBP) and Ca<sub>V</sub>1.3 channels. We first examined the number of presynapses pa<br>212 with Ca<sub>V</sub>1.3 puncta (exam 212 with Ca<sub>V</sub>1.3 puncta (example images, Fig 4E,G). We found a similar number of CTBP-Ca<sub>V</sub>1.3<br>213 paired puncta per hair cell in *nrxn3a; nrxn3b* mutants compared to controls (Fig 4I). We then<br>214 examined the number of 212 with Ca<sub>V</sub>1.3 puncta (example images, Fig 4E,G). We found a similar number of CTBP-Ca<sub>V</sub>1.3<br>213 paired puncta per hair cell in *nrxn3a; nrxn3b* mutants compared to controls (Fig 4I). We then<br>214 examined the number of 213 paired puncta per half cell in *malibu*, *malibb* mutants compared to controls (Fig 4I). We then<br>214 examined the number of postsynapses paired with Ca<sub>V</sub>1.3 puncta using an immunostain to la<br>215 postsynapses (MAGUK) 215 postsynapses (MAGUK) and Ca<sub>V</sub>1.3 channels (example images, Fig 4F, H). Here we found a<br>216 reduced number of MAGUK-Ca<sub>V</sub>1.3 paired puncta per hair cell in *nrxn3a; nrxn3b* double<br>217 mutants compared to controls (Fig 216 reduced number of MAGUK-Ca<sub>V</sub>1.3 paired puncta per hair cell in *nrxn3a; nrxn3b* double<br>217 mutants compared to controls (Fig 4J). This latter reduction mirrors the reduction in comp<br>218 synapses in *nrxn3a; nrxn3b* m 216 reduced number of MAGOK CaV1.3 paired puncta per hancen in *mansu, mansu* double<br>217 mutants compared to controls (Fig 4J). This latter reduction mirrors the reduction in com<br>218 synapses in *nrxn3a; nrxn3b* mutants ( 218 synapses in  $nrxn3a$ ;  $nrxn3b$  mutants (Fig 2H). Our examination of Ca<sub>V</sub>1.3 pairing indicates that<br>219 Nrxn3 is not required for presynapses to couple with Ca<sub>V</sub>1.3 channels. Instead Nrxn3 may be<br>220 required to pair a 218 Synapses in *mxh3d*, *mxh3b* mutants (Fig 2H). Our examination of Ca<sub>V</sub>1.3 pairing indicates that<br>219 Nrxn3 is not required for presynapses to couple with Ca<sub>V</sub>1.3 channels. Instead Nrxn3 may be<br>220 required to pair a 220 required to pair a presynapse and its associated  $Ca_V1.3$  channels to an adjacent postsynapse.<br>221 Lastly, we examined the size and distribution of  $Ca_V1.3$  channels within each CTBP-<br>222  $Ca_V1.3$  paired puncta. We found 221 Lastly, we examined the size and distribution of Ca<sub>V</sub>1.3 channels within each CTBP-<br>222 Ca<sub>V</sub>1.3 paired puncta. We found that the area of Ca<sub>V</sub>1.3 puncta was significantly reduced in<br>223 nrxn3a; nrxn3b double mutants 221 Lastly, we examined the size and distribution of Ca<sub>V</sub>1.3 channels within each CTBP-<br>222 Ca<sub>V</sub>1.3 paired puncta. We found that the area of Ca<sub>V</sub>1.3 puncta was significantly reduced in<br>223 nrxn3a; nrxn3b double mutants 223 *nrxn3a; nrxn3b* double mutants compared to controls (Fig 4K). Although we observed smalle<br>224 Ca<sub>V</sub>1.3 puncta, the average intensity of each Ca<sub>V</sub>1.3 punctum was unchanged in *nrxn3a; nrx*<br>225 mutants compared to con 223 *manod, manob* double mutants compared to controls (Fig 4K). Although we observed shaller<br>224 Ca<sub>V</sub>1.3 puncta, the average intensity of each Ca<sub>V</sub>1.3 punctum was unchanged in *nrxn3a; nrxn3*<br>225 mutants compared to co 224 Cav1.5 puncta, the average intensity of each Cav1.5 punctum was unchanged in *mxn5u*, *mxn5b*<br>225 mutants compared to controls (Fig 4L). This indicates that on average, fewer Ca<sub>V</sub>1.3 channels<br>226 may reside within ea 226 may reside within each Ca<sub>v</sub>1.3 puncta in *nrxn3a; nrxn3b* mutants compared to controls.<br>
227 Together, this analysis of synapse morphology demonstrates that in addition to proper pre- and<br>
228 post-synapse pairing, N 228 may reside within each Cay1.5 paneta in mansa, mansa mitatins compared to controls.<br>227 Together, this analysis of synapse morphology demonstrates that in addition to proper post-synapse pairing, Nrxn3 is important to 228 post-synapse pairing, Nrxn3 is important to establish proper pre- and post-synapse size.<br>229<br>230 **Nrxn3 plays a conserved role in synapse organization in mice**<br>231 Many of the core genes required at hair-cell synapses

230 **Nrxn3 plays a conserved role in synapse organization in mice**<br>231 Many of the core genes required at hair-cell synapses are conserved between zebrafish and<br>232 mammals (Sheets et al., 2021). Like zebrafish hair cells, 230<br>231<br>232<br>233 230 Nrxn3 plays a conserved role in synapse organization in mice<br>231 Many of the core genes required at hair-cell synapses are cons<br>232 mammals (Sheets et al., 2021). Like zebrafish hair cells, scRNAs<br>233 that *Nrxn3* is e mammals (Sheets et al., 2021). Like zebrafish hair cells, scRNAseq studies in mice have shown<br>that *Nrxn3* is expressed in hair cells in both the auditory and vestibular system (Cai et al., 2015<br>8 that Nrxn3 is expressed in hair cells in both the auditory and vestibular system (Cai et al., 2015; 233 that Nrxn3 is expressed in hair cells in both the auditory and vestibular system (Cai et al., 2015;

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9235 organization has not yet been demonstrated.<br>
236 To determine whether Nrxn3 is required for synapse assembly in mice we used a<br>
237 conditional inactivation strategy. The Nrxn3<sup>flox</sup> strain (Aoto et al., 2015) was b 236 To determine whether Nrxn3 is require<br>237 conditional inactivation strategy. The Nrxn3<sup>flox</sup><br>238 Atoh1-Cre driver (Matei et al., 2005) to abroga<br>239 postmitotic hair cells. We examined ribbon sy 237 conditional inactivation strategy. The *Nrxn3<sup>flox</sup>* strain (Aoto et al., 2015) was bred with the<br>238 Atoh1-Cre driver (Matei et al., 2005) to abrogate both the  $\alpha$ - and  $\beta$ -isoforms of NRXN3 in<br>239 postmitotic hair 237 conditional inactivation strategy. The *Nrxn3<sup>nox</sup>* strain (Aoto et al., 2015) was bred with the<br>238 Atoh1-Cre driver (Matei et al., 2005) to abrogate both the α- and β-isoforms of NRXN3 in<br>239 postmitotic hair cells 238 Atoh1 Cre driver (Matei et al., 2005) to abrogate both the α- and β-isoforms of NRXN3 in<br>239 postmitotic hair cells. We examined ribbon synapses in auditory inner hair cells (IHCs) of *Cre; Nrxn<sup>flox/flox</sup>* mutants ( 239 postmitotic hair cells. We examined ribbon synapses in auditory inner hair cells (IHCs) of Atoh1-<br>240 Cre; Nrxn<sup>flox/flox</sup> mutants (Nrxn3 mutants) and control animals by immunolabeling both<br>241 presynapses (CTBP2) and Cre; Nrxn<sup>noxynox</sup> mutants (Nrxn3 mutants) and control animals by immunolabeling both<br>241 presynapses (CTBP2) and postsynapses (GluR2). We examined IHCs at 4 (P28) and 6 (P4<br>242 of age. At both ages we found a significant 242 of age. At both ages we found a significant reduction (20-25%) in the number of complete<br>243 synapses (paired CTBP2-GluR2 puncta) per IHC in 3 distinct tonotopic regions of the mouse<br>244 cochlea (apex, mid and base thi synapses (paired CTBP2-GluR2 puncta) per IHC in 3 distinct tonotopic regions of the mouse<br>cochlea (apex, mid and base thirds) in *Nrxn3* mutants compared to controls (Fig 5A-E, Fig S7A-<br>E). These results indicate that NRXN cochlea (apex, mid and base thirds) in *Nrxn3* mutants compared to controls (Fig 5A-E, Fig S7A-<br>245 E). These results indicate that NRXN3 plays a conserved role in synapse organization in mouse<br>246 HCs.<br>247 244 cochiea (apex, mid and base thirds) in *WXN3* mutants compared to controls (Fig 5A-E, Fig 57A-<br>245 E). These results indicate that NRXN3 plays a conserved role in synapse organization in mouse<br>247<br>248 Nrxn3 disrupts ri

246 EIHCs.<br>246 EIHCs.<br>247 **E). These results indicate that NRXN3 plays a conserved role in synapse organization**<br>249 After verifying that Nrxn3 is essential for proper synapse organization in both zebrafish and 247<br>248 **Nrxn**3<br>249 After<br>250 mice, 248<br>249<br>250<br>251 248 Nrxn3 disrupts ribbon-synapse runction in han cells in zebrafish<br>249 After verifying that Nrxn3 is essential for proper synapse organiza<br>250 mice, we next assessed the functional impact of fewer synapses. I<br>251 establi 249 After verifying that Nrxn3 is essential for proper synapse organization in both zebrafish and<br>250 mice, we next assessed the functional impact of fewer synapses. Previous work from our group<br>251 established assays to a established assays to assess hair-cell function in living zebrafish (Lukasz and Kindt, 2018). These<br>assays rely on transgenic fish expressing GCaMP6s in hair cells or afferent neurons of the<br>lateral-line system (Zhang et a 252 assays rely on transgenic fish expressing GCaMP6s in hair cells or afferent neurons of the<br>253 lateral-line system (Zhang et al., 2018). A fluid jet is used to stimulate hair cells, and GCaMP6s-<br>254 dependent calcium s lateral-line system (Zhang et al., 2018). A fluid jet is used to stimulate hair cells, and GCaMP6s-<br>254 dependent calcium signals are imaged during stimulation. To assay mechanotransduction and<br>255 presynaptic calcium resp 257 laternalioselisoly hall buildies and at the presynapse (Fig OA, Fig SoA (Zhang et al., 2016)). To 255 presynaptic calcium responses, we used a transgenic line expressing a membrane-localized<br>256 GCaMP6s (memGCaMP6s) in lateral-line hair cells. We imaged calcium signals both at the<br>257 mechanosensory hair bundles and at 256 GCaMP6s (memGCaMP6s) in lateral-line hair cells. We imaged calcium signals both at the<br>257 mechanosensory hair bundles and at the presynapse (Fig 6A; Fig S8A (Zhang et al., 2018)). T<br>258 assay postsynaptic activity, we 257 mechanosensory hair bundles and at the presynapse (Fig 6A; Fig S8A (Zhang et al., 2018)).<br>258 assay postsynaptic activity, we used a transgenic line expressing GCaMP6s in afferent neural-<br>259 and imaged calcium signals 258 assay postsynaptic activity, we used a transgenic line expressing GCaMP6s in afferent neurons<br>259 and imaged calcium signals in the afferent terminals beneath lateral-line hair cells (Fig 6A<br>260 (Zhang et al., 2018)).<br> 259 and imaged calcium signals in the afferent terminals beneath lateral-line hair cells (Fig 6A<br>260 (Zhang et al., 2018)).<br>261 Using GCaMP6s in hair cells, we first assessed whether *nrxn3a; nrxn3b* mutants have<br>262 norma

260 (Zhang et al., 2018)).<br>261 Using GCaMP6s in hair cells, we first assessed whether nrxn3a; nrxn3b mutants hair<br>262 normal mechanotransduction (i.e. normal ability to detect sensory stimuli). We found that 261 (Enang et al., 2022),<br>261 Using GCaMP<br>262 normal mechanotran 261 Using Geam os in han cells, we first assessed whether *mxh3d*, *mxh3b* mutants have<br>262 normal mechanotransduction (i.e. normal ability to detect sensory stimuli). We found that the<br>9 262 normal mechanotransduction (i.e. normal ability to detect sensory stimuli). We found that the

r<br>,<br>; 263 264 *nrxn3a; nrxn3b* mutants compared to controls (Fig S8B-C). After verifying normal<br>265 mechanotransduction, we next assessed the magnitude of evoked GCaMP6s signals measured in the presynaptic region of hair cells. We f 264 *manda, manda* mutants compared to controls (Fig 38B-C). After verifying normal<br>265 mechanotransduction, we next assessed the magnitude of evoked GCaMP6s signi<br>266 in the presynaptic region of hair cells. We found that 266 in the presynaptic region of hair cells. We found that the magnitude of presynaptic GCaMP6s<br>267 signals was reduced by ~35% in *nrxn3a; nrxn3b* mutants compared to controls (Fig 6C-F and Fig<br>268 S9A-F). These signals w 266 in the presynaptic region of hair cells. We found that the magnitude of presynaptic GCaMP6s<br>267 signals was reduced by ~35% in *nrxn3a; nrxn3b* mutants compared to controls (Fig 6C-F and Fig<br>268 S9A-F). These signals 267 signals was reduced by 35% in mailsd, mails indicated compared to controls (Fig 6C-F and Fig<br>268 S9A-F). These signals were measured at the level of individual hair cells, not individual synapses<br>269 Thus, a reduction 269 Thus, a reduction in presynaptic calcium signals could be due to fewer complete synapses,<br>270 smaller presynapses, altered calcium channel density, or some combination of these<br>271 possibilities.<br>272 lt is important to

270 Smaller presynapses, altered calcium channel density, or some combination of these<br>271 possibilities.<br>272 It is important to understand how changes in presynaptic calcium activity are reflec<br>273 in the afferent neurons 271 possibilities.<br>272 let is important to understand how changes in presynaptic calcium activity are<br>273 in the afferent neurons, as afferents encode information that is ultimately carried to<br>274 downstream circuitry. The 272 It is in<br>273 in the afferer<br>274 downstream<br>275 terminals of 273 In the afferent neurons, as afferents encode information that is ultimately carried to the<br>274 downstream circuitry. Therefore, we next examined evoked GCaMP6s signals in the afferent<br>275 terminals of *nrxn3a; nrxn3b* 273 in the afferent neurons, as afferents encode information that is ultimately carried to the<br>274 downstream circuitry. Therefore, we next examined evoked GCaMP6s signals in the afferent<br>275 terminals of  $nrxn3a$ ;  $nrxn3b$ 275 terminals of *nrxn3a; nrxn3b* mutants. We observed a ~50% reduction in the magnitude of<br>276 evoked GCaMP6s signals in the terminals of *nrxn3a; nrxn3b* mutants compared to controls (F<br>277 6G-J and Fig S9G-L). Overall, evoked GCaMP6s signals in the terminals of *nrxn3a; nrxn3b* mutants compared to controls<br>277 6G-J and Fig S9G-L). Overall, our calcium imaging experiments indicate that the synapse lo<br>278 *nrxn3a; nrxn3b* mutants result in 276 evoked Gcami os signals in the terminals of *mxn5d*, *mxn5b* mutants compared to controls (Fig.<br>277 6G-J and Fig S9G-L). Overall, our calcium imaging experiments indicate that the synapse loss in<br>278 *nrxn3a; nrxn3b* m

nrxn3a; nrxn3b mutants result in a dramatic reduction in afferent terminal responses.<br>
279 After assessing how synapse loss in nrxn3a; nrxn3b mutants impacts synapse function,<br>
280 we next examined how these deficits impac 278 *ITARISB* mutants result in a dramatic reduction in afferent terminal responses.<br>279 After assessing how synapse loss in *nrxn3a; nrxn3b* mutants impacts synapse fu<br>280 we next examined how these deficits impacted beha 279 After assessing now synapse loss in *mansu, mansu* mutants impacts synapse function,<br>280 we next examined how these deficits impacted behavior. One robust behavioral assay to asses<br>281 hair-cell function in zebrafish i 281 hair-cell function in zebrafish is the acoustic startle response. In response to an<br>282 acoustic/vibrational stimulus, zebrafish will exhibit a stereotyped startle response (Kimmel et<br>283 al., 1974). This behavior reli acoustic/vibrational stimulus, zebrafish will exhibit a stereotyped startle response (Kimmel et<br>al., 1974). This behavior relies on hair cells in both the inner ear and the lateral line and is<br>appropriate to use because we 283 al., 1974). This behavior relies on hair cells in both the inner ear and the lateral line and is<br>284 appropriate to use because we observed synapse loss in  $nrxn3a$ ;  $nrxn3b$  mutants in both<br>285 sensory systems (Figs 2, 284 appropriate to use because we observed synapse loss in  $nrxn3a$ ;  $nrxn3b$  mutants in both sensory systems (Figs 2, Fig S5, S6). For an in-clutch comparison, we compared  $nrxn3a$ ;  $nrx$  double mutants to  $nrxn3a^{+/-}$ ;  $nrxn3$ 284 appropriate to use because we observed synapse loss in *manda*; *manda*; mandants in both<br>285 sensory systems (Figs 2, Fig S5, S6). For an in-clutch comparison, we compared *nrxn3a; nr*<br>286 double mutants to *nrxn3a<sup>+*</sup> 285 sensory systems (Figs 2, Fig 35, 36). For an in-clude comparison, we compared *manda*, *manda*<br>286 double mutants to  $nran3a^{t/2}$ ;  $nran3b^{t/2}$  double heterozygotes. We assayed the acoustic startle<br>287 response using an double mutants to *nrxn3a'' ; nrxn3b''* double heterozygotes. We assayed the acoustic startle<br>287 response using an automated Zantiks behavioral system at three different intensities. Using the approach, we observed no dif 288 approach, we observed no difference in the proportion of animals startling at any stimulus<br>289 intensity between the two genotypes (Fig 6B). A surprisingly normal acoustic startle response in<br>290 *nrxn3a; nrxn3b* mutan approach, we observed no difference in the proportion of animals startling at any stimulus<br>
intensity between the two genotypes (Fig 6B). A surprisingly normal acoustic startle response in<br> *nrxn3a; nrxn3b* mutants shows t 289 *nrxn3a; nrxn3b* mutants shows that a dramatic reduction in synapse numbers is not sufficient to<br>291 impact this behavior. It remains possible that other lateral-line-mediated behaviors are more<br>291 impact this behavio 290 *manda, manda* mutants shows that a dramatic reduction in synapse numbers is not sufficient to<br>291 impact this behavior. It remains possible that other lateral-line-mediated behaviors are more<br>10 291 impact this behavior. It remains possible that other lateral-line-mediated behaviors are more

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| sensitive to reductions in hair-cell synapse number. However, our results broadly suggest that 293 hair-cell sensory systems are extremely robust and may not require all synapses to function.<br>294 **Discussion**<br>296 however, our find a dramatic reduction in the number of ribbon synapses in hair

hair-cell sensory systems are extremely robust and may not require all synapses to function.<br>294<br>**Discussion**<br>296 have and mice, we find a dramatic reduction in the number of ribbon synapses in hair<br>297 cells when Nrxn3 is 296<br>297<br>298 255 Discussion<br>296 In zebrafish<br>297 cells when<br>298 zebrafish, v<br>299 coupled to cells when Nrxn3 is absent (mouse Nrxn3 cKO and *nrxn3a; nrxn3b* zebrafish mutants). In<br>298 zebrafish, we show that presynapses are present in normal numbers and are still tightly<br>299 coupled to Ca<sub>v</sub>1.3 channels in *nrxn* 297 cells when NRXIIS is absent (mouse NRXIIS CKO and *IIP XIISU, IIP XIISU* zebrafish mutants). In<br>298 zebrafish, we show that presynapses are present in normal numbers and are still tightly<br>299 coupled to Ca<sub>V</sub>1.3 chann 299 coupled to Ca<sub>V</sub>1.3 channels in *nrxn3* mutants, but they fail to pair with postsynapses. Furtuary we demonstrate that both pre- and post-synaptic calcium responses are reduced in the are of Nrxn3. Overall, this work 299 coupled to Cay1.9 channels in *mails* mutants, but they fail to pair with postsynapses. Further,<br>300 we demonstrate that both pre- and post-synaptic calcium responses are reduced in the absen<br>301 of Nrxn3. Overall, thi 301 of Nrxn3. Overall, this work highlights a conserved role for Nrxn3 in the organization of ribbon<br>302 synapses in hair cells.<br>303 **Postsynaptic partners for Nrxn3 at the ribbon synapse in hair cells** 302 of Nrxnamer 2004 of Nrxn3. Overall, the organization of the organization of Nrxn3.<br>303<br>304 **Postsynaptic partners for Nrxn3 at the ribbon synapse in hair cells**<br>305 Our work demonstrates that Nrxn3 is required in zebra

303<br>304 **Postsynaptic partners**<br>305 Our work demonstrate<br>306 organization of ribbon 303<br>304<br>305<br>306<br>307 305 **Dur work demonstrates that Nrxn3** is required in zebrafish and mou<br>306 **Dur work demonstrates that Nrxn3** is required in zebrafish and mou<br>306 **Demonstrates in the represent of actival** serve as presynaptic rece<br>307 306 organization of ribbon synapses. Neurexins serve as presynaptic receptors for several<br>307 extracellular binding partners to facilitate synapse assembly. For  $\alpha$ -neurexins these partner<br>308 include: secreted cerebelli 307 extracellular binding partners to facilitate synapse assembly. For α-neurexins these pa<br>308 include: secreted cerebellins and neuroexophilins, as well as transmembrane proteins<br>309 neuroligins, Dystroglycan, leucine-308 include: secreted cerebellins and neuroexophilins, as well as transmembrane proteins such a<br>309 neuroligins, Dystroglycan, leucine-rich repeat transmembrane proteins (LRRTM), and<br>310 Calsyntenin-3 (Boucard et al., 2005 include: secreted cerebellins and neuroexophilins, as well as transmembrane proteins such as<br>309 neuroligins, Dystroglycan, leucine-rich repeat transmembrane proteins (LRRTM), and<br>310 Calsyntenin-3 (Boucard et al., 2005; D 310 Calsyntenin-3 (Boucard et al., 2005; Dai et al., 2022; Hauser et al., 2022; Kim et al., 20<br>311 al., 2009; Sugita et al., 2001; Trotter et al., 2023). Recent work in mice has demonstra<br>312 for  $\alpha$ -neurexin binding par al., 2009; Sugita et al., 2001; Trotter et al., 2023). Recent work in mice has demonstrated a role<br>312 for α-neurexin binding partners NLGN1 and NLGN3 at the postsynapses of mouse auditory IHCs<br>313 (Ramirez et al., 2022). 312 for  $\alpha$ -neurexin binding partners NLGN1 and NLGN3 at the postsynapses of mouse auditory IHCs<br>
313 (Ramirez et al., 2022). When NLGN1 and NLGN3 are lost, there is a ~25% loss of ribbon<br>
314 synapses in IHCs (Ramirez e 313 (Ramirez et al., 2022). When NLGN1 and NLGN3 are lost, there is a ~25% loss of ribbon<br>314 synapses in IHCs (Ramirez et al., 2022). In our work, we also observed a ~20-25% reduction in<br>315 ribbon synapses in *Nrxn3* mo 314 synapses in IHCs (Ramirez et al., 2022). In our work, we also observed a ~20-25% reduction in<br>315 ribbon synapses in *Nrxn3* mouse mutants (Fig 5, Fig S7). Based on these results, it is possible<br>316 that α-Nrxn3 is a 316 that  $\alpha$ -Nrxn3 is a presynaptic binding partner for NLGN1 and NLGN3 at auditory IHCs synapses<br>317 in mice. In the future, it will be interesting to examine zebrafish mutants lacking NLGN1 and<br>318 NLGN3 to understand 315 The Hording synapses in MARS mouse mutants (Fig 3, Fig 37). Based on these results, it is possible<br>316 that  $\alpha$ -Nrxn3 is a presynaptic binding partner for NLGN1 and NLGN3 at auditory IHCs synapse<br>317 in mice. In the 317 in mice. In the future, it will be interesting to examine zebrafish mutants lacking NLGN1 and NLGN3 to understand whether this complex plays a conserved role in vertebrate species. In addition, it will be interesting t 318 NLGN3 to understand whether this complex plays a conserved role in vertebrate species. In<br>319 addition, it will be interesting to examine both mice and zebrafish lacking both Nrxn3 and 319 addition, it will be interesting to examine both mice and zebrafish lacking both Nrxn3 and<br>319 and it will be interesting to examine both mice and zebrafish lacking both Nrxn3 and<br>311  $31$ 

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321 detail.<br>322 In mice lacking NLGN1 and NLGN3 or NRXN3 and zebrafish lacking  $\alpha$ -Nrxn3 there is a<br>323 clear reduction in the number of complete ribbon synapses-yet intact synapses remain. In our<br>324 zebrafish *nrxn3* m 322<br>323 clear re<br>324 zebrafi<br>325 of α-Nı 323 clear reduction in the number of complete ribbon synapses–yet intact synapses remain. In or<br>324 zebrafish *nrxn3* mutants, β-Nrxn3 is still present and could potentially compensate for the lo:<br>325 of α-Nrnx3. In cont 323 clear reduction in the number of complete ribbon synapses-yet intact synapses remain. In our<br>324 zebrafish *nrxn3* mutants, β-Nrxn3 is still present and could potentially compensate for the loss<br>325 of α-Nrnx3. In co 324 zebrafish *nrxn3* mutants, β-Nrxn3 is still present and could potentially compensate for the loss<br>325 of α-Nrnx3. In contrast, our mouse *Nrxn3* mutant lacks both α- and β-Nrxn3 (Aoto et al., 2015).<br>326 Therefore, in 325 of α-Nrnx3. In contrast, our mouse *Nrxn3* mutant lacks both α- and β-Nrxn3 (Aoto et al., 2015).<br>326 Therefore, in mice neither NRXN3 nor NLGN1/3 are strictly required to organize all ribbon<br>327 synapses in IHCs. Whet 327 Synapses in IHCs. Whether β-Nrxn3 (in zebrafish) or other neurexins (in zebrafish or mice)<br>328 partially compensate when NRXN3 is lost, or whether these remaining synapses simply rel<br>329 completely different synaptic 327 synapses in IHCs. Whether β-Nrxn3 (in zebrafish) or other neurexins (in zebrafish or mice) can<br>328 partially compensate when NRXN3 is lost, or whether these remaining synapses simply rely on<br>329 completely different s 329 completely different synaptic adhesion complex, remains to be determined.<br>330<br>331 **A role for Nrxn3 in hair-cell synapse specificity?**<br>332 If Nrxn3 and Nlgn1/3 are not required to organize all ribbon synapses in mice o

330<br>331 **A role for Nrxn3 in hair-cell synapse specificity?**<br>332 If Nrxn3 and Nlgn1/3 are not required to organize all ribbon synapses in mice<br>333 possible that this complex is only required for a specific subset of synaps 330<br>331<br>332<br>333<br>334 332 If Nrxn3 and Nlgn1/3 are not required to organize<br>333 possible that this complex is only required for a s<br>334 subset specificity in the lateral line is selective inr<br>335 (Nagiel et al., 2008). Here, afferent neurons in possible that this complex is only required for a specific subset of synapses. One example of<br>334 subset specificity in the lateral line is selective innervation based on the orientation of hair cell.<br>335 (Nagiel et al., 2 334 subset specificity in the lateral line is selective innervation based on the orientation of hair complex is<br>335 (Nagiel et al., 2008). Here, afferent neurons innervate lateral-line hair cells based on the<br>336 direction (Nagiel et al., 2008). Here, afferent neurons innervate lateral-line hair cells based on the<br>336 direction of fluid that they sense (for example, anterior or posterior flow in the primary<br>337 posterior-lateral line, Fig S4 direction of fluid that they sense (for example, anterior or posterior flow in the primary<br>337 (1992). Here, Fig S4, (1992-1999). Here, 2008). Here, we observe<br>338 similar loss of ribbon synapses in hair cells that sense 337 posterior-lateral line, Fig S4, (López-Schier and Hudspeth, 2006)). In our work, we obser<br>338 similar loss of ribbon synapses in hair cells that sense anterior and posterior fluid flow (F<br>339 This indicates that  $\alpha$ -338 similar loss of ribbon synapses in hair cells that sense anterior and posterior fluid flow (Fig S4)<br>339 This indicates that  $\alpha$ -Nrnx3 is not required for selective innervation based on hair-cell<br>340 orientation.<br>341 339 This indicates that  $\alpha$ -Nrnx3 is not required for selective innervation based on hair-cell<br>340 orientation.<br>341 Another level of innervation specificity in the lateral line is reflected in the wiring<br>342 pattern each

340 orientation.<br>341 Another level of innervation specificity in the lateral line is reflected in the wiri<br>342 pattern each neuron makes within the afferent terminals beneath neuromast hair cell<br>343 Numerous studies have 340 orientation. 342 pattern each neuron makes within the afferent terminals beneath neuromast hair cells.<br>343 Numerous studies have demonstrated that in the lateral line, hair cells are redundantly<br>344 innervated by multiple neurons (Haeh State Mumerous studies have demonstrated that in the lateral line, hair cells are redundantly<br>343 bumerous studies have demonstrated that in the lateral line, hair cells are redundantly<br>345 connectomic work has shown that 344 innervated by multiple neurons (Haehnel et al., 2012; Nagiel et al., 2008). More recent<br>345 connectomic work has shown that each neuromast contains a dominant afferent neurol<br>346 innervates nearly all hair cells (one f 345 connectomic work has shown that each neuromast contains a dominant afferent neuro<br>346 innervates nearly all hair cells (one for each orientation); these neurons form 75% of th<br>347 synapses (Dow et al., 2018). In additi 346 innervates nearly all hair cells (one for each orientation); these neurons form 75% of the<br>347 synapses (Dow et al., 2018). In addition to these dominant neurons, 1-3 additional afferent<br>348 neurons form the remaining 347 synapses (Dow et al., 2018). In addition to these dominant neurons, 1-3 additional affere<br>348 meurons form the remaining synapses within the neuromast. Therefore, it is possible that<br>12 348 section of the remaining synapses within the neuromast. Therefore, it is possible that in<br>348 sections form the remaining synapses within the neuromast. Therefore, it is possible that in  $348$  neuromast. The remaining synapses with intervals with intervals with intervals  $12$ 

 $\begin{array}{c} \n\frac{1}{2} & \frac{1}{2} \\ \n\frac{1}{2} & \frac{1}{$ Interal line, 2<sup>9</sup>-Nrnx3 is required specifically to form synapses made by the dominant neurons.<br>
350 This is consistent with the 60% loss in synapses we observed in zebrafish *nrxn3* mutants. In this<br>
351 scenario, a sepa 349 353 Senario, a separate synaptic adhesion complex could be used to form synapses in the less<br>352 dominant neurons. This would suggest that there may be distinct subsets of neurons in the<br>353 dominant neurons. This would su dominant neurons. This would suggest that there may be distinct subsets of neurons in the<br>353 posterior-lateral line. While studies have shown that there are both functionally (spike rate<br>354 and morphologically (innervati 353 posterior-lateral line. While studies have shown that there are both functionally (spike rate)<br>354 and morphologically (innervation pattern) distinct neurons within the lateral-line nerve,<br>355 currently there are no mo 354 and morphologically (innervation pattern) distinct neurons within the lateral-line nerve,<br>355 currently there are no molecular markers to distinguish these subtypes (Dow et al., 2018; Lia<br>356 and Haehnel, 2012). In the 255 currently there are no molecular markers to distinguish these subtypes (Dow et al., 2018<br>356 and Haehnel, 2012). In the future, it will be important to investigate the role Nrxn3 plays<br>357 lateral line with regards to 356 and Haehnel, 2012). In the future, it will be important to investigate the role Nrxn3 plays in the<br>357 lateral line with regards to these potential subtypes.<br>358 While neuronal subtypes are less defined in the lateral

357 ateral line with regards to these potential subtypes.<br>358 While neuronal subtypes are less defined in the lateral line, in the mouse auditory<br>359 system, molecular studies (scRNAseq) have demonstrated that there are at 358 CHT While neuronal subtypes are less defined in the system, molecular studies (scRNAseq) have demonst<br>360 Subtypes of afferent neurons (Type I spiral ganglion r<br>361 Within the mouse cochlea (Shrestha et al., 2018; Sun 358 While neuronal subtypes are less defined in the lateral line, in the mouse auditory<br>359 system, molecular studies (scRNAseq) have demonstrated that there are at least three main<br>360 subtypes of afferent neurons (Type I 360 subtypes of afferent neurons (Type I spiral ganglion neurons) that innervate auditory IHCs<br>361 within the mouse cochlea (Shrestha et al., 2018; Sun et al., 2018). Each subtype synapses ont<br>362 hair cells at roughly dis 361 within the mouse cochlea (Shrestha et al., 2018; Sun et al., 2018). Each subtype synapses c<br>362 hair cells at roughly distinct spatial locations (across the pillar and modiolar faces of the ce<br>363 and likely correspond 362 hair cells at roughly distinct spatial locations (across the pillar and modiolar faces of the cell)<br>363 and likely corresponds to a distinct functional profile (Liberman et al., 2011; Petitpré et al.,<br>364 2018; Taberne 363 and likely corresponds to a distinct functional profile (Liberman et al., 2011; Petitpré et al.,<br>364 2018; Taberner and Liberman, 2005). Work on NLGN1 and NLGN3 in the mouse cochlea has<br>365 shown that NLGN1 is localize and likely corresponds to a distinct functional profile (Liberman et al., 2011; Petitpré et al.,<br>364 2018; Taberner and Liberman, 2005). Work on NLGN1 and NLGN3 in the mouse cochlea has<br>365 shown that NLGN1 is localized to 365 shown that NLGN1 is localized to postsynapses contacting the modiolar face of the auditory<br>366 HCs (Ramirez et al., 2022). In contrast, the pillar face of auditory IHCs is populated by<br>367 postsynapses containing NLGN3 366 HCs (Ramirez et al., 2022). In contrast, the pillar face of auditory IHCs is populated by<br>367 postsynapses containing NLGN3 or NLGN1/3. Whether a particular combination of NRXN3 ar<br>368 NLGN1 or 3 are required to form s 367 postsynapses containing NLGN3 or NLGN1/3. Whether a particular combination of NR.<br>368 NLGN1 or 3 are required to form synapses at distinct spatial locations on mouse auditorium remains to be determined.<br>370 368 NLGN1 or 3 are required to form synapses at distinct spatial locations on mouse auditory IHCs<br>369 remains to be determined.<br>370 Nrxn3 Interactions with core presynaptic components 369 nemains to be determined.<br>370<br>371 Nrxn3 Interactions with core presynaptic components<br>372 Although neurexins are considered classic synaptic adhesion molecules, genetic loss of

370<br>371 **Nrxn3 Interactions with co**<br>372 Although neurexins are con<br>373 neurexins does not confer s 371<br>372<br>373<br>374 372 Although neurexins with core presymaptic components<br>372 Although neurexins are considered classic synaptic adhe<br>373 neurexins does not confer synapse loss in all contexts. F<br>374 Nrxn1/2/3 triple KO mice, there is no l 372 Although neurexins are considered classic synaptic adhesion molecules, genetic loss of<br>373 neurexins does not confer synapse loss in all contexts. For example, in the brainstem of α-<br>374 Nrxn1/2/3 triple KO mice, the 373 Interexins does not comer synapse loss in all contexts. For example, in the brainstem of α-<br>374 *Nrxn1/2/3* triple KO mice, there is no loss of glutamatergic synapses (Missler et al., 2003).<br>375 Instead, Missler et al 374 *Nrxn1/2/3* triple KO mice, there is no loss of glutamatergic synapses (Missler et al., 2003).<br>375 Instead, Missler et al. found that  $\alpha$ -NRXNs play a key role in calcium-mediated<br>376 neurotransmission; this work fou 376 Instead, Missler et al. found that a change play a key role in calcium channel function<br>377 despite a normal number of synapses and channels. These data led to the hypotology<br>377 313 377 despite a normal number of synapses and channels. These data led to the hypothesis that  $\alpha$ -<br>13

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| 379 *Nrxn1/2/3* triple KO mice were shown to impact presynaptic Ca<sub>V</sub>2.1 channel clustering at a<br>380 central auditory synapse, the calyx of Held (Luo et al., 2020). This study also found that loss<br>381 α-NRXNs did not res 379 MXM1/2/3 triple KO mice were shown to impact presynaptic CaV2.1 channel clustering at a<br>380 central auditory synapse, the calyx of Held (Luo et al., 2020). This study also found that loss<br>381 a-NRXNs did not result in 381 α-NRXNs did not result in synapse loss. Instead, mutant synapses contained fewer Ca<sub>V</sub>2.1<br>382 channels. These defects ultimately led to impaired neurotransmission.<br>383 In zebrafish hair cells, we found that loss of N

382 channels. These defects ultimately led to impaired neurotransmission.<br>383 In zebrafish hair cells, we found that loss of Nrxn3 also impacts Ca<sub>V</sub>1.3 channels at<br>384 hair-cell presynapse. Our work on zebrafish *nrxn3* 383 channels, we found that loss of Nrxn3 also impacts<br>384 chair-cell presynapse. Our work on zebrafish nrxn3 mutants found small<br>385 subunit of Ca<sub>v</sub>1.3 channels (Fig 4E-H, K), and smaller presynaptic calciu<br>386 cells of 384 hair-cell presynapse. Our work on zebrafish *nrxn3* mutants found smaller clusters of the  $\alpha$ -<br>385 subunit of Ca<sub>V</sub>1.3 channels (Fig 4E-H, K), and smaller presynaptic calcium responses in the hair<br>386 cells of the la 385 subunit of Ca<sub>V</sub>1.3 channels (Fig 4E-H, K), and smaller presynaptic calcium responses in the cells of the lateral line (Fig 6E-F). In hippocampal neurons,  $\alpha$ -NRXNs can regulate presynaptic calcium responses via inte 386 cells of the lateral line (Fig 6E-F). In hippocampal neurons,  $\alpha$ -NRXNs can regulate presynaptic<br>387 calcium responses via interactions with  $\alpha$ 261 auxiliary subunits of Ca<sub>V</sub>2.1 channels (Brockhaus<br>388 et al., 2018 386 cells of the lateral line (Fig 6E-F). In hippocampal neurons, α-NRXNs can regulate presynaptic<br>387 calcium responses via interactions with α2δ1 auxiliary subunits of Ca<sub>v</sub>2.1 channels (Brockhaus<br>388 et al., 2018). Ba 388 et al., 2018). Based on this work, it is possible that the reduced presynaptic calcium signal that<br>389 we observed in *nrxn3* mutants could result, at least in part, from loss of interactions between<br>390 Nrxn3 and an 389 we observed in *nrxn3* mutants could result, at least in part, from loss of interactions between<br>390 Nrxn3 and an  $\alpha$ 26 subunit of Ca<sub>V</sub>1.3 channels in hair cells. Other studies have found that  $\alpha$ 26<br>391 subunits ac 389 we observed in *mand* mutants could result, at least in part, from loss of interactions between<br>390 Nrxn3 and an  $\alpha$ 26 subunit of Ca<sub>V</sub>1.3 channels in hair cells. Other studies have found that  $\alpha$ 26<br>391 subunits act 391 subunits act as trans-synaptic organizers of glutamatergic synapses by aligning the pre- and<br>392 post-synaptic active zones (Schöpf et al., 2021). Consistent with this idea, work in mouse<br>393 auditory IHCs has shown t 392 post-synaptic active zones (Schöpf et al., 2021). Consistent with this idea, work in mouse<br>393 auditory IHCs has shown that  $\alpha$ 262 subunits are required for proper gating properties of Ca<br>394 channels as well as alig 392 post-synaptic active zones (Schöpf et al., 2021). Consistent with this idea, work in mouse<br>393 auditory IHCs has shown that  $\alpha$ 262 subunits are required for proper gating properties of Ca<sub>V</sub>1.3<br>394 channels as well a 394 channels as well as alignment of these channels with the postsynapse (Fell et al., 2016). In<br>395 future work, it will be interesting to pursue both the physical and functional link between Nrxn3<br>396 and all subunits o 395 future work, it will be interesting to pursue both the physical and functional link between M<br>396 and all subunits of Ca<sub>V</sub>1.3 channels.<br>397 The most straightforward way to understand the interaction between Nrxn3 and

396 and all subunits of Ca<sub>v</sub>1.3 channels.<br>397 **function of Cavit Up 1.5 future and the interaction between Nrxn3 and other**<br>398 synaptic components is to visualize Nrxn3 localization. Unfortunately, neurexin molecules ar 397 and all subunity of Cay<sub>1</sub>.2 channels.<br>398 synaptic components is to visualize l<br>399 notoriously difficult to label either u<br>400 future it will be important to genera 398 synaptic components is to visualize Nrxn3 localization. Unfortunately, neurexin molecules are<br>399 notoriously difficult to label either using immunohistochemistry or via tagged proteins. In the<br>300 future it will be im 399 notoriously difficult to label either using immunohistochemistry or via tagged proteins. In the<br>300 future it will be important to generate endogenously tagged Nrxn3 animal models to visualize<br>301 whether Nrxn3 is pres 399 1999 100 100 future it will be important to generate endogenously tagged Nrxn3 animal models to visualize<br>399 401 whether Nrxn3 is present at ribbons synapses in hair cells. In recent years, adding endogenous<br>402 tags 401 whether Nrxn3 is present at ribbons synapses in hair cells. In recent years, adding endogenous<br>402 tags to proteins in mice and zebrafish has become more straightforward, making this approach<br>403 more straightforward ( whether Nrxn3 is present at ribbons synapses in hair cells. In recent years, adding endogenous<br>
402 tags to proteins in mice and zebrafish has become more straightforward, making this approach<br>
403 more straightforward (Ca 403 more straightforward (Carrington et al., 2022; Morrow et al., 2021).<br>404 **Functional consequences of synapse loss in Nrxn3 mutants** 

404<br>405 Functional consequences of synapse loss in Nrxn3 mutants<br>Al., 2022; Morrow et al., 2021 405 405 Functional consequences of synapse loss in Nrxn3 mutants

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| 407 (Fig 2: 60% lateral line; Fig S6: 27% medial crista, 44% anterior macula). Along with this synapions, we also observed a dramatic decrease in the evoked pre- and post-synaptic calcium signiant in hair cells of *nrxn3* (Fig 2: 60% lateral line; Fig S6: 27% medial crista, 44% anterior macula). Along with this synapse<br>
408 loss, we also observed a dramatic decrease in the evoked pre- and post-synaptic calcium signals<br>
409 in hair cells of 409 in hair cells of *nrxn3* mutants (Fig 6, 35% presynaptic and 45% postsynaptic reduction). Despite<br>410 this decrease in synaptic function, *nrxn3* homozygotes were viable as adults and did not have<br>411 any overt vestibu 410 this decrease in synaptic function, *nrxn3* homozygotes were viable as adults and did not have<br>411 any overt vestibular (circling behavior or difficultly remaining upright) or auditory defects (Fig<br>412 6B). This sugges 410 this decrease in synaptic function, *mails* homozygotes were viable as adults and did not have<br>411 any overt vestibular (circling behavior or difficultly remaining upright) or auditory defects (Fig<br>412 6B). This sugges 6B). This suggests that in zebrafish, there is enough redundancy built into hair-cell sensory<br>systems that fewer synapses and decreased synaptic function do not affect these behaviors.<br>This result it in line with work on a 413 systems that fewer synapses and decreased synaptic function do not affect these behaviors.<br>414 This result it in line with work on auditory synaptopathy in mammals where a loss of IHC ribbo<br>415 synapses does impact hea 414 This result it in line with work on auditory synaptopathy in mammals where a loss of IHC ribb<br>415 synapses does impact hearing thresholds, but instead is linked to more subtle hearing deficit<br>416 (Liberman et al., 2016 synapses does impact hearing thresholds, but instead is linked to more subtle hearing deficits<br>416 (Liberman et al., 2016). Based on this work on auditory synaptopathy, it is possible that more<br>417 subtle behavioral defici 416 (Liberman et al., 2016). Based on this work on auditory synaptopathy, it is possible that more<br>417 subtle behavioral deficits exist in *nrxn3* zebrafish mutants. For example, *nrxn3* mutants may no<br>418 be able to rheot 417 subtle behavioral deficits exist in *nrxn3* zebrafish mutants. For example, *nrxn3* mutants may n<br>418 be able to rheotax (use their lateral line to orient in a constant flow), or they may not be able<br>419 detect a parti 418 be able to rheotax (use their lateral line to orient in a constant flow), or they may not be able to<br>419 detect a particular auditory frequency or threshold. In the future, it will be interesting to study<br>420 more comp detect a particular auditory frequency or threshold. In the future, it will be interesting to study<br>420 more complex hair-cell mediated behaviors in nrxn3 zebrafish mutants in more detail.<br>421 In humans, NRXN3 sequence var

420 more complex hair-cell mediated behaviors in *nrxn3* zebrafish mutants in more detail.<br>421 In humans, *NRXN3* sequence variants and mutations have been linked to alcohol and<br>422 drug abuse, obesity and autism (Heard-Co 420 more complex half cell mediated behaviors in mains zebrafish mutants in more detail.<br>421 In humans, NRXN3 sequence variants and mutations have been linked to alcohe<br>422 drug abuse, obesity and autism (Heard-Costa et al 422 In Humans, MANO sequence variants and mutations have been linked to alcohol and<br>422 In drug abuse, obesity and autism (Heard-Costa et al., 2009; Hishimoto et al., 2007; Lachman et<br>423 In humans to the<br>424 *NRXN3* locus al., 2007; Vaags et al., 2012). Currently no studies have linked hearing loss in humans to the<br>424 MRXN3 locus, or any other NRXN locus. In mice, a genome-wide association study identified a<br>425 locus near Nlgn1 that was l MRXN3 locus, or any other NRXN locus. In mice, a genome-wide association study identified<br>425 locus near Nlgn1 that was linked to compromised auditory brain-stem responses (Ramirez e<br>426 al., 2022). In addition, Nlgn1/3 mu Fig. 24 THINN3 locus, or any other NRXN locus. In the earth of a genome-wide association study identified a<br>425 Iocus near NIgn1 that was linked to compromised auditory brain-stem responses (Ramirez et<br>426 Iocus near NIgn 425 locus near *Nlgn1* that was linked to compromised auditory brain-stem responses (Namiez et al., 2022). In addition, *Nlgn1/3* mutant mice have impaired hearing and are more vulnerable to noise trauma. In the future it ally 2022). In addition, *Nlgn1/3* mutant mice have impaired hearing and are more vulnerable to<br>
427 noise trauma. In the future it will be important to further examine *Nrxn3* mutant mice to<br>
428 investigate whether they 427 and the future it will be important to further examine MXID mutant mice to<br>428 investigate whether they have hearing and balance defects and whether they are more<br>430 Our work demonstrates that Nrxn3 is critical for sy investigate whether they have hearing and balance defects and whether they are more<br>
429 vulnerable to noise trauma.<br>
430<br>
431 Our work demonstrates that Nrxn3 is critical for synapse assembly in both mouse and zebrafish<br>

430<br>431 Our work demonstrates that<br>432 hair cells. However, the maj<br>433 study and in other studies re 431<br>432<br>433<br>434 432 hair cells. However, the majority of work on the assembly of ribbon synapses in our current<br>433 study and in other studies relies on information obtained from static images. It is important to<br>434 note that development study and in other studies relies on information obtained from static images. It is important to<br>note that development is not static but is instead dynamic and is best studied in living tissue<br>15 434 note that development is not static but is instead dynamic and is best studied in living tissue<br>15  $434$ 

435 over time. In future work, we will continue to use the zebrafish system-which is ideal for live

(<br>i<br>. 436 imaging–to determine the exact role that Nrxn3 plays in synapse assembly *in vivo*. Overall,<br>437 elucidating how hair-cell synapses assemble is essential to understand how to re-form synaps<br>438 when they are lost after imaging–to determine the exact role that Nrxn3 plays in synapse assembly *in vivo*. Overall,<br>
437 elucidating how hair-cell synapses assemble is essential to understand how to re-form synapses<br>
438 when they are lost after

when they are lost after noise- or age-related hearing loss.<br>439<br>440 **Acknowledgements:** The Zebrafish International Resource Center (ZIRC) provided the<br>441 *nrxn3d<sup>sa11330</sup>* and *nrxn3b<sup>sa36960</sup>* ENU alleles used in this

439<br>440 Acknowledgements: The Zebrafish International Resource<br>441 *nrxn3d<sup>sa11330</sup>* and *nrxn3b<sup>sa36960</sup>* ENU alleles used in this stud<br>442 Science group at The Jackson Laboratory for cryorecovery c 440<br>441<br>442<br>443 Science group at The Jackson Laboratory for cryorecovery of the Nrxn3<sup>flox</sup> strain. This work was<br>
443 made possible in part by software funded by the NIH: Fluorender (VVDViewer) "An Imaging<br>
444 Tool for Visualization and

 $nrxn3a^{sal1330}$  and  $nrxn3b^{sal36060}$  ENU alleles used in this study. We thank the Reproduct<br>Science group at The Jackson Laboratory for cryorecovery of the *Nrxn3<sup>flox</sup>* strain. This v<br>made possible in part by software fund

442 Science group at The Jackson Laboratory for cryorecovery of the Nrxn3<sup>nox</sup> strain. This work was<br>443 made possible in part by software funded by the NIH: Fluorender (VVDViewer) "An Imaging<br>444 Tool for Visualization an 444 Tool for Visualization and Analysis of Confocal Data as Applied to Zebrafish Research", R01-<br>445 GM098151-01. We thank Drs. Katie Drerup and Candy Wong for their thoughtful comments<br>447 445 GM098151-01. We thank Drs. Katie Drerup and Candy Wong for their thoughtful comments<br>446 our manuscript.<br>447 **Competing interests:** The authors declare no competing financial interests. 446 our manuscript.<br>447<br>448 **Competing interests:** The authors declare no competing financial interests.<br>449

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111<br>447<br>448 **Competing inter**<br>449<br>450 **Funding:** This we

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450 Francing: This work was supported by National Institute on Deamess and Other Communication<br>451 Disorders (NIDCD) Intramural Research Program Grant 1ZIADC000085-01 to KK and Grants R01s<br>453 **Data availability:** All raw 1912 Discrete Controllers (NIDCD) Intramum Research Program Grant 12000 Control 1214 Minimum Control<br>453<br>454 Data availability: All raw data for this work is posted on Dryad (link to be added upon<br>455 completion). 453<br>454 **Data availability:** All raw data fo<br>455 completion).<br>456 454<br>455<br>456<br>457

454 Data availability: All raw data for this work is posted on Dryad (link to be added upon<br>456 completion).<br>457 Contributions: AJ, ZCL, KK and NM performed immunohistochemistry and confocal im<br>458 examine *nrxn3* zebrafis 456<br>457 **Contribution**<br>458 examine *nrxn*<br>459 code to quan 458<br>459<br>460 457 Contributions: AJ, 2CL, KK and NWI performed immunionstochemistry and confocal imaging to<br>458 examine *nrxn3* zebrafish mutants. BT did the mouse immunohistochemistry. ZCL wrote the<br>459 code to quantify all immunohisto 459 cxannie *mans* zebrafish mutants. BT did the mouse immunohistochemistry, 201 wrote the<br>460 code to quantify all immunohistochemistry and RNA FISH images. SD did the behavioral<br>461 analyses. KP performed RNA FISH to exa analyses. KP performed RNA FISH to examine *nrxn3* expression in zebrafish hair cells and<br>assisted with genotyping and husbandry. EC and KK performed the calcium imaging for post-synaptic responses respectively. AJ, ZCL, E 461 analyses. KP performed RNA FISH to examine *HIXHS* expression in zebrafish han cells and<br>461 assisted with genotyping and husbandry. EC and KK performed the calcium imaging for pr<br>462 manuscript. All authors edited the 162 assisted with genotyping and husbandry. EC and KK made figures and wrote the<br>163 manuscript. All authors edited the manuscript.<br>16 463 manuscript. All authors edited the manuscript.<br>16

463 manuscript. All authors edited the manuscript.

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 $\frac{1}{2}$ 465<br>466<br>467<br>468 Example 103 Diversity and inclusive statement: We strive to make our group the most inclusive, diverse,<br>466 and equitable space possible. We acknowledge the inequities that exist within STEM. We ho<br>467 ourselves accountabl ourselves accountable and continuously recognize and work against these inequities. All<br>468 scientists on this project had different levels of training, knowledge, personal circumstances,<br>469 and backgrounds. Our lab seeks ourselves accountable and continuously recognize and work against these inequities. All<br>scientists on this project had different levels of training, knowledge, personal circumstances,<br>and backgrounds. Our lab seeks to prov and backgrounds. Our lab seeks to provide scientists with an academic space that<br>470 acknowledges, supports, and celebrates these differences.<br>471 Materials and Methods 470 acknowledges, supports, and celebrates these differences.<br>471<br>472 Materials and Methods<br>473

# 471<br>472 Materials and Methods<br>473 Zebrafish strains and husbandry

472<br>473<br>474<br>475 473<br>474 **Zebrafish strains and hu<br>475 Zebrafish (Danio rerio) w<br>476 raised in E3 embryo med** ---<br>474<br>475<br>476 The Manusian Strains and husbandry<br>475 Zebrafish (*Danio rerio*) were grow<br>476 Traised in E3 embryo medium (5<sup>2</sup><br>477 MgSO<sub>4</sub>, pH 7.2). Zebrafish work p<br>478 Trapproved by the Animal Use Com 475 Zebrahsh (*Dulho Ferlo)* were grown at 30°C using a 14 hr light, 10 hr dark cycle. Larvae were<br>476 anised in E3 embryo medium (52 mM NaCl, 0.172 mM KCl, 0.332 mM CaCl<sub>2</sub>, and 0.332 mM<br>477 MgSO<sub>4</sub>, pH 7.2). Zebrafish w 477 MgSO<sub>4</sub>, pH 7.2). Zebrafish work performed at the National Institute of Health (NIH) was<br>478 approved by the Animal Use Committee at the NIH under animal study protocol #1362-1.<br>479 Larvae were examined at either 3 da approved by the Animal Use Committee at the NIH under animal study protocol #1362-2<br>479 Larvae were examined at either 3 days post fertilization (dpf) or 5 dpf unless stated othe<br>480 The following previously established li 179 Larvae were examined at either 3 days post fertilization (dpf) or 5 dpf unless stated otherw<br>180 The following previously established lines were used in this study: myo6b:memGCaMP6s<sup>idci</sup><br>181 and en.sill,hsp70l:GCaMP6s 480 The following previously established lines were used in this study: myo6b:memGCaMP6s<sup>idcTg1</sup><br>481 and en.sill,hsp70l:GCaMP6s<sup>idcTg8</sup> (Jiang et al., 2017; Zhang et al., 2018). In addition to these lines<br>482 two sanger mu The following previously established lines were used in this study: myo6b:memGCaMP6s<sup>'dc1g1</sup><br>
481 and en.sill,hsp70l:GCaMP6s<sup>idcTg8</sup> (Jiang et al., 2017; Zhang et al., 2018). In addition to these lines,<br>
482 two sanger mu 481 and *en.sill,hsp70l:GCaMP6s<sup>idcryg</sup>* (Jiang et al., 2017; Zhang et al., 2018). In addition to these lines,<br>482 two sanger mutants were obtained from the Zebrafish International Resource Center (ZIRC) and<br>483 used in t used in this study:  $nrxn3a^{s011330}$  and  $nrxn3b^{s036960}$ . The  $nrxn3a^{s011330}$  mutant results in a<br>484 premature stop codon in the second LNS domain (C to stop at amino acid 455/1697 in the  $\alpha$ <br>485 isoform, ENSDART00000 483 used in this study:  $nrxn3a^{341330}$  and  $nrxn3b^{335330}$ . The  $nrxn3a^{341330}$  mutant results in a<br>484 premature stop codon in the second LNS domain (C to stop at amino acid 455/1697 in<br>485 isoform, ENSDART00000088179.5 485 isoform, ENSDART00000088179.5). This allele was genotyped using standard PCR and<br>486 sequencing with the following primer sets: FWD: 5'-AATGAACTCTTTAAAAGGAGCA-3' and RE<br>487 5'-TCCACTTTTGTGTTCTTCTGGC-3'. The *nrxn3b<sup>sa3*</sup> 486 sequencing with the following primer sets: FWD: 5'-AATGAACTCTTTAAAAGGAGCA-3' a<br>487 5'-TCCACTTTTGTGTTCTTCTGGC-3'. The nrxn3b<sup>sa36960</sup> mutants results in a point mutatic<br>488 to a premature stop codon in the first LNS dom 487 5'-TCCACTTTTGTGTTCTTCTGGC-3'. The  $nrxn3b^{5036960}$  mutants results in a point mutation leading<br>488 to a premature stop codon in the first LNS domain (R to stop at amino acid 135/1687 in the  $\alpha$  isoform, ENSDART000001 5'-TCCACTTTTGTGTTCTTCTGGC-3'. The *nrxn3b*<sup>5036960</sup> mutants results in a point mutation leading<br>to a premature stop codon in the first LNS domain (R to stop at amino acid 135/1687 in the  $\alpha$ <br>isoform, ENSDART00000127050.3  $489$  isoform, ENSDART00000127050.3). This allele was genotyped using standard PCR and<br>490 sequencing with the following primer sets: FWD: 5'-TCACTGGCACTTTGCTACAATC-3' and REV:<br>491 5'-GTTGGAACCTTATTGCCGTAAC-3'. Each mutan 490 sequencing with the following primer sets: FWD: 5'-TCACTGGCACTTTGCTACAATC-3' are<br>491 5'-GTTGGAACCTTATTGCCGTAAC-3'. Each mutant line was outcrossed 3 times before u<br>492 outcrossing, the  $nrxn3a^{s}^{a11330}$  and  $nrxn3b^{s}$ 491 5'-GTTGGAACCTTATTGCCGTAAC-3'. Each mutant line was outcrossed 3 times before use. After outcrossing, the  $nrxn3\alpha^{sat1330}$  and  $nrxn3b^{s36960}$  mutants were crossed to produce double 492 outcrossing, the  $nrxn3a^{s\sigma11330}$  and  $nrxn3b^{s\sigma36960}$  mutants were crossed to produce double<br>17 and nrxn3a and nrxn3b and mutants were crossed to produce double<br>and the nrxn3bs and nrxn3bs mutants were crossed to produce double<br>17

r<br>r 493 mutants:  $nrxn3a'$ ;  $nrxn3b'$ . For comparisons,  $nrxn3a'$ , and  $nrxn3b'$ , and  $nrxn3a'$ ;  $nrxn3b'$ <br>494 mutants were either compared to their respective wild-type siblings or to wild-type larvae<br>625 collected and grown at th  $495$  collected and grown at the same time as double mutants. For behavioral experiments, *nrxn*<br>496 ;  $nrxn3b<sup>2/-</sup>$  double heterozygotes were compared to double mutants obtained from the san<br>497 clutch of embryos to di

495 collected and grown at the same time as double mutants. For behavioral experiments, nrxn3a<sup>3</sup><br>496 ferry nrxn3b<sup>+/-</sup> double heterozygotes were compared to double mutants obtained from the same<br>497 clutch of embryos to ;  $n$ rxn3b<sup>+/-</sup> double heterozygotes were compared to double mutants obtained from the same

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- quared to double mutants obtained from the space of the terrozygotes were compared to double mutants obtained from the same of the of embryos to directly compare siblings.<br> **A98** Mouse strains and husbandry<br>
The Nrxn3<sup>flo</sup>
- 497 clutch of embryos to directly compare sibilities.<br>498 **Mouse strains and husbandry**<br>500 The Nrxn3<sup>flox</sup> strain was cryorecovered at The Ja<br>501 (B6;129-Nrxn3<sup>tm3Sud/J</sup>; MGI:5437468) (Aoto et al 501<br>502
- 499 Mouse strains and husbandry<br>500 The Nrxn3<sup>flox</sup> strain was cryored<br>501 (B6;129-Nrxn3<sup>tm3Sud/J</sup>; MGI:543<br>502 the  $\alpha$  and  $\beta$  transcripts (exon 1<br>503 inactivate Nrxn3 in post-mitotic
- 500 The Nrxn3<sup>nox</sup> strain was cryorecovered at The Jackson Laboratory from stock JR#014157<br>501 (B6;129-Nrxn3<sup>tm3Sud/J</sup>; MGI:5437468) (Aoto et al 2015). In this strain, the first common ex<br>502 the  $\alpha$  and  $\beta$  transcripts 502 the α and β transcripts (exon 18) is flanked by *loxP* sites. The Atoh1-*Cre* driver used to<br>503 inactivate *Nrxn3* in post-mitotic hair cells is stock JR#011104 (*B6.Cg-Tg(Atoh1-cre)1Bfr*<br>504 MGI:3775845) (Matei et a
- 
- 501 (B6;129-*Nrxn3<sup>an3343</sup>)*; MGI:5437468) (Aoto et al 2015). In this strain, the first common exon for<br>502 the α and β transcripts (exon 18) is flanked by *loxP* sites. The Atoh1-Cre driver used to<br>503 inactivate *Nrxn3*
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- 503 inactivate *NNAS* in post-mitotic hangers is stock JNPO11104 (B0.Cg-Tg(Atoh1-cre)1Bfri/J;<br>504 MGI:3775845) (Matei et al., 2005). Atoh1-Cre; Nrxn3<sup>flox/flox</sup> mutants were compared to con<br>505 littermates of the followin 504 MGI:3775845) (Matei et al., 2005). *Atoh1-Cre; Nrxn3<sup>noxy</sup>m* mutants were compared to control<br>505 littermates of the following genotypes: *Atoh1-Cre; Nrxn3<sup>flox/+</sup>* (this control genotype is depicted<br>506 in the Figures SO5 littermates of the following genotypes: Atoh1-Cre; Nrxn3<sup> $f$ ox/+</sup> (this control genotype is depicted<br>506 in the Figures), Nrxn3<sup> $f$ ox/fox</sup>, Nrxn3<sup> $f$ ox/+</sup>. Both males and females were included in the study.<br>507 Animal in the Figures), Nrxn3<sup>nox</sup>, Nrxn3<sup>nox</sup>, Both males and females were included in the study.<br>507 Animals were maintained under standard housing conditions (14h light/10h dark cycle, amb<br>508 temperature and normal humidity). 508 temperature and normal humidity). All mouse work was reviewed for compliance and approved<br>509 by the Animal Care and Use Committee of The Jackson Laboratory.<br>510 **Zebrafish immunohistochemistry and imaging**
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- 509 by the Animal Care and Use Committee of The Jackson Laboratory.<br>510<br>511 **Zebrafish immunohistochemistry and imaging**<br>512 Immunohistochemistry was performed on whole larvae at either 3 dpf or 5 dpf. Whole larvae 510<br>511 **Zebrafish immunohistochemistry and imaging**<br>512 Immunohistochemistry was performed on whole larvae at either 3<br>513 were fixed with paraformaldehyde (PFA 4%; Thermoscientific; 2890 510 512 Immunohistochemistry was performed on whol<br>513 were fixed with paraformaldehyde (PFA 4%; The<br>514 For Ca<sub>V</sub>1.3 labeling (Ca<sub>V</sub>1.3, Otoferlin, MAGUK o<br>515 and antibody solutions were prepared with PBS
- 513 were fixed with paraformaldehyde (PFA 4%; Thermoscientific; 28906) in PBS at 4°C for 3.5 hr.<br>514 For Ca<sub>V</sub>1.3 labeling (Ca<sub>V</sub>1.3, Otoferlin, MAGUK or Ca<sub>V</sub>1.3, Paravalbumin, CTBP), all wash, block<br>515 and antibody sol
- 514 For Ca<sub>V</sub>1.3 labeling (Ca<sub>V</sub>1.3, Otoferlin, MAGUK or Ca<sub>V</sub>1.3, Paravalbumin, CTBP), all wash, block<br>515 and antibody solutions were prepared with PBS + 0.1% Tween (PBST). For pre- and post-<br>516 synaptic labeling (rabb
- 515 and antibody solutions were prepared with PBS + 0.1% Tween (PBST). For pre- and post-<br>516 synaptic labeling (rabbit anti-MYO7A, CTBP, MAGUK), all wash, block and antibody solutions<br>517 were prepared with PBS at 21% DM
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- 515 and antibody solutions were prepared with PBS + 0.1% Tween (PBST). For pre- and post-S17 were prepared with PBS $\overline{a}$ + $\overline{a}$ 1% DMSO, 0.5% Triton-X100, 0.1% Tween-20 (PBDTT). After<br>518 fixation, larvae were washed 4 × 5 min in PBST or PBDDT. For Ca<sub>V</sub>1.3 labeling, prior to block,<br>519 larvae were permea 517 were prepared with PBSD+D1% DMSO, 0.5% Triton-X100, 0.1% Tween-20 (PBDTT). After<br>518 fixation, larvae were washed 4 × 5 min in PBST or PBDDT. For Ca<sub>V</sub>1.3 labeling, prior to block,<br>519 larvae were permeabilized with a 519 larvae were permeabilized with acetone. For this permeabilization, larvae were washed for 5 min with H<sub>2</sub>O in glass vials. The H<sub>2</sub>O was removed and replaced with ice-cold acetone and larvae placed at  $-20^{\circ}$ C for 5
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- 520 min with H<sub>2</sub>O in glass vials. The H<sub>2</sub>O was removed and replaced with ice-cold acetone and<br>521 larvae placed at -20°C for 5 min, followed by a 5 min H<sub>2</sub>O wash. The larvae were then washed<br>18 521 larvae placed at -20°C for 5 min, followed by a 5 min H<sub>2</sub>O wash. The larvae were then was<br>18 521 min placed at −20°C for 5 min, for 5 min H<sub>2</sub>O wash. The larvae were then washed then washed<br>18

 $\begin{array}{c} \uparrow \uparrow \quad \ \ \, \circ \\ \downarrow \quad \ \ \, \circ \quad \$ 522 523 solution (2% goat serum, 1% bovine serum albumin, 2% fish skin gelatin in PBST or PBDTT).<br>524 After block, larvae were incubated in primary antibodies in antibody solution (1% bovine seru<br>525 albumin in PBST or PBDTT) 524 After block, larvae were incubated in primary antibodies in antibody solution (1% bovine se<br>525 albumin in PBST or PBDTT) overnight, nutating at 4°C. The next day, the larvae were washed<br>526 4 × 5 min in PBST or PBDTT 524 After block, larvae were incubated in primary antibodies in antibody solution (1% bovine serum<br>525 albumin in PBST or PBDTT) overnight, nutating at 4°C. The next day, the larvae were washed for<br>526 4 × 5 min in PBST o 526 4 × 5 min in PBST or PBDTT to remove the primary antibodies. Secondary antibodies in antibody<br>527 solution were added and larvae were incubated for 2 hrs at room temperature, with minimal<br>528 exposure to light. Seconda 527 solution were added and larvae were incubated for 2 hrs at room temperature, with minimal<br>528 exposure to light. Secondary antibodies were removed by washes with PBST or PBDTT for 4 x 5<br>529 min. Larvae were mounted on exposure to light. Secondary antibodies were removed by washes with PBST or PBDTT for 4 x<br>529 min. Larvae were mounted on glass slides with Prolong Gold (ThermoFisher Scientific) using N<br>530 1.5 coverslips.<br>531 Fixed sampl 529 min. Larvae were mounted on glass slides with Prolong Gold (ThermoFisher Scientific) using No.<br>530 1.5 coverslips.<br>531 Fixed samples were imaged on an upright LSM 980 laser-scanning confocal microscope<br>532 with an Airy

1.5 coverslips.<br>531 Fixed samples were imaged on an upright LSM 980 laser-scanning confocal microscope<br>532 with an Airyscan 2 attachment using Zen Blue 3.4 (Carl Zeiss) and a 63x/1.4 NA Plan Apo oil<br>533 immersion objectiv 531 Fixed sa<br>
532 with an Airysca<br>
533 immersion obje<br>
534 for lateral-line with an Airyscan 2 attachment using Zen Blue 3.4 (Carl Zeiss) and a 63x/1.4 NA Plan Apo oil<br>533 immersion objective lens. Z-stacks were acquired every 0.15 $\mathbb{E}$ µm with a 0.043 µm X-Y pixel size<br>534 for lateral-line and 533 immersion objective lens. Z-stacks were acquired every 0.15 $\frac{1}{2}$ um with a 0.043  $\mu$ m X-Y pixel stand tor lateral-line and medial-crista hair cells, and every 0.15 $\frac{1}{2}$ um with a 0.067  $\mu$ m X-Y pixel size<br>535 for lateral-line and medial-crista hair cells, and every 0.15<sub>μμm</sub> with a 0.067 μm X-Y pixel size for<br>535 hair cells in the anterior macula. The Airyscan z-stacks were autoprocessed in 2D. Experiments<br>536 were imaged with 535 hair cells in the anterior macula. The Airyscan z-stacks were autoprocessed in 2D. Experiments<br>536 were imaged with the same acquisition settings to maintain consistency between comparisons.<br>537 For presentation in fig 536 bair were imaged with the same acquisition settings to maintain consistency between comparisons<br>537 For presentation in figures, images were further processed using Fiji.<br>538 **Mouse immunohistochemistry and imaging** 537 For presentation in figures, images were further processed using Fiji.<br>538 Mouse immunohistochemistry and imaging<br>540 Temporal bones were isolated, and an insulin syringe was used to gently flush cold

538<br>539 Mouse immunohistochemistry and imaging<br>540 Temporal bones were isolated, and an insulin syringe was used to ger<br>541 paraformaldehyde (PFA 4%; Electron Microscopy Sciences; 15710) thi 538<br>539<br>540<br>541<br>542 533 Mouse Immunonistochemistry and imaging<br>540 Temporal bones were isolated, and an insulir<br>541 paraformaldehyde (PFA 4%; Electron Microso<br>542 round windows after poking a small hole at tl<br>543 immersion-fixed in PFA for 1 For the strategies were increasing that the internal syring cluster is gently fluster to a<br>541 Teamporal delay in paraformal bones are reading to an insulin synthetic synthetic synthetic reading<br>543 Temporal bones were imm 542 round windows after poking a small hole at the cochlear apex. Temporal bones were then<br>543 immersion-fixed in PFA for 1 hour at 4°C, washed in PBS, and rotated overnight in EDTA 4% for<br>544 decalcification. The next day 542 round windows after poking a small hole at the cochlear apex. Temporal bones were then<br>543 immersion-fixed in PFA for 1 hour at 4°C, washed in PBS, and rotated overnight in EDTA 4% for<br>544 decalcification. The next day 546 bovine serum albumin; 0.5% Triton X-100). The following primary antibodies were used: CTBP2,<br>547 GluR2 and mouse anti-MYO7A. Primary and secondary antibodies were incubated overnight at apex) before blocking and permeabilization for 1 hr at room temperature under agitation (1%<br>546 bovine serum albumin; 0.5% Triton X-100). The following primary antibodies were used: CTBP<br>547 GluR2 and mouse anti-MYO7A. Pri 546 bovine serum albumin; 0.5% Triton X-100). The following primary antibodies were used: CTBP:<br>547 GluR2 and mouse anti-MYO7A. Primary and secondary antibodies were incubated overnight at<br>548 4°C in PBS. Samples were wash 547 GluR2 and mouse anti-MYO7A. Primary and secondary antibodies were incubated overnight at<br>548 4°C in PBS. Samples were washed 3 times in PBS + 0.05% Triton X-100 after each antibody<br>549 incubation and finally post-fixed 548 4°C in PBS. Samples were washed 3 times in PBS + 0.05% Triton X-100 after each antibody<br>549 incubation and finally post-fixed in PFA 4% for at least 1 hr at room temperature. Samples were<br>550 then mounted flat in Mowio 548 4°C in PBS. Samples were washed 3 times in PBS + 0.05% Triton X-100 after each antibody<br>549 incubation and finally post-fixed in PFA 4% for at least 1 hr at room temperature. Samples were<br>550 then mounted flat in Mowio 550 then mounted flat in Mowiol mounting medium (Calbiochem/MilliporeSigma 4759041) using<br>19  $19$ 

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- of the coveral of the coveral and the coveral of the coveral prepared in (25% w/v) glycerol and 0.1M Tris-Cl pH8.5.<br>
553 Mounted samples were imaged on an upright LSM 980 laser-scanning confocal<br>
554 microscope with using microscope with using Zen Blue 3.4 (Carl Zeiss) and an 63x 1.4 NA oil objective lens. Z-s<br>555 were acquired every 0.250 lum with an 0.085 µm X-Y pixel size in confocal mode. For<br>556 presentation in figures, imaged were fur
- were acquired every 0.250 km with an 0.085 km X-Y pixel size in confocal mode. For<br>556 presentation in figures, imaged were further processed using Fiji.<br>557 Primary antibody list
- Mounted samples were imaged on an upright LS<br>553 Mounted samples were imaged on an upright LS<br>554 microscope with using Zen Blue 3.4 (Carl Zeiss) and an 6<br>555 were acquired every 0.250. presentation in figures, imaged were
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Z-stack image acquisitions from zebrafish and mouse confocal images were processed in Fiji 583<br>584<br>585 583 Z-stack image acquisitions from zebrafish and mouse confocal images were<br>584 (Schindelin et al., 2012). Researchers were blinded to genotype during an<br>585 neuromasts, hair-bundle orientation was scored manually (neurom 584 (Schindelin et al., 2012). Researchers were blinded to genotype during analyses. In zebrafish<br>585 neuromasts, hair-bundle orientation was scored manually (neuromasts L1-L4) relative to the<br>586 midline of the muscle som neuromasts, hair-bundle orientation was scored manually (neuromasts L1-L4) relative to the<br>586 midline of the muscle somites. Hair-cell numbers were counted manually based on MYO7a,<br>587 Paravalbumin, or Otoferlin labeling. 586 midline of the muscle somites. Hair-cell numbers were counted manually based on MYO7a,<br>587 Paravalbumin, or Otoferlin labeling. Prior to automated puncta quantification, each channel<br>588 was background subtracted using 587 Paravalbumin, or Otoferlin labeling. Prior to automated puncta quantification, each channel<br>588 was background subtracted using rolling-ball radius background subtraction. Then each z-sta<br>21 588 Paravalabumin, or Ottoferminia emigration to automated puncta quantification, Then each z-stated was background subtracted using rolling-ball radius background subtraction. Then each z-stated using  $21$  $5888$  was background subtracted using rolling-ball radius background subtraction. Then each z-stackground subtraction. Then each z-stackground subtraction. Then each z-stackground subtraction. Then each z-stackground sub

\<br>(<br>s 590 (ie: hair cells) in the reference channel. This mask was then applied to the z-projection of each<br>591 synaptic component or RNA FISH channel.<br>592 An automated synapse quantification was then performed using a customize

synaptic component or RNA FISH channel.<br>592 An automated synapse quantification was then performed using a customized Fiji-base<br>593 macro, "Complete Synapse Counter v5.2". In this macro, each masked image was thresholded<br>5 592 An automated synapse quantifications<br>593 macro, "Complete Synapse Counter v5.2".<br>594 using an adaptive thresholding plugin by Q.<br>595 (https://sites.google.com/site/qingzongtse matricology. The substitute of the synapse Counter v5.2". In this macro, each masked image was thresholded<br>594 Institute synapse Counter v5.2". In this macro, each masked image was thresholded<br>595 Institute the puncta (pre

594 using an adaptive thresholding plugin by <u>Qingzong TSENG</u><br>595 (https://sites.google.com/site/qingzongtseng/adaptivethreshold) to generate a binary image of<br>596 the puncta (presynaptic, postsynaptic, Ca<sub>V</sub>1.3 cluster o 595 (https://sites.google.com/site/qingzongtseng/adaptivethresholding the puncta (presynaptic, postsynaptic, Ca<sub>v</sub>1.3 cluster or RN<br>596 the puncta (presynaptic, postsynaptic, Ca<sub>v</sub>1.3 cluster or RN<br>598 analysis, the follo 596 the puncta (presynaptic, postsynaptic, Ca<sub>V</sub>1.3 cluster or RNA FISH puncta). Individual synaptic or<br>597 RNA FISH puncta were then segmented using the particles analysis function in Fiji. For particle<br>598 analysis, the 597 RNA FISH puncta were then segmented using the particles analysis function in Fiji. For particle<br>598 analysis, the following minimum size thresholds were applied: zebrafish lateral-line images –<br>599 CTBP: 0.025  $\mu$ m<sup>2</sup> 598 analysis, the following minimum size thresholds were applied: zebrafish lateral-line images –<br>599 CTBP: 0.025  $\mu$ m<sup>2</sup>, MAGUK: 0.04  $\mu$ m<sup>2</sup>, Ca<sub>V</sub>1.3 0.025  $\mu$ m<sup>2</sup>, Nrxn3a and Nrxn3b RNA FISH particles<br>600 0.03  $\mu$ 599 CTBP: 0.025 μm<sup>2</sup>, MAGUK: 0.04 μm<sup>2</sup>, Ca<sub>V</sub>1.3 0.025 μm<sup>2</sup>, Nrxn3a and Nrxn3b RNA FISH particle<br>600 0.03 μm<sup>2</sup> and 0.01 μm<sup>2</sup>, zebrafish inner ear images – CTBP: 0.025 μm<sup>2</sup>, MAGUK: 0.025 μm<sup>2</sup>,<br>601 mouse IHCs – CTBP CTBP: 0.025 μm<sup>-</sup>, MAGUK: 0.04 μm<sup>-</sup><br>0.03 μm<sup>2</sup> and 0.01 μm<sup>2</sup>, zebrafish in<br>mouse IHCs – CTBP: 0.025 μm<sup>2</sup>, GluF<br>applied to particle analysis. A waters<br>apart overlapping synaptic compone 599 CTBP: 0.025 μm<sup>-</sup>, MAGUK: 0.04 μm<sup>-</sup>, Ca<sub>V</sub>1.3 0.025 μm<sup>-</sup>, Nrxn3a and Nrxn3b RNA FISH particles:<br>600 0.03 μm<sup>2</sup> and 0.01 μm<sup>2</sup>, zebrafish inner ear images – CTBP: 0.025 μm<sup>2</sup>, MAGUK: 0.025 μm<sup>2</sup>,<br>601 mouse IHCs – CT 600  $0.03 \mu$ m<sup>2</sup> and  $0.01 \mu$ m<sup>2</sup>, zebrafish inner ear images – CTBP: 0.025 μm<sup>2</sup>, MAGUK: 0.025 μm<sup>2</sup>, mouse IHCs – CTBP: 0.025 μm<sup>2</sup>, GluR2: 0.025 μm<sup>2</sup>. A circularity factor between 0.1-0.5 was applied to particle anal applied to particle analysis. A watershed was applied to the particle analysis result to break<br>apart overlapping synaptic components. After the watershed, the particle analysis was rerur<br>with size and circularity threshold 601 mouse IHCs – CTBP: 0.025 μm<sup>2</sup>, GluR2: 0.025 μm<sup>2</sup>. A circularity factor between 0.1-0.5 was also<br>602 applied to particle analysis. A watershed was applied to the particle analysis result to break<br>603 apart overlappin 602 applied to particle analysis. A watershed was applied to the particle analysis result to break<br>603 apart overlapping synaptic components. After the watershed, the particle analysis was rerun<br>604 with size and circulari 604 with size and circularity thresholds to generate ROIs and measurements of each synaptic or<br>605 RNA FISH component. The ROIs were applied to the original z-projection to get the average<br>606 intensity and area of each pu

607 To recognize paired synaptic components, images were further processed using<br>608 Complete Synapse Counter v5.2". Here, the overlap and proximity of ROIs from different 609 channels (ex: pre- and post-synaptic puncta) was calculated. ROIs with positive overlap or ROIs 607 10 recognize paired synaptic<br>1608 1608 608 Ecomplete Synapse Counter v5.2". H<br>1609 channels (ex: pre- and post-synaptic<br>1610 within 2 pixels were counted as paire Complete Synapse Counter v5.2". Here, the overlap and proximity of ROIs from differe<br>609 channels (ex: pre- and post-synaptic puncta) was calculated. ROIs with positive overlap<br>610 within 2 pixels were counted as paired or channels (ex: pre- and post-synaptic puncta) was calculated. ROIs with positive overlap or<br>610 within 2 pixels were counted as paired or partner components. The ROIs and synaptic<br>611 component measurement (average intensit 610 within 2 pixels were counted as paired or partner components. The ROIs and synaptic<br>611 component measurement (average intensity, area) and pairing results were then saved as Fiji<br>612 ROIs, jpg images and csv files. Fo 611 component measurement (average intensity, area) and pairing results were then save<br>612 ROIs, jpg images and csv files. For puncta counts the total number of ROIs was plotted<br>613 neuromast or per hair cell.<br>614 Some ima For the paint measurement (average internet), and pairing results into another and resp.<br>
612 ROIs, jpg images and csv files. For puncta counts the total number of ROIs was plotted per<br>
613 The mage datasets required a pre

For Section<br>613 The uromast or per hair cell.<br>614 Some image datasets required a pre-processing step prior to entry into the "Comple<br>615 Synapse Counter v5.2". This includes zebrafish samples imaged at 3 dpf and our mouse Some image datase<br>
614 Some image datase<br>
615 Synapse Counter v5.2". Thi:<br>
616 datasets. For the pre-proce<br>
617 (https://github.com/Janelia 615 Synapse Counter v5.2". This includes zebrafish samples imaged at 3 dpf and our mouse IHCs<br>616 datasets. For the pre-processing step, the volumes were segmented in VVDviewer<br>617 (https://github.com/JaneliaSciComp/VVDVi datasets. For the pre-processing step, the volumes were segmented in VVDviewer<br>617 (https://github.com/JaneliaSciComp/VVDViewer). Staining outside of the hair cell was manual<br>72 617 (https://github.com/JaneliaSciComp/VVDViewer). Staining outside of the hair cell in  $\frac{22}{\sqrt{1 - \frac{1}{\sqrt{1 - \$ 

 $617.77$  (https://github.com/ $\frac{1}{2}$ ). Statining outside of the hair cell was manually  $\frac{1}{2}$ 

618 segmented or removed using VVDviewer. After this segmentation, the z-stacks were then max-

 $rac{1}{2}$ 

622 For functional imaging, 4-6 dpf larvae were anesthetized in 0.04% Tricaine-S (tricaine 620<br>620 Calcium imaging of lateral-line hair cells and afferents<br>622 For functional imaging, 4-6 dpf larvae were anesthetized in 0.04% Tricaine-S (tricaine<br>623 methanesulfonate, Western chemical, TRS1), pinned to a Sylgard 620<br>621<br>622<br>623<br>624 622 For functional imaging 4-6 dpf larvae were anesthetized<br>623 methanesulfonate, Western chemical, TRS1), pinned to<br>624 the head and tail, and paralyzed by injection of 125  $\mu$ M<br>625 heart cavity, as previously described 623 For Form Formuist 10.07 Trical imaging, 1932 Form of 23 and the head and tail, and paralyzed by injection of 125  $\mu$ M  $\alpha$ -bungarotoxin (Tocris, 2133)<br>625 heart cavity, as previously described (Lukasz and Kindt, 2018 623 methanesulfonate, Western chemical, TRS1), pinned to a Sylgard-filled perfusion chamber at<br>624 the head and tail, and paralyzed by injection of 125  $\mu$ M  $\alpha$ -bungarotoxin (Tocris, 2133) into the<br>625 heart cavity, as 624 the head and tail, and paralyzed by injection of 125 μM α-bungarotoxin (Tocris, 2133) into the<br>625 heart cavity, as previously described (Lukasz and Kindt, 2018). Larvae were then rinsed three<br>626 times in E3 embryo 626 times in E3 embryo media to remove the tricaine. Next, larvae were rinsed three times with<br>627 extracellular imaging solution (in mM: 140 NaCl, 2 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 10 HEPES, pH 7.3<br>628 OSM 310±10) and all extracellular imaging solution (in mM: 140 NaCl, 2 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 10 HEPES, pH 7.<br>628 OSM 310±10) and allowed to recover. Stimulation was achieved by a fluid jet, which consiste<br>629 of a pressure clamp (HS 628 CSM 310±10) and allowed to recover. Stimulation was achieved by a fluid jet, which consisted<br>629 of a pressure clamp (HSPC-1, ALA Scientific) and glass pipette, pulled and broken to an inner<br>630 diameter 40-50 µm, and 629 of a pressure clamp (HSPC-1, ALA Scientific) and glass pipette, pulled and broken to an inner<br>630 diameter 40-50 μm, and filled with extracellular imaging solution. A 500-ms pulse of positive of<br>631 negative pressure diameter 40-50  $\mu$ m, and filled with extracellular imaging solution. A 500-ms pulse of positive<br>631 origative pressure was used to deflect the hair bundles of mechanosensitive hair cells along t<br>632 anterior-posterior ax 630 diameter 40-50  $\mu$ m, and filled with extracellular imaging solution. A 500-ms pulse of positive or<br>631 negative pressure was used to deflect the hair bundles of mechanosensitive hair cells along the<br>632 anterior-post anterior-posterior axis of the fish. Hair cells of the two orientations (anterior and posterior)<br>633 were stimulated separately. Stimuli that deflected kinocilia 5-15 µm were included in the<br>634 analysis, as these deflecti were stimulated separately. Stimuli that deflected kinocilia 5-15  $\mu$ m were included in the<br>634 analysis, as these deflections represent saturating stimuli that do not induce damage.<br>635 Hair-cell responses to stimuli we

635 Hair-cell responses to stimuli were imaged with an A1R laser-scanning confocal scan<br>636 head on an upright Nikon NI-E microscope with a resonant scanner and a 60x/1.0 NA CFI Fluor<br>637 water immersion objective equipped For a statuture interaction of process to stimuli were imaged with an A1R laser-scanning confoca<br>636 bead on an upright Nikon NI-E microscope with a resonant scanner and a 60x/1.0 NA (<br>637 water immersion objective equippe 636 head on an upright Nikon NI-E microscope with a resonant scanner and a 60x/1.0 NA CFI Flu<br>637 water immersion objective equipped with a z-piezo. Acquisition was controlled with Nikon<br>638 Elements Advanced Research v. 5 water immersion objective equipped with a z-piezo. Acquisition was controlled with Nikon<br>638 Elements Advanced Research v. 5.20.02. GCaMP6s fluorescence was excited with a 488 nm<br>639 solid-state laser passed through a stan 638 Elements Advanced Research v. 5.20.02. GCaMP6s fluorescence was excited with a 488 nm<br>639 solid-state laser passed through a standard 405/488/561/640 BS20/80 dichroic and collected<br>640 with a 560 nm low-pass dichroic a solid-state laser passed through a standard 405/488/561/640 BS20/80 dichroic and collecte<br>640 with a 560 nm low-pass dichroic and 525/50 emission filter. Images were acquired using a<br>641 GaAsP PMT and 4x averaging. Pixel s 640 with a 560 nm low-pass dichroic and 525/50 emission filter. Images were acquired using a<br>641 GaAsP PMT and 4x averaging. Pixel size for presynaptic imaging was 0.28 µm; pixel size for MI<br>642 imaging was 0.14 µm. Each 641 GaAsP PMT and 4x averaging. Pixel size for presynaptic imaging was 0.28 µm; pixel size for<br>642 imaging was 0.14 µm. Each neuromast (L2 or L3) was stimulated four times (starting with a<br>643 posterior-to-anterior stimulu Example 18 and 18 averaging. Pixel size prespinging was transmitted for the sixter present in the posterior stimulus and alternating between the two directions) with an inter-<br>643 posterior-to-anterior stimulus and altern Follow the metal of the metal of the two directions) with an inter-<br>
643 imaginal of the metal of the mandled us to collect presynaptic responses (collected first)<br>
645 hair-bundle responses to both stimulus directions fo For a constraint measure of collections in the collect present present present of constraining and<br>645 hair-bundle responses to both stimulus directions for each neuromast. 3 z-slices (1.5 µm step<br>646 size for presynaptic 645 hair-bundle responses to both stimulus directions for each neuromast. 3 z-slices (1.5  $\mu$ m step 646 size for presynaptic responses; 0.5  $\mu$ m step size for hair bundle responses) were collected per<br>23  $646$  size for presentation responses; 0.5  $\mu$ m step size for hair bundle per collected per co

t<br>t 647 timepoint for 110 timepoints at a frame rate of 33 ms for a total of ~100 ms per z-stack and a<br>648 total acquisition time of ~11 sec. Stimulation began at timepoint 31; timing of the stimulus was<br>649 triggered by an o 647

Frame of action time of action time of action time of any time point 31; tiggered by an outgoing voltage signal from Nikon Elements.<br>
G50 Calcium responses in the afferent process were acquired on a Swept-field confocal<br>
s 650 Calcium responses in the afferent process were acquir<br>651 system built on a Nikon FN1 upright microscope (Bruker) with<br>652 immersion objective. The microscope was equipped with a Ro<br>653 (Qlmaging), controlled using Pra system built on a Nikon FN1 upright microscope (Bruker) with a 60x/1.0 NA CFI Fluor water-<br>immersion objective. The microscope was equipped with a Rolera EM-C2 EMCCD camera<br>(Qlmaging), controlled using Prairie view 5.4 (Br 655 for postsynaptic imaging was 0.27 µm. Stimuli were delivered as outlined above for hair-cell FERRET 1999 (Qlmaging), controlled using Prairie view 5.4 (Bruker). GCaMP6s was excited using a 488 r<br>654 solid state laser. We used a dual band-pass 488/5612 nm filter set (59904-ET, Chroma). Pix<br>655 for postsynaptic imag 654 solid state laser. We used a dual band-pass 488/5612 mm filter set (59904-ET, Chroma). Pixel<br>655 for postsynaptic imaging was 0.27 μm. Stimuli were delivered as outlined above for hair-cell<br>656 responses. Each neuroma 655 for postsynaptic imaging was 0.27  $\mu$ m. Stimuli were delivered as outlined above for hair-cell<br>656 responses. Each neuromast (L2, L3 or L4) was stimulated two times with an inter-stimulus<br>657 interval of ~2 min. 5 z-656 responses. Each neuromast (L2, L3 or L4) was stimulated two times with an inter-stimulus<br>657 interval of ~2 min. 5 z-slices (1.0 µm step) were collected per timepoint for 80 timepoints at frame rate of 20 ms for a tot 657 interval of  $\approx$  2 min. 5 z-slices (1.0 µm step) were collected per timepoint for 80 timepoints a<br>658 frame rate of 20 ms for a total of  $\approx$  100 ms per Z-stack and a total acquisition time of  $\approx$  8 see<br>659 Stimulati 658 frame rate of 20 ms for a total of ~100 ms per Z-stack and a total acquisition time of ~8 sec.<br>659 Stimulation began at timepoint 31; timing of the stimulus was triggered by an outgoing voltage<br>660 signal from Prairie 659 Stimulation began at timepoint 31; timing of the stimulus was triggered by an outgoing volta<br>660 signal from Prairie view.<br>661 Acquired images were converted into TIFF series for processing. Researchers were bl<br>662 to

660 signal from Prairie view.<br>661 Acquired images were converted into TIFF series for processing. Researchers were blind<br>662 to genotype during analysis. Z-stacks were average projected, registered, and spatially<br>663 smoot 661 Acquired images<br>662 to genotype during analy<br>663 smoothed with a Gaussia<br>664 described previously (Zh: to genotype during analysis. Z-stacks were average projected, registered, and spatially<br>663 Smoothed with a Gaussian filter (size = 3, sigma = 2) in custom-written MatLab software as<br>664 described previously (Zhang et al., 663 smoothed with a Gaussian filter (size = 3, sigma = 2) in custom-written MatLab softwar<br>664 described previously (Zhang et al., 2018). The first 10 timepoints (~1 sec) were remove<br>665 reduce the effect of initial photo described previously (Zhang et al., 2018). The first 10 timepoints (~1 sec) were removed to<br>665 reduce the effect of initial photobleaching. Registered average projections were then open<br>666 Fiji for intensity measurements Fig. 1986 of the effect of initial photobleaching. Registered average projections were then open<br>666 Fiji for intensity measurements. Using the Time Series Analyzer V3 plugin, circular ROIs (18)<br>667 pixels for presynaptic France in Entertainment photoblead ingless of ingless analyzer V3 plugin, circular ROIs (18x18<br>pixels for presynaptic responses; 8x8 pixels for hair-bundle responses, 12x12 pixels for afferent<br>process) were placed on hair Fiji for intensity measurements. Using the Time Series Analyzer V3 plugin, circular ROIs (18x18<br>
pixels for presynaptic responses; 8x8 pixels for hair-bundle responses, 12x12 pixels for afferent<br>
process) were placed on ha present presponses process were placed on hair bundles or synaptic sites; average intensity measurements over<br>669 time were measured for each ROI, as described previously (Lukasz and Kindt, 2018).<br>670 Neuromasts were exclu time were measured for each ROI, as described previously (Lukasz and Kindt, 2018).<br>670 between the synaptic sites of motion artifacts. Hair-bundles responses were<br>671 excluded if they responded to stimuli of both direction ROI Neuromasts were excluded in the case of motion artifacts. Hair-bundles responses were excluded if they responded to stimuli of both directions. All other data was included Presynaptic responses were defined as >10% ΔF EXTE STALL EXERCIAE AT All other data was included in an excluded if they responded to stimuli of both directions. All other data was included in artifacts. Presynaptic responses were defined as >10%  $\Delta$ F/F0 within the c Frame in the year of the stimula of the March Christman in the March direction mass of the stimulus or  $>20\%$  within<br>1 sec of stimulus onset. Hair-bundle responses were defined as  $>15\%$   $\Delta F$ /F0 within the 500 ms<br>674 s Fresynaptic responses were defined as >10%  $\Delta$ F/F0 within the 500 ms stimulus or >20% within<br>1 sec of stimulus onset. Hair-bundle responses were defined as >15%  $\Delta$ F/F0 within the 500 ms<br>574 stimulus and >15% in the 500 674 stimulus and >15% in the 500 ms after the stimulus. Postsynaptic responses were defined as  $>$ 5%  $\Delta$ F/F0 and a minimum duration of 500 ms. Square wave responses indicate movement  $675$  >5%  $\Delta$ F/FO and a minimum duration of 500 ms. Square wave responses indicate movement<br>24  $675$   $\frac{1}{24}$ 

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677 (Graphpad). The first 20 timepoints were averaged to generate an F0 value, and all re<br>678 were calculated as  $\Delta F/F0$ . Responses presented in figures represent average response<br>679 synaptically active cells within a ne were calculated as  $\Delta F/F0$ . Responses presented in figures represent average responses of<br>679 synaptically active cells within a neuromast. The max  $\Delta F/F0$  was compared between wild-type<br>680 animals and double mutants.<br>68 ockeen by the calculate of the calculate of the Section of the Section of the Section of the Section animals and double mutants.<br>
680 animals and double mutants.<br>
681 Zebrafish startle behavior o synaptically active cells within a neuromast. The max ∆F/F0 was compared between wild-type<br>680 animals and double mutants.<br>681 Zebrafish startle behavior<br>683 A Zantiks MWP behavioral system was used to examine acoustic

681<br>682 Zebrafish startle behavior<br>683 A Zantiks MWP behavioral sys<br>684 trials were performed at 5 dpi 683<br>684<br>685 683 A Zantiks MWP behavioral<br>684 trials were performed at 5<br>685 compared  $nrxn3a^{+/}$ ;  $nrxn3a^{+}$ trials were performed at 5 dpf, on three independent days. For this behavioral analysis, we<br>685 compared  $nrxn3a^{t/}$ ;  $nrxn3b^{t/}$  double heterozygotes to  $nrxn3a^{t/}$ ;  $nrxn3b^{t/}$  double mutants for<br>686 an in-clutch, sibli 685 compared  $nrxn3a^{t/2}$ ;  $nrxn3b^{t/2}$  double heterozygotes to  $nrxn3a^{t/2}$ ;  $nrxn3b^{t/2}$  double mutants for an in-clutch, sibling comparison.  $Nrxn3a^{t/2}$ ;  $nrxn3b^{t/2}$  double heterozygotes showed a slight (12%) yet sig 685 compared  $nrxn3a''$ ;  $nrxn3b''$  double heterozygotes to  $nrxn3a'$ ;  $nrxn3b''$  double mutants for<br>686 an in-clutch, sibling comparison.  $Nrxn3a^{t/2}$ ;  $nrxn3b^{t/2}$  double heterozygotes showed a slight<br>687 (12%) yet significant 686 an in-clutch, sibling comparison. Nrxn3a"; nrxn3b" double heterozygotes showed a slight<br>687 (12%) yet significant reduction in complete synapses compared to wild-type controls.<br>688 Therefore, we compared nrxn3a<sup>+/-</sup>; Therefore, we compared  $nrxn3a^{+/}$ ;  $nrxn3b^{+/}$  double heterozygotes and  $nrxn3a^{+/}$ ;  $nrxn$ <br>double mutants sibling to wild-type animals born the same day; this analysis revealed<br>difference in startle response between these g Therefore, we compared  $nrxn3a''$ ;  $nrxn3b''$  double heterozygotes and  $nrxn3a''$ ;  $nrxn3b''$ <br>
double mutants sibling to wild-type animals born the same day; this analysis revealed no<br>
difference in startle response between these

double mutants sibling to wild-type animals born the same day; this analysis revealed no<br>difference in startle response between these genotypes.<br>The Zantiks system tracked and monitored behavioral responses via a built-in 690 difference in startle response between these genotypes.<br>691 The Zantiks system tracked and monitored behavioral responses via a built-in infra<br>692 camera at 30 frames per second. A 12-well plate was used to house larva For the matrice in startle response to these difference in the game of the candidary<br>692 difference in startle response between the camera at 30 frames per second. A 12-well plate was use<br>693 analysis. Each well was filled 692 camera at 30 frames per second. A 12-well plate was used to house larvae during behavioral<br>693 analysis. Each well was filled with E3 and 1 larva. All fish were acclimated in the plate within th<br>694 Zantiks chamber in analysis. Each well was filled with E3 and 1 larva. All fish were acclimated in the plate within t<br>694 Zantiks chamber in the dark for 15 min before each test. To induce startle, an integrated<br>695 stepper motor was used to Franks chamber in the dark for 15 min before each test. To induce startle, an integrated<br>695 stepper motor was used to drive a vibration-induced startle response. A vibrational stimulus<br>696 that triggered a maximal % of an stepper motor was used to drive a vibration-induced startle response. A vibrational stimution that triggered a maximal % of animals startling in controls without any tracking artifacts (<br>the vibration), was used for our st 696 that triggered a maximal % of animals startling in controls without any tracking artifacts (due<br>697 the vibration), was used for our strongest stimuli. Each larva was presented with each<br>698 vibrational stimulus 5 time the vibration), was used for our strongest stimuli. Each larva was presented with each<br>698 vibrational stimulus 5 times with 100 s between trials. For each animal, the proportion of startle<br>699 responses out of the 5 trial 698 vibrational stimulus 5 times with 100 s between trials. For each animal, the proportion<br>699 responses out of the 5 trials was plotted. During the tracking and stimulation, a Cisco r<br>700 connected to the Zantiks system For the conservation of the 5 trials was plotted. During the tracking and stimulation, a Cisco router<br>
connected to the Zantiks system was used to relay x, y coordinates of each larva every frame.<br>
To qualify as a startle FRAND 1990 1991 responses out of the Zantiks system was used to relay x, y coordinates of each larva every frame<br>
1991 1991 1991 To qualify as a startle response, a distance above 4 pixels or  $\sim$ 1.9 mm was required withi 700 connected to the Zantiks system was used to relay x, y coordinates or each larva every frame.<br>701 To qualify as a startle response, a distance above 4 pixels or ~1.9 mm was required within 2<br>702 frames after stimulus o Frames after stimulus onset. Animals were excluded from our analysis if no tracking data wa<br>703 Frames after stimulus onset. Animals were excluded from our analysis if no tracking data was recorded for the animal. 102 Frames after stimulus onest. Animals were excluded for the animals were excluded for the animal.<br>704<br>25  $704$ 

 $\begin{bmatrix} 1 \\ 2 \\ 3 \end{bmatrix}$ 205 Experimental design and statistical analysis<br>
706 Statistical analyses and data plots were performed to estimate approximate sample of<br>
709 performed on a minimum of 4 animals, 7 net 207 with error bars on graphs and in text are expressed as mean  $\pm$  SEM. A power analysis was<br>208 performed to estimate approximate sample sizes needed. All zebrafish experiments were<br>209 performed on a minimum of 4 anim 708 performed to estimate approximate sample sizes needed. All zebrafish experiments were<br>709 performed on a minimum of 4 animals, 7 neuromasts. Primary posterior lateral-line neuro<br>710 with A-P orientations L1-L4 were us For a performed on a minimum of 4 animals, 7 neuromasts. Primary posterior lateral-line neuro<br>
710 with A-P orientations L1-L4 were used for all experiments except Ca<sub>V</sub>1.3 immunostains wh<br>
711 examined L1, L2 and DV1 neu 710 with A-P orientations L1-L4 were used for all experiments except Ca<sub>V</sub>1.3 immunostains which<br>711 examined L1, L2 and DV1 neuromasts. For 5 dpf larvae, each neuromast represents analysis<br>712 from 12 to 20 hair cells an examined L1, L2 and DV1 neuromasts. For 5 dpf larvae, each neuromast represents analysis<br>
712 from 12 to 20 hair cells and 41-68 synapses. For mouse studies all experiments were perform<br>
713 on at least 4 mutants and 4 sib 22 From 12 to 20 hair cells and 41-68 synapses. For mouse studies all experiments were perform<br>213 on at least 4 mutants and 4 siblings at P28 and P42. For synapse quantification, at least one<br>214 containing 8 IHCs were ex on at least 4 mutants and 4 siblings at P28 and P42. For synapse quantification, at least one ROI<br>714 containing 8 IHCs were examined from each region of the cochlea (apex, mid, base) for each<br>715 animal. All replicates ar 2008<br>214 containing 8 IHCs were examined from each region of the cochlea (apex, mid, base) for each<br>215 animal. All replicates are biological. Samples were scored and imaged blind to genotype<br>216 whenever possible. Where a 215 animal. All replicates are biological. Samples were scored and imaged blind to genotype<br>716 whenever possible. Where appropriate, data was confirmed for normality using a D'Agostino<br>717 Pearson normality test. For pair The Universal Community of the setted are biology of the vertex whenever possible. Where appropriate, data was confirmed for normality using a D'Agoret Pearson normality test. For pairwise comparisons, an unpaired t-test w  $720$ Pearson normality test. For pairwise comparisons, an unpaired t-test was used if data passed<br>T18 normality tests. If the data failed normality tests, a Mann-Whitney U test was used. For multiple<br>T19 comparisons, a one-way 719 comparisons, a one-way or two-way ANOVA was used.<br>720<br>721 Code accessibility: The code Complete Synapse Counter v5.2 and Matlab software to visualize<br>722 calcium signals will be deposited on Github. Frame comparisons, a one-may or the may the contract acts.<br>720<br>721 **Code accessibility:** The code Complete Synapse Counte<br>722 calcium signals will be deposited on Github.<br>723 721<br>722<br>723<br>724 721 Code accessibility: The code Complete Synapse Counter v5.2 and Matlab software to visualize<br>22 calcium signals will be deposited on Github.<br>723<br>725 723<br>724<br>725<br>726 - - -<br>724<br>725<br>726<br>727 725<br>726<br>727<br>728 725

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739 and presynaptic activity of cochle<br>
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| (A) Schematic showing a larval zebrafish at 5 days post fertilizational (A) Schematic showing a larval zebrafish at 5 days post fertilization<br>934 bair cells are in the inner ear and posterior lateral line (neuroma<br>935 late (A) Schematic showing a larval zebrafish at 5 days post fertilization (5 dpf). Clusters of sensory<br>934 hair cells are in the inner ear and posterior lateral line (neuromasts, pink). Hair cells of the<br>935 lateral line are i atteral line are innervated by neurons (green) that project from the posterior lateral-line<br>936 ganglion (pLLg, blue). (B) A lateral-line neuromast organ viewed from the side. Hair cells ar<br>937 labeled in gray, presynapses ganglion (pLLg, blue). (B) A lateral-line neuromast organ viewed from the side. Hair cells a<br>937 labeled in gray, presynapses or ribbons in magenta, and afferent processes beneath the h<br>938 cells in green. The dashed box i ganghon (pLLg, blue). (**D**) A lateral-line neuromast organ viewed from the side. Half cells are<br>937 labeled in gray, presynapses or ribbons in magenta, and afferent processes beneath the hair<br>938 layer, viewed from the top 937 labeled in gray, presynapses or ribbons in magenta, and afferent processes beneath the hair<br>938 cells in green. The dashed box indicates the synaptic layer. (C) Immunostaining of the synaptic<br>939 layer, viewed from the ens in green. The dashed box indicates the synaptic layer. (C) immunostaning of the synaptic<br>939 layer, viewed from the top down. CTBP labels the presynapses or ribbons (magenta), pan-<br>940 MAGUK labels the postsynapses (gr 945 latter to primarily or niveye, a splice variant or ctbP2. The moduli is surrounded by gluta 942 highlights the main components of a hair-cell ribbon synapse. The presynapse, or ribbon is<br>943 composed primarily of Ribeye, a splice variant of CtBP2. The ribbon is surrounded by glutamate<br>944 filled synaptic vesicles 942 highlights the main components of a hair-cell ribbon synapse. The presynapse, or ribbon is<br>943 composed primarily of Ribeye, a splice variant of CtBP2. The ribbon is surrounded by glutamate-<br>944 filled synaptic vesicl composed primarily of Ribeye, a splice variant of CtBP2. The ribbon is surrounded by glutar<br>944 filled synaptic vesicles (SVs). Ca<sub>V</sub>1.3 channels (purple) are clustered beneath the ribbon. AN<br>945 receptors are clustered w 947 composition prioritic veces annihed zebitansh indicaties that are predicted to distupt the  $\alpha$  form 945 receptors are clustered within the postsynaptic density (PSD). (E) There are 2 orthologues of<br>946 Nrxn3 in zebrafish, Nrxn3a and Nrxn3b. Similar to mammals, each neurexin has a long  $\alpha$  form<br>947 and a shorter  $\beta$  fo 945 receptors are clustered within the postsynaptic density (PSD). (L) mere are 2 orthologies of<br>946 Nrxn3 in zebrafish, Nrxn3a and Nrxn3b. Similar to mammals, each neurexin has a long α form<br>947 and a shorter β form. We 947 and a shorter β form. We examined zebrafish mutants that are predicted to disrupt the α form<br>948 of each orthologue (C455stop and R134stop). The α and β forms each have a unique start and<br>949 signal peptide (SP). Eac 948 of each orthologue (C455stop and R134stop). The α and β forms each have a unique start and<br>949 signal peptide (SP). Each α form has 6 Laminin G-like domains (LNS) and 3 epidermal growth<br>950 factor-like domains (EGF). 949 signal peptide (SP). Each α form has 6 Laminin G-like domains (LNS) and 3 epidermal growth<br>950 factor-like domains (EGF). The red dashed line indicates the location of the RNA FISH probes<br>951 used in F-H. (F-H) RNA F 949 signal peptide (SP). Each α form has 6 Laminin G-like domains (LNS) and 3 epidermal growth<br>950 factor-like domains (EGF). The red dashed line indicates the location of the RNA FISH probes<br>951 used in F-H. (F-H) RNA F 954 independent experiments. The dashed lines in F-H outline the locations of hair cells. All images essam F-H. (F-H) RNA FISH analysis reveals that both α-mxh3a (F, orange) and α-mxh3b (G,<br>952 cyan) mRNAs are present in lateral-line hair cells at 5 dpf. In H, hair cells<br>953 (*myo6b:memGCαMP6s*) are shown in grayscale. R 955 are from larvae at 5 dpf. Scale bars = 5  $\mu$ m in C and F, 1  $\mu$ m in the inset in C.<br>956 953 (*myoob:memocum os)* are shown in grayscale. RNA FISH labeling was committed in 3<br>954 independent experiments. The dashed lines in F-H outline the locations of hair cells. A<br>955 are from larvae at 5 dpf. Scale bars = 955 are from larvae at 5 dpf. Scale bars = 5  $\mu$ m in C and F, 1  $\mu$ m in the inset in C.<br>956<br>957<br>958 956<br>957<br>958<br>959 ---<br>957<br>958<br>959<br>960 958<br>959<br>960 ---<br>959<br>960

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- 973 Indifferent postsynapses (J) per half cell is decreased in *mxh5d*, *mxh5b* mutants compared to<br>974 Indifferent wild-type controls. N = 10 wild-type and 11 *nrxn3a; nrxn3b* mutant neuromasts at 5 dpf.<br>975 Inspired to 974 wild-type controls. N = 10 wild-type and 11 *mxh3d*, *mxh3b* mutant neuromasts at 3 dpf.<br>975 Synapse quantifications were replicated in at least three separate experiments. An unpair<br>976 test (2-tailed) was used in G
- 977 bites (2-tailed) was used in A, and a 2-way and a 2-way and a 2-way ANOVA was used in C.<br>978<br>979<br>980 978<br>979<br>980<br>981
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986<br>987<br>988<br>989<br>990<br>991 988 (A-F) Confocal images of developing hair cells (3 dpf) from wild-type controls (A<br>989 *nrxn3b* mutants (D-F). CTBP is used to label the presynapses (A,D), and MAGUK<br>990 the postsynapses (B,E). Merged images are shown i (A-F) confocal images of developing handers (3 dpf) from wild type controls (A-C) and *mxn3b*<br> *nrxn3b* mutants (D-F). CTBP is used to label the presynapses (A,D), and MAGUK is used to label<br>
the postsynapses (B,E). Merged the postsynapses (B, E). Merged images are shown in C and F. The insets to the side in C and F<br>show 3 examples of individual synapses. Dashed lines in C and F outline the hair-cell region in<br>each image. (G-J) Quantificati 990 the postsynapses (B, E). Merged images are shown in C and F. The insets to the side in C and F<br>991 show 3 examples of individual synapses. Dashed lines in C and F outline the hair-cell region in<br>992 each image. (G-J) Q each image. (**G-J**) Quantification reveals that wild-type controls and *nrxn3a; nrxn3b* mutants<br>
have a similar number of hair cells per neuromast (G). There are significantly fewer complete<br>
synapses per hair cell in *nrx* 993 have a similar number of hair cells per neuromast (G). There are significantly fewer complete<br>994 synapses per hair cell in *nrxn3a; nrxn3b* mutants compared to wild-type controls (H). Along w<br>995 fewer complete synaps synapses per hair cell in *nrxn3a; nrxn3b* mutants compared to wild-type controls (H). Along with fewer complete synapses, there are significantly more unpaired presynapses (I) and postsynapses (J) per hair cell in *nrxn3a* 995 fewer complete synapses, there are significantly more unpaired presynapses (I) and<br>996 fewer complete synapses, there are significantly more unpaired presynapses (I) and<br>996 postsynapses (J) per hair cell in *nrxn3a;* 996 postsynapses (J) per hair cell in  $nrxn3a$ ;  $nrxn3b$  mutants compared to wild-type controlled presynapses (I), or pothere is no change in the total number of presynapses (I), or pothermore under the significant cells th 997 bostsynapses (J) per han cell in mxn3d, mxn3b mutants compared to wild-type controls. In<br>997 developing hair cells there is no change in the total number of presynapses (I), or postsynap<br>37

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- 999 and 13  $nrxn3a$ ;  $nrxn3b$  mutant neuromasts. Synapse quantifications were replicated in three<br>900 separate experiments. An unpaired t-test (2-tailed) was used in G and H, and a 2-way ANOVA<br>901 was used in I and J. ns P 9999 and 19 *mxh3d*, *mxh3b* mutant neuromasts. Synapse quantifications were replicated in three<br>000 separate experiments. An unpaired t-test (2-tailed) was used in G and H, and a 2-way ANOVA<br>001 was used in I and J. ns P 1001 was used in I and J. ns P > 0.05, \*P < 0.05, \*\*\*\*P < 0.0001. Scale bars = 5 µm in C and F, 0.5 µm<br>1002 in the insets.<br>1003 1002 in the insets.<br>1003
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1005 Fig 4. Loss of Nrxn3 impacts pre- and post-synapse size and Cay1.3 channel localization in<br>
1006 **alteral-line hair cells**<br>
1007 **(A-D)** There is a significant increase in the area of paired (A,C) but not unpaired (B, 1007 **(A-D)** There is a signif<br>1008 post-synapses in *nrxn*<br>1009 images of mature neu<br>1010 (G,H). An immunostai 1008 post-synapses in *nrxn3a; nrxn3b* mutants compared to wild-type controls. (**E-H**) Confocal<br>1009 images of mature neuromasts (5 dpf) from wild-type controls (E,F) and *nrxn3a; nrxn3b* mutant<br>1010 (G,H). An immunostain 1009 images of mature neuromasts (5 dpf) from wild-type controls (E,F) and *nrxn3a; nrxn3b* mutants (G,H). An immunostain for CTBP is used to label the presynapses along with Ca<sub>V</sub>1.3 (E,G), immunostain for MAGUK is used 1010 (G,H). An immunostain for CTBP is used to label the presynapses along with Ca<sub>V</sub>1.3 (E,G), or an<br>1011 immunostain for MAGUK is used to label the postsynapse and Ca<sub>V</sub>1.3 (F,H). Merged images are<br>1012 shown in E-H. Th 1011 immunostain for MAGUK is used to label the postsynapse and Ca<sub>V</sub>1.3 (F, H). Merged images are<br>1012 shown in E-H. The inset to the right of each merged image shows 3 examples of individual<br>1013 synapses. (I-L) Quantif 1012 shown in E-H. The inset to the right of each merged image shows 3 examples of individual<br>1013 synapses. (I-L) Quantification reveals that the number of Ca<sub>V</sub>1.3-CTBP paired puncta per hair<br>1014 cell is the same in *n* 1013 synapses. (I-L) Quantification reveals that the number of Ca<sub>V</sub>1.3-CTBP paired puncta per ha<br>1014 cell is the same in *nrxn3a; nrxn3b* mutants compared to wild-type controls (I). Compared to<br>1015 wild-type, *nrxn3a;* 1013 synapses. ( $I-L$ ) Quantification reveals that the number of Cay1.3 CTBP pairce puncta per hair<br>1014 cell is the same in  $nrxn3a$ ;  $nrxn3b$  mutants compared to wild-type controls (I). Compared to<br>1015 wild-type,  $nrxn3a$ ; 1015 wild-type, *nrxn3a; nrxn3b* mutants compared to wild type controls (i). Compared to<br>1015 wild-type, *nrxn3a; nrxn3b* mutant neuromasts have a dramatically fewer Ca<sub>V</sub>1.3-MAGUK pair<br>1016 puncta per hair cell (J). The 1015 wild-type, *mando*, *mando* mutant neuromasts have a dramatically fewer Cay1.3 MAGOK paired<br>
1016 puncta per hair cell (J). The area (K) but not the average intensity (L) of Ca<sub>V</sub>1.3 puncta<br>
1017 associated with CTBP 1017 associated with CTBP puncta are significantly lower in  $nrxn3a$ ;  $nrxn3b$  mutants compa<br>1018 wild-type controls. N = 10 wild-type and 11  $nrxn3a$ ;  $nrxn3b$  mutant neuromasts in A-D<br>1019 and L and n = 10 wild-type and 8 usism associated with CTBP puncta are significantly lower in *manda*; *nranda* matallities compared to<br>1018 wild-type controls. N = 10 wild-type and 11 *nrxn3a; nrxn3b* mutant neuromasts in A-D and I,<br>1019 and L and n = 1 1019 and L and n = 10 wild-type and 8 nrxn3a; nrxn3b mutant neuromasts in A-D and I, K<br>1019 and L and n = 10 wild-type and 8 nrxn3a; nrxn3b mutant neuromasts in J. Images and<br>1020 quantification are from larvae at 5 dpf. quantification are from larvae at 5 dpf. Ca<sub>v</sub>1.3 labeling was confirmed in two separate<br>
1021 experiments. An unpaired t-test (2-tailed) was used in A-D and I-L. ns P > 0.05, \*\*P < 0.0001. Scale bars = 5 µm in E-H, 1 µm 1021 experiments. An unpaired t-test (2-tailed) was used in A-D and I-L. ns P > 0.05, \*\*P < 0.<br>1022 \*\*\*\*P < 0.0001. Scale bars = 5  $\mu$ m in E-H, 1  $\mu$ m in the inset in E-H.<br>1023<br>1024 1022 \*\*\*\*P < 0.0001. Scale bars = 5  $\mu$ m in E-H, 1  $\mu$ m in the inset in E-H.<br>1023<br>1024<br>1025  $1023$ <br>1024<br>1025<br>1026 1023 1025<br>1026<br>1027<br>1028 1026<br>1027<br>1028<br>1029 1027<br>1028<br>1029<br>1030 1028<br>1029<br>1030<br>1031 1029<br>1030<br>1031<br>1032 1030<br>1031<br>1032<br>1033 1031<br>1032<br>1033

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1034<br>1035<br>1036<br>1037<br>1038 1035 Fig 5. NRXN3 is required at 6 weeks for proper synapse number in mouse auditory inner hair<br>1036 cells<br>1037 (A-B) Confocal images of mouse IHCs at 6 weeks (P42) from control (A) and *Nrxn3* mutant<br>1038 animals (Atoh1-1030 cells<br>
1037 (A-B)<br>
1038 anim<br>
1039 GluR:<br>
1040 regio 2037 (A-B) confocal miages of mouse ines at 6 weeks (P42) from control (A) and NYXIS mutant<br>2038 animals (Atoh1-Cre; Nrxn3<sup>flox/flox</sup>) (B). CTBP2 is used to label the presynapses (magenta), an<br>2039 GluR2 is used to label animals (Atoh1-Cre; Nrxn3<sup>noxynox</sup>) (B). CTBP2 is used to label the presynapses (magenta), and<br>1039 GluR2 is used to label the postsynapses (green). Merged images show 4 IHCs from 3 differen<br>1040 regions of the cochlea (ap 1039 GluR2 is used to label the postsynapses (green). Merged images show 4 IHCs from 3 different<br>
1040 regions of the cochlea (apex, middle, basal thirds) for each genotype. Dashed lines indicate the<br>
1041 outlines of hai 1041 outlines of hair-cell bodies in each image. (C-E) Quantification reveals that compared to<br>1042 controls, *Nrxn3* mutants have significantly fewer complete synapses per IHC at the apex (C) mid<br>1043 (D) and base (E). N 1041 controls, *Nrxn3* mutants have significantly fewer complete synapses per IHC at the apex<br>1043 (D) and base (E). N = 70 control and 58 *Nrxn3* IHCs for the apex region, 70 control and 64<br>1044 IHCs for the for mid regio 1042 controls, *NRXIS* mutants have significantly fewer complete synapses per ITC at the apex (C) find<br>1043 (D) and base (E). N = 70 control and 58 *Nrxn3* IHCs for the apex region, 70 control and 64 *Nrxn3*<br>1044 IHCs for 1043 (D) and base (E). N = 70 control and 58 *Nrxn3* IHCs for the apex region, 70 control and 64 *Nrxn3*<br>1044 IHCs for the for mid region, 75 control and 57 *Nrxn3* IHCs for the for base region. These findings<br>1045 were c 1044 IHCs for the for mid region, 75 control and 57 MXh3 IHCs for the for base region. These findings<br>1045 were compiled from 4 animals from each genotype and from 2 independent liters and<br>1046 Immunostains. An unpaired t 1046 immunostains. An unpaired t-test (2-tailed) was used in C-E. \*P < 0.05, \*\*P < 0.01. Scal<br>1047  $\mu$ m in A. 1047  $\mu$ m in A.  $\frac{1047}{41}$ 

1047 µm in A.



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1049<br>1050<br>1051<br>1052<br>1053 1049 Fig 6. NEXTEST is required for proper nan-cell synapse function in the lateral line.<br>
1050 (A) Schematic of a neuromast shown from the side. The pre- and post-synaptic re<br>
1051 measure GCaMP6s responses is indicated 1051 measure GCaMP6s responses is indicated with a dashed box. (**B**) A vibrational acoustic tap<br>1052 stimulus was used at three stimuli of decreasing intensity to trigger an escape response in<br>1053 nrxn3a<sup>+/-</sup>; nrxn3b<sup>+/-</sup> 1052 stimulus was used at three stimuli of decreasing intensity to trigger an escape response in<br>
1053 nrxn3a<sup>+/-</sup>; nrxn3b<sup>+/-</sup> double heterozygotes and nrxn3a<sup>-/-</sup>; nrxn3b<sup>-/-</sup> double mutants. The<br>
1054 proportion of tim 1053  $nrxn3a<sup>+/</sup>; nrxn3b<sup>+/</sup> double heterozygotes and nrxn3a<sup>-/-</sup>; nrxn3b<sup>-/-</sup> double mutants. The proportion of times (out of 5 trials) an animal responded to each stimulus is shown. N = 18  
42$ 1054 proportion of times (out of 5 trials) an animal responded to each stimulus is shown. N = 18<br> $42$  $1054$  proportion of  $42$ 

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| 1055 *IITARISU, HERISB* double heterozygotes, and 21 *IITARISU*, *IITARISU* double mutants at 5 dpf.<br>1056 Behavioral data was acquired from two independents clutches and experimental days.<br>1057 heatmaps show spatial patter 1057 behavioral data was acquired from two independents clutches and experimental days. (C-D) Δ1<br>1057 heatmaps show spatial patterns of presynaptic GCaMP6s increases in hair cells before (C) and<br>1058 during (D) a 500 ms f 1058 during (D) a 500 ms fluid-jet stimulation in a wild-type neuromast. ROIs indicate synaptically<br>1059 active hair cells and examples of regions used to measure the average response per neuromas<br>1060 (E)  $\Delta F/FO$  GCaMP6s 1059 active hair cells and examples of regions used to measure the average response per neuroma<br>1060 (E) ΔF/F0 GCaMP6s traces showing average presynaptic GCaMP6 response during stimulation<br>1061 for wild-type controls (bla 1060 (E)  $\Delta F/F0$  GCaMP6s traces showing average presynaptic GCaMP6 response during stimulation<br>1061 for wild-type controls (black) and *nrxn3a; nrxn3b* mutants (blue). Traces are displayed as mean,<br>1062 dashed lines are S 1061 for wild-type controls (black) and *nrxn3a; nrxn3b* mutants (blue). Traces are displayed as mear<br>1062 dashed lines are SEM, shaded gray represents the timing of the stimulus. (F) Maximum ΔF/F0<br>1063 presynaptic calciu 1062 dashed lines are SEM, shaded gray represents the timing of the stimulus. (F) Maximum ΔF/F0<br>1063 presynaptic calcium responses to stimulation for wild-type controls (black) and *nrxn3a; nrxn3b*<br>1064 mutants (blue). N 1062 dashed lines are SEM, shaded gray represents the timing of the stimulus. (F) Maximum ΔF/F0<br>1063 presynaptic calcium responses to stimulation for wild-type controls (black) and *nrxn3a; nrxn3l*<br>1064 mutants (blue). N presynaptic calcium responses to sumulation for wild type controls (black) and *mxn5d*, *mxn5b*<br>1064 mutants (blue). N = 15 wild-type and 14 *nrxn3a; nrxn3b* mutant neuromasts at 5-6 dpf. (G-H) Δl<br>1065 heatmaps show spatia 1065 heatmaps show spatial patterns of postsynaptic GCaMP6s increases in the afferent terminal<br>1066 before (G) and during (H) a 500 ms fluid-jet stimulation in a wild-type neuromast. ROIs indicate<br>1067 synaptically active 1066 before (G) and during (H) a 500 ms fluid-jet stimulation in a wild-type neuromast. ROIs indica<br>
1067 synaptically active terminals and examples of regions used to measure the average active<br>
1068 postsynaptic respons 1067 synaptically active terminals and examples of regions used to measure the average active<br>
1068 postsynaptic response per neuromast. (I) ΔF/FO GCaMP6s traces showing average postsynaptic<br>
1069 GCaMP6 response during 1068 postsynaptic response per neuromast. (I)  $\Delta F/FO$  GCaMP6s traces showing average postsyn<br>1069 GCaMP6 response during stimulation for wild-type controls (black) and  $nrxn3a$ ;  $nrxn3b$  mu<br>1070 (blue). Traces are displayed 1069 postsynaptic response per neuromast. (1) Δ1710 GCaMP os traces showing average postsynaptic<br>1069 GCaMP6 response during stimulation for wild-type controls (black) and *nrxn3a; nrxn3b* mutants<br>1070 (blue). Traces are CCAMP of Exportse during stimulation for wild type controls (black) and *inxh3d*, *inxh3b* mutants<br>1070 (blue). Traces are displayed as mean, dashed lines are SEM. (J) Maximum ΔF/F0 postsynaptic<br>1071 calcium responses to 1070 (blue). Traces are displayed as mean, dashed lines are SEM. (J) Maximum ∆F/F0 postsynaptic<br>1071 calcium responses to stimulation for wild-type controls (black) and *nrxn3a; nrxn3b* mutants<br>1072 (blue). N = 13 wild-typ 1072 (blue). N = 13 wild-type and 22 *nrxn3a; nrxn3b* mutant neuromasts at 4-5 dpf. Each dot in F<br>1073 I represents the average response from a single neuromast. Calcium imaging findings were<br>1074 acquired from two indepe 1072 (blue). N = 13 who type and 22 *mxh3b*, *mxh3b* mutant neuromasts at 4-5 dpf. Each dot in F and<br>1073 I represents the average response from a single neuromast. Calcium imaging findings were<br>1074 acquired from two ind 1074 acquired from two independents clutches and experimental days. A two-way ANOVA was u<br>1075 in B, a Mann-Whitney test in F and an unpaired t-test was used in J. ns P > 0.05, \*\*\*P < 0.00<br>1076 \*\*P < 0.01. Scale bars = 5 1075 in B, a Mann-Whitney test in F and an unpaired t-test was used in J. ns P > 0.05, \*\*\*P < 0.001,<br>1076 \*\*P < 0.01. Scale bars = 5 µm in D and H.<br>1077<br>1078 1076  $*$  $P < 0.01$ , Scale bars = 5  $\mu$ m in D and H.<br>1077<br>1078 1077<br>1078<br>1079<br>1080

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- 1086<br>1087<br>1088<br>1089<br>1090 1087 (A) Schematic showing a larval zebrafish inner ear. Within the inner ear, cluster<br>1088 present in 3 cristae and 2 maculae. Each macula is associated with an otolith (o<br>1089 analysis reveals that both  $\alpha$ -nrxn3a (ora
- 1088 present in 3 cristae and 2 maculae. Each macula is associated with an otolith (o). (**B**) RNA FISH<br>
1089 analysis reveals that both  $\alpha$ -nrxn3a (orange) and  $\alpha$ -nrxn3b (cyan) mRNAs are present in inner-<br>
1090 ear hai
- 1089 present in 3 cristae and 2 maculae. Each macula is associated with an otomic (o). (B) NNA FISH<br>1089 analysis reveals that both  $\alpha$ -nrxn3a (orange) and  $\alpha$ -nrxn3b (cyan) mRNAs are present in inner-<br>1090 ear hair cel
- 1090 analysis reveals that both α-*nrxn3d* (orange) and α-*nrxn3b* (cyan) mRNAs are present in inner-<br>1090 ear hair cells. The dashed line in B outlines the locations of hair cells within the sensory<br>1091 epithelium. Imag
- 1091 epithelium. Images are from larvae at 5 dpf. Scale bars = 5  $\mu$ m in B. 111 epithelium. Images are from larvae at 5 dpf. Scale bars = 5 µm in B.<br>1091 epithelium. Images at 5 dpf. Scale bars = 5 years = 5 y



1092<br>1093<br>1094<br>1095<br>1096<br>1097 Fig S2. nrxn3a and nrxn3b mRNAs are reduced in lateral-line hair cells in zebrafish nrxn3a;<br>nrxn3b mutants<br>(A-C) RNA FISH reveals that both  $\alpha$ -nrxn3a (A, orange) and  $\alpha$ -nrxn3b (B, cyan) mRNAs are<br>present in lateral-li

1095 (A-C) RNA FISH re<br>1096 present in lateral<br>1097 (*myo6b:memGCc*<br>1098 hair cells. (D-G) C

1095 (A-C) RNA FISH reveals that both α-*nrxn3a*; *nrxn3b* mutants. In C, hair cells<br>1097 (*myo6b:memGCaMP6s*) are labeled in grayscale. The dashed lines in A-C outline the locati<br>1098 hair cells. (D-G) Quantification re

1098 hair cells. (**D-G**) Quantification reveals that the number of α-nrxn3a (D) and α-nrxn3b (F) puncta<br>1099 are reduced in nrxn3a; nrxn3b mutants compared to wild-type controls. In addition, the size of<br>1100 a-nrxn3b (G

1098 hair cells. (**D-G**) Quantification reveals that the number of  $\alpha$ -nrxn3a (D) and  $\alpha$ -nrxn3b (F) puncta<br>1099 are reduced in nrxn3a; nrxn3b mutants compared to wild-type controls. In addition, the size of<br>1100  $\alpha$ -n

1100 a-mxn3b (G), but not a-mxn3d (E) puncta are slightly larger in mxn3d, mxn3b mutants<br>
1101 compared to wild-type controls. An unpaired t-test was used in D-G, n = 12 wild-type ar<br>
1102 nrxn3a; nrxn3b mutant neuromasts

1099 are reduced in *nrxn3a; nrxn3b* mutants compared to wild-type controls. In addition, the size of  $\alpha$ -*nrxn3b* (G), but not  $\alpha$ -*nrxn3a* (E) puncta are slightly larger in *nrxn3a; nrxn3b* mutants compared to wild-t 2009 are reduced in mandal, mando inductive compared to wild type controls. In addition, the size of<br>
2100 a-nrxn3b (G), but not a-nrxn3a (E) puncta are slightly larger in nrxn3a; nrxn3b mutants<br>
2101 compared to wild-typ 1102 *nrxn3a; nrxn3b* mutant neuromasts at 5 dpf. ns P > 0.05, \*P < 0.05, \*\*\*\*P < 0.0001. Scale band-<br>1103 5 µm in C.<br>1104 1102 *mxn3a*; *mxn3b* mutant neuromasts at 5 dpf. ns P  $> 0.05$ ,  $P \le 0.05$ ,  $P \le 0.0001$ . Scale bar =<br>1103 5  $\mu$ m in C.<br>1104<br>1105

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1107<br>1108<br>1109<br>1110<br>1111<br>1111 Fig S3. Minor defects in synapse organization are observed in *nrxn3a* and *nrxn3b* single<br>mutants in mature hair cells at 5 dpf.<br>(A-F) Quantification reveals that both *nrxn3a* and *nrxn3b* single mutants have a similar 1109 mutants in mature han cells at 5 dpf.<br>1110 (A-F) Quantification reveals that both is<br>1111 of hair cells per neuromast compared<br>1112 complete synapses per hair cell in nrxr<br>1113 controls (B). The total number of pre-s (A-F) Quantification reveals that both *nrxn3a* and *nrxn3b* single mutants have a similar number<br>1111 of hair cells per neuromast compared to wild-type controls (A). There are significantly fewer<br>1112 complete synapses pe 1112 complete synapses per hair cell in *nrxn3b* and *nrxn3a* single mutants compared to wild-type<br>1113 controls (B). The total number of pre-synapses are the same across all genotypes but there a<br>1114 significantly more u complete synapses per hair cell in *nrxn3b*<br>controls (B). The total number of pre-syna<br>significantly more unpaired presynapses i<br>postsynapses per hair cell is significantly r<br>compared to wild-type controls. In contra complete synapses per hair cell in *nrxn3b* and *nrxn3d* single mutants compared to wild-type<br>1113 controls (B). The total number of pre-synapses are the same across all genotypes but there a<br>1114 significantly more unpair 1114 significantly more unpaired presynapses in  $nrxn3b$  mutants (C). The total number of<br>1115 postsynapses per hair cell is significantly reduced in both in  $nrxn3b$  and  $nrxn3a$  single mutants<br>1116 compared to wild-type co 1114 significantly more unpaired presynapses in *mando* mutants (c). The total number of<br>1115 postsynapses per hair cell is significantly reduced in both in *nrxn3b* and *nrxn3a* single<br>1116 compared to wild-type controls. compared to wild-type controls. In contrast, the number of unpaired postsynapses per hair cell<br>is the same across all genotypes (D). N = 12 wild-type, 8 nrxn3a and 12 nrxn3b mutant<br>neuromasts in A-D at 5 dpf. A one-way AN 1115 postsynapses per hair cell is significantly reduced in both in  $nrxn3b$  and  $nrxn3a$  single mutants<br>1116 compared to wild-type controls. In contrast, the number of unpaired postsynapses per hair ce<br>1117 is the same acr 1117 is the same across all genotypes (D). N = 12 wild-type, 8 nrxn3a and 12 nrxn3b mutant<br>
1118 neuromasts in A-D at 5 dpf. A one-way ANOVA was used in A-B, while a 2-way ANOVA was used<br>
1119 in C-D. ns P > 0.05, \*P < 0. 1117 is the same across an genotypes (D).  $N = 12$  wild-type, 8 mxh3d and 12 mxh3b mutant<br>1118 neuromasts in A-D at 5 dpf. A one-way ANOVA was used in A-B, while a 2-way ANOVA<br>1119 in C-D. ns P > 0.05, \*P < 0.05, \*\*\*P < 0. 1119 in C-D. ns P > 0.05, \*P < 0.05, \*\*\*P < 0.001, \*\*\*\*P < 0.0001.<br>1120 1120<br>1120<br>1120





1121<br>1122<br>1123<br>1124<br>1125 1122 Fig S4. Synapse loss in mature han cells is not linked to hair-cell orientation<br>1123 (A-B) In primary posterior lateral-line neuromasts there are two populations of<br>1124 responds to anterior flow (blue, A), while the 1123 (A-B) In primary posterior lateral-line neuromasts there are two populations of hair cens. One<br>1124 responds to anterior flow (blue, A), while the other responds to posterior flow (orange, A). Eac<br>1125 population is s 1125 population is selectively innervated by distinct afferent neurons (blue and orange processes).<br>
1126 There is a significant and equivalent reduction in the number of complete synapses in hair cel<br>
1127 that respond t 1126 There is a significant and equivalent reduction in the number of complete synapses in hair cells<br>
1127 that respond to anterior and posterior flow in  $nrxn3a$ ;  $nrxn3b$  mutants compared to wild-type<br>
1128 controls (B). 1127 that respond to anterior and posterior flow in  $nrxn3a$ ;  $nrxn3b$  mutants compared to wild-type<br>1128 controls (B). N = 10 wild-type and 11 in  $nrxn3a$ ;  $nrxn3b$  mutant neuromasts at 5 dpf. A 2-way<br>1129 ANOVA was used in controls (B). N = 10 wild-type and 11 in  $nrxn3a$ ;  $nrxn3b$  mutant neuromasts at 5 dpf. A 2-way 1128 controls (B). N = 10 wild-type and 11 in *m* xii 3d, *m* xii 3d mutant neuromasts at 5 dpf. A 2-way<br>1129 ANOVA was used in B. ns P > 0.05, \*\*\*\*P < 0.0001.<br>1130 1130<br>1131<br>1131

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1142 Fig So. Quantification of synapse loss in anterior macula and medial crista in *m* xh3b, *m* xh3b<br>1143 **mutants**<br>1144 Quantification reveals that wild-type controls and *nrxn3a; nrxn3b* mutants have a similar<br>1145 num 1143 mutants<br>1144 Quantific<br>1145 number of<br>1146 complete<br>1147 wild-type 1144 Quantification reveals that wild type controls and *mxhod*, *mxhod* mutants have a similar<br>1145 number of hair cells per anterior macula and medial crista. There are significantly fewer<br>1146 complete synapses per hair 1146 complete synapses per hair cell in each epithelium in *nrxn3a; nrxn3b* mutants compared<br>1147 wild-type controls. Along with fewer complete synapses, there are significantly more un<br>1148 presynapses per hair cell in *n* unique to the synapses per hair cell in each epithelium in *mandu, mandu* mutants compared to<br>1147 wild-type controls. Along with fewer complete synapses, there are significantly more unpair<br>1148 presynapses per hair cell presynapses per hair cell in *nrxn3a; nrxn3b* mutants compared to wild-type controls in both the<br>1149 anterior macula and medial crista. There are also more unpaired postsynapses per hair cell in<br>1150 the anterior macula, 2148 presynapses per hair cell in *mxn5a*; *mxn5b* mutants compared to wild type controls in both the<br>
2149 anterior macula and medial crista. There are also more unpaired postsynapses per hair cell in<br>
2150 the anterior 1150 the anterior macula, but not the medial crista in  $nrxn3a$ ;  $nrxn3b$  mutants compared to wild-ty<br>
1151 controls. N = 8 wild-type and n = 6  $nrxn3a$ ;  $nrxn3b$  mutant anterior maculae, n = 8 wild-type an<br>
1152 n = 7  $nrxn3a$ 1150 the anterior macula, but not the medial crista in *mxn5u*, *mxn5b* mutants compared to what type<br>
1151 controls. N = 8 wild-type and n = 6 *nrxn3a; nrxn3b* mutant anterior maculae, n = 8 wild-type and<br>
1152 n = 7 *nr* 1151 controls. N = 8 wild-type and n = 6 *mxh3d*, *mxh3b* mutant anterior maculae, n = 8 wild-type and<br>1152 n = 7 *nrxn3a; nrxn3b* mutant medial cristae. Quantifications are from larvae at 5 dpf. An<br>1153 unpaired t-test w 1152  $n = 7$  mansu, mansu mutant medial cristae. Quantifications are from larvae at 5 dpf. An<br>1153 unpaired t-test was used for comparisons. ns P > 0.05, \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.00<br>1154 49 1154  $^{1154}$ 



1156<br>1157<br>1158<br>1159 1156 Fig S7. NRXN3 is required at 4 weeks for proper synapse numbers in mouse auditory inner<br>1157 hair cells<br>1158 (A-B) Confocal images of 4-week old (P28) mouse inner hair cells from control (A) and Nrxn3<br>1159 mutant anim 1157 han cens<br>1158 (A-B) Con<br>1159 mutant and<br>1160 and GluR?<br>1161 different 1158 (A-B) Confocal images of 4 week old (P28) mouse inner han cens from control (A) and MXh3<br>1159 mutant animals (Atoh1-Cre; Nrxn3<sup>flox/flox</sup>) (B). CTBP2 is used to label the presynapses (magent<br>1160 and GluR2 is used to mutant animals (Atoh1-Cre; Nrxn3<sup>noxylox</sup>) (B). CTBP2 is used to label the presynapses (magenta),<br>1160 and GluR2 is used to label the postsynapses (green). Merged images show 4 IHCs from 3<br>1161 different regions of the coc 1160 and GluR2 is used to label the postsynapses (green). Merged images show 4 IHCs from 3<br>1161 different regions of the cochlea (apex, middle, basal thirds) for each genotype. Dashed lines<br>1162 indicate the outlines of h indicate the outlines of hair-cell bodies in each image. (C-E) Quantification reveals that<br>1163 compared to controls, *Nrxn3* mutants have significantly fewer complete synapses per inner l<br>1164 cell at the mid (D) and base 1163 compared to controls, *Nrxn3* mutants have significantly fewer complete synapses per in<br>1164 cell at the mid (D) and base (E), and a reduced but not significant decrease at the apex (<br>1165 61 control and 58 *Nrxn3* IH 1163 compared to controls, *NRXn3* mutatics have significantly fewer complete synapses per inner hair<br>1164 cell at the mid (D) and base (E), and a reduced but not significant decrease at the apex (C). N =<br>1165 61 control 1165 61 control and 58 *Nrxn3* IHCs for the apex region, 65 control and 59 *Nrxn3* IHCs for the for mid<br>1166 region, 50 control and 60 *Nrxn3* IHCs for the for base region. These findings were compiled<br>1167 from 4 animals 1165 of control and 58 MXh3 IHCs for the apex region, 65 control and 59 MXh3 IHCs for the for line<br>
1166 region, 50 control and 60 Nrxn3 IHCs for the for base region. These findings were compiled<br>
1167 from 4 animals from 1166 region, 50 control and 60 MXM3 IHCs for the for base region. These imaings were compiled<br>1167 from 4 animals from each genotype. An unpaired t-test was used in C-E. \*\*P < 0.01, \*\*\*P <<br>1168 0.001. Scale bar = 5 µm in 1168  $0.001$ . Scale bar = 5  $\mu$ m in A.<br>1169  $1169$ 

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1171 Fig S8. Loss of Nrxn3 does not impact the magnitude of mechanosensitive responses in<br>
1172 lateral-line hair cells.<br>
1173 (A) Schematic of a neuromast shown from the side. The region used to measure<br>
1174 mechanosens

1172 lateral-line han cells.<br>1173 (A) Schematic of a net<br>1174 mechanosensitive GCa<br>1175 box. (B) ΔF/F0 GCaMP<br>1176 jet stimulation (grey a

1173 (A) Schematic of a heardmast shown from the side. The region used to measure<br>1174 mechanosensitive GCaMP6 responses (MET) in apical hair bundles is indicated w<br>1175 box. (B) ΔF/F0 GCaMP6s traces showing average MET G 1175 box. (B)  $\Delta F$ /FO GCaMP6s traces showing average MET GCaMP6 response during a 500 ms fluid<br>1176 in extendination (grey area) for wild-type controls (black) and  $nrxn3a$ ;  $nrxn3b$  mutants (blue).<br>1177 Traces are display 1175 box. (**B**/21710 GcaMP6s traces showing average MET GcaMP6 response during a 500 ms fiuld<br>1176 iet stimulation (grey area) for wild-type controls (black) and *nrxn3a; nrxn3b* mutants (blue).<br>1177 Traces are displayed 1176 Jet stimulation (grey area) for wild type controls (black) and *mxh3d*, *mxh3b* mutants (blue).<br>
1177 Traces are displayed as mean, dashed lines are SEM. (C) Maximum  $\Delta F/FO$  MET calcium GCaN<br>
1178 during stimulation

1177 Traces are displayed as mean, dashed lines are SEM. (C) Maximum ∆F/F0 MET calcium GCaMF6<br>1178 during stimulation for wild-type controls (black) and *nrxn3a; nrxn3b* mutants (blue). N = 15<br>1179 wild-type and 13 *nrxn3* 1178 during stimulation for wild-type controls (black) and *inxh3d*, *mxh3d* mutants (blue). N = 15<br>1179 wild-type and 13 *nrxn3a; nrxn3b* mutant neuromasts at 5-6 dpf. An unpaired t-test was used<br>1180 C. ns P > 0.05.<br>118 1179 wild-type and 13 *mxn3a*; *mxn3b* mutant neuromasts at 5-6 dpf. An unpaired t-test was used in<br>1180 C. ns P > 0.05.<br>1181<br>1182

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- **1191 Fig S9. Nrxn3 is required for proper hair-cell synapse function in the lateral line.**<br> **1192** (A-F)  $\Delta$ F heatmaps show spatial patterns of presynaptic GCaMP6s increases in hair cells before<br> **1193** (A,D) and during 1193 (A,D) and during (B,E) a 500 ms fluid-jet stimulation in a wild-type (A,D) and *mxh3a*; *mxh3b*<br>1194 mutant (D,E) neuromast. ROIs indicate synaptically active hair cells and examples of regions<br>1195 used to measure t
- (A-F) ΔF heatmaps show spatial patterns of presynaptic GCaMP6s increases in hair cells before<br>1193 (A,D) and during (B,E) a 500 ms fluid-jet stimulation in a wild-type (A,B) and *nrxn3a; nrxn3b*<br>1194 mutant (D,E) neuromas 1194 mutant (D,E) neuromast. ROIs indicate synaptically derive han eens and examples of regions<br>1195 used to measure the average response per neuromast. Traces in C and F show  $\Delta F/F$  response<br>1196 from ROIs in A and D. Gr
- 1196 from ROIs in A and D. Gray area indicates timing of stimulus. (G-L)  $\Delta F$  heatmaps show spatial<br>52 1196 From ROIs in A and D. Gray area indicates timing of stimulus. (G-L)  $\Delta$ F heatmaps show spatial<br>52

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- |}<br>|}<br>| indiget stimulation in a wild-type (G,H) and *inxh3d*, *inxh3d* mutant (J,K) neuromast. ROIs<br>1199 indicate synaptically active terminals and examples of regions used to measure the averag<br>1200 terminal response per neuroma
- 1198 fluid-jet stimulation in a wild-type (G, H) and  $nrxn3a$ ;  $nrxn3b$  mutant (J, K) neuromast. ROIs<br>1199 indicate synaptically active terminals and examples of regions used to measure the average<br>1200 terminal response pe 1200 terminal response per neuromast. Traces in I and J show  $\Delta F/F$  responses from ROIs in G and<br>1201 Gray area indicates timing of stimulus. Wild-type examples in A-B and G-H correspond to the<br>1202 same example in Fig 6
- 1201 Gray area indicates timing of stimulus. Wild-type examples in A-B and G-H correspond to the<br>1202 same example in Fig 6 C,D and G,H.<br>1203<br>1204
- 1202 same example in Fig 6 C,D and G,H.<br>1203<br>1204  $1203$ <br>1204

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