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1 **Neuronal overexpression of Alzheimer's disease and Down's syndrome associated**  
2 ***DYRK1A/minibrain* gene alters motor decline, neurodegeneration and synaptic**  
3 **plasticity in *Drosophila***

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14

15

16 Keywords: Down's syndrome, Alzheimer's disease, *DYRK1A/minibrain*, *Drosophila*,  
17 neuromuscular junction, synaptic transmission, neurodegeneration, motor decline, short-term  
18 synaptic depression, spontaneous vesicular transmitter release

19

20 **Abstract**

21 Down syndrome (DS) is characterised by abnormal cognitive and motor development, and  
22 later in life by progressive Alzheimer's disease (AD)-like dementia, neuropathology, declining  
23 motor function and shorter life expectancy. It is caused by trisomy of chromosome 21 (Hsa21),  
24 but how individual Hsa21 genes contribute to various aspects of the disorder is incompletely  
25 understood. Previous work has demonstrated a role for triplication of the Hsa21 gene *DYRK1A*  
26 in cognitive and motor deficits, as well as in altered neurogenesis and neurofibrillary  
27 degeneration in the DS brain, but its contribution to other DS phenotypes is unclear. Here we  
28 demonstrate that overexpression of *minibrain (mnb)*, the *Drosophila* ortholog of *DYRK1A*, in  
29 the *Drosophila* nervous system accelerated age-dependent decline in motor performance and  
30 shortened lifespan. Overexpression of *mnb* in the eye was neurotoxic and overexpression in  
31 ellipsoid body neurons in the brain caused age-dependent neurodegeneration. At the larval  
32 neuromuscular junction, an established model for mammalian central glutamatergic synapses,  
33 neuronal *mnb* overexpression enhanced spontaneous vesicular transmitter release. It also  
34 slowed recovery from short-term depression of evoked transmitter release induced by high-  
35 frequency nerve stimulation and increased the number of boutons in one of the two  
36 glutamatergic motor neurons innervating the muscle. These results provide further insight into  
37 the roles of *DYRK1A* triplication in abnormal aging and synaptic dysfunction in DS.

39 **Highlights**

- 40 • Overexpression of *minibrain* (*DYRK1A*) causes Down's relevant phenotypes including:
- 41 • Age-dependent degeneration of brain neurons
- 42 • Accelerated age-dependent decline in motor performance and shorted lifespan
- 43 • Modified presynaptic structure and enhanced spontaneous transmitter release
- 44 • Slowed recovery from short-term depression of synaptic transmission

45

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49 (*minibrain-H*, CG42273) and Dr Frank Hirth (Kings College London) for *EB1-Gal4; UAS-*  
50 *mCD8-GFP* flies.

51

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54

55 **Introduction**

56 Down syndrome (DS, also known as Down's syndrome) or trisomy 21 is caused by the  
57 presence of three copies of chromosome 21 (Hsa21) instead of the usual two (Herault et al.,  
58 2017). It is characterised by cognitive impairment (Lott and Dierssen, 2010) and the delayed  
59 and incomplete acquisition of motor skills (Malak et al., 2015) as a result of abnormal  
60 development of the nervous system (Stagni et al., 2018). Individuals with DS almost invariably  
61 develop Alzheimer's disease (AD)-like symptoms (AD-DS). These include progressive  
62 dementia after 40 years of age, the onset of amyloid plaques, neurofibrillary tangles (NFTs)  
63 and neurodegeneration after 10 – 20 years (Wiseman et al., 2015; Zigman, 2013), faster age-  
64 dependent motor decline that is an early marker for the onset of cognitive decline and health  
65 deterioration (Anderson-Mooney et al., 2016; Buchman and Bennett, 2011), and a shorter

66 mean life expectancy by approximately 28 years (O'Leary et al., 2018). Currently there is no  
67 treatment for DS or AD; our understanding of the mechanisms of the disorder is incomplete  
68 and this hampers the development of effective therapies.

69

70 One of the Hsa21 genes, *DYRK1A* (dual specificity tyrosine-phosphorylation-regulated kinase  
71 1A), is a candidate causative gene for the structural and functional changes that occur in the  
72 DS brain, and for the associated cognitive and motor deficits (Herault et al., 2017; Stagni et  
73 al., 2018). *DYRK1A/Dyrk1a* mRNA and protein are expressed throughout the brain in humans  
74 and rodents, wherein *DYRK1A* controls aspects of neuronal development and function  
75 (Duchon and Herault, 2016; Kay et al., 2016; Stringer et al., 2017). *DYRK1A/Dyrk1a* mRNA  
76 and protein expression is increased in DS brain and in the brain of different mouse models of  
77 DS (Duchon and Herault, 2016; Garcia-Cerro et al., 2018; Kay et al., 2016; Stringer et al.,  
78 2017), whether the gene is triplicated as part of a genomic segment, as in Dp1Yey, Ts65Dn,  
79 Ts1Cje and Tc1 mice, or alone as in TgDyrk1a and TgDYRK1A mice (Herault et al., 2017).

80

81 DS-associated cognitive and motor deficits are replicated by overexpression of *Dyr1ka* in mice  
82 (Ahn et al., 2006; Altafaj et al., 2001; Altafaj et al., 2013; Arque et al., 2013; García-Cerro et  
83 al., 2014; Garcia-Cerro et al., 2018; Martínez de Lagrán et al., 2004; Ortiz-Abalia et al., 2008;  
84 Souchet et al., 2014; Watson-Scales et al., 2018). However, the contribution of *DYRK1A*  
85 overexpression to the shorter life expectancy, faster age-dependent decline in cognitive and  
86 motor function, and development of AD-like pathology in DS is unclear. It is predicted to play  
87 a role as it both phosphorylates tau and alters its splicing (Shi et al., 2008; Woods et al., 2001),  
88 promoting its self-aggregation (Liu et al., 2008) into NFTs. *Dyrk1A* is found physically  
89 associated with NFTs in the brain to a greater level in DS-AD than non-DS associated AD  
90 (Wegiel et al., 2008; Wegiel et al., 2011). In the Ts65Dn and Ts1Cje mouse models of DS,  
91 *Dyrk1a* overexpression in the brain intensifies with age (Ahmed et al., 2017; Creau et al., 2016;  
92 Stringer et al., 2017; Watson-Scales et al., 2018), and this is associated with AD-DS-like  
93 histopathological changes in the aged Ts65Dn brain (García-Cerro et al., 2017; Wiseman et

94 al., 2015). However, there is insufficient behavioural data from aged animals to directly assess  
95 the impact of *DYRK1A* overexpression in inducing DS-AD phenotypes.

96

97 Cognitive and motor dysfunction in individuals with DS and in mouse models of DS are  
98 associated with changes in synaptic plasticity and with changes in the number and structure  
99 of GABAergic and glutamatergic brain neurons and synapses (Battaglia et al., 2008;  
100 Contestabile et al., 2017; Duchon and Herault, 2016). Such modifications have been linked to  
101 *Dyrk1a* overexpression (Duchon and Herault, 2016; García-Cerro et al., 2017; Garcia-Cerro  
102 et al., 2018; Ruiz-Mejias et al., 2016), but the effects of *Dyrk1a* overexpression on the basic  
103 properties of synaptic function have rarely been explored. In one study, there was no change  
104 in the frequency of miniature excitatory synaptic currents (mEPSCs) or the probability of  
105 electrically-evoked glutamate release in the prefrontal cortex of TgDyrk1a mice (Thomazeau  
106 et al., 2014). Nevertheless, since *Dyrk1a* controls the activity of proteins that regulate  
107 endocytosis (Murakami et al., 2012) and *DYRK1A* overexpression slows endocytosis of  
108 transmitter vesicles in hippocampal presynaptic membranes from TgDYRK1A mice (Kim et  
109 al., 2010), modulation of transmitter release at other glutamatergic synapses is likely.

110

111 To investigate the contribution of *DYRK1A* overexpression in the nervous system to various  
112 aspects of DS, we overexpressed *minibrain (mnb)*, the *Drosophila* ortholog of *DYRK1A*  
113 (Duchon and Herault, 2016), in the *Drosophila* nervous system and implemented well-  
114 established assays in larvae and adult flies (Bykhovskaia and Vasin, 2017; Lenz et al., 2013;  
115 McGurk et al., 2015). The assays monitored motor impairment and its development with age,  
116 lifespan, age-related neurodegeneration, and synaptic dysfunction. Due to their short lifecycle,  
117 *Drosophila* are one of the pre-eminent models for aging and neurodegeneration (Jones and  
118 Grotewiel, 2011), both aspects of DS that are more difficult to investigate in mice. The  
119 *Drosophila* larval neuromuscular junction (NMJ) is a well-established model for mammalian  
120 central glutamatergic synapses and is easily accessible to electrophysiology (Bykhovskaia

121 and Vasin, 2017). *Mnb* is expressed presynaptically at larval NMJs and reducing its expression  
122 changes motor nerve terminal structure and impairs recycling of transmitter vesicles, while  
123 overexpression of one isoform, *mnb-F*, has no effect on basal transmission but ameliorates  
124 the effects of reduced *mnb* expression (Chen et al., 2014). Five *mnb* isoforms, *E-I*, have been  
125 identified, all of which share a highly conserved kinase domain (Gramates et al., 2017; Hong  
126 et al., 2012). Regions of DYRK1A outside the kinase domain also appear to play important  
127 roles, but which areas exactly and how they impact function is thus far incompletely  
128 understood (Jin et al., 2015; Kelly and Rahmani, 2005; von Groote-Bidlingmaier et al., 2003).  
129 We therefore utilised *mnb-H*, the isoform with the longest coding region (Gramates et al., 2017;  
130 Zerbino et al., 2018). Here we report the effects of neuronal overexpression of *mnb-H* on motor  
131 function, the rate of motor decline with age, lifespan, age-related neurodegeneration,  
132 presynaptic structure, spontaneous transmitter release and recovery from frequency-  
133 dependent depression of electrically-evoked transmitter release.

134

135 **Results**

136

137 **Neuronal overexpression of *mnb* produced motor deficits in larvae, accelerated age-**  
138 **dependent motor decline in adult flies and shortened adult lifespan**

139 The effect of *mnb* overexpression in the nervous system on motor function (specifically the  
140 *mnb-H* splice variant) was tested using two assays of fly larval locomotion. *Elav>mnb* larvae,  
141 overexpressing *mnb* throughout the nervous system under the control of the *Elav-Gal4* driver  
142 (Robinow and White, 1991), did not move as far as control larvae (*Elav/+*) in a free movement  
143 assay (Fig. 1A), which measures the ability of larvae to perform rhythmic muscle contractions  
144 necessary for gross locomotion (Kohsaka et al., 2017). They also took longer to complete a  
145 self-righting assay (Fig. 1B), which is a more complex motor task requiring larvae to enact a  
146 co-ordinated sequence of movements to right themselves after being rolled onto their backs  
147 (Picao-Osorio et al., 2015). To assess the impact of neuronal *mnb* overexpression on age-  
148 related decline in locomotor function, the performance of the same cohorts of adult flies was  
149 assessed in a negative geotaxis assay at different ages (Jones and Grotewiel, 2011). This  
150 showed acceleration in *Elav>mnb* flies of the usual age-related decline in performance  
151 (*Elav/+*). There was also evident shortening of the lifespan of *Elav>mnb* flies, so that the  
152 median lifespan was reduced by almost 50% (*Elav/+*, 73 days; *Elav>mnb*, 38 days; Fig. 1D).  
153 These results indicate that neuronal overexpression of *mnb* alone produced a motor deficit  
154 and abnormal aging characterised by accelerated age-related locomotor impairment and a  
155 shorter lifespan.

156

157 **Overexpression of *mnb* caused neurodegeneration in adult flies**

158 As *DYRK1A* triplication has been linked to degeneration of brain neurons in AD-DS and in  
159 Ts65Dn mice (García-Cerro et al., 2017; Wegiel et al., 2008), we tested the possibility that  
160 neuronal overexpression of *mnb* is sufficient to cause neurotoxicity and age-related  
161 neurodegeneration using two established assays of neurodegeneration in adult flies (Lenz et  
162 al., 2013; McGurk et al., 2015). In the first, *mnb* was overexpressed in the eye through



163 development and adulthood using the *Glass multimer reporter* driver (*GMR-Gal4*) (Ellis et al.,  
164 1993). The *GMR>mnb* flies, but not control flies (*GMR/+*), had a reduced eye surface area  
165 and a visible “rough eye” phenotype (Fig. 2A), both of which indicate neural death and the  
166 resultant breakdown of the regularly spaced array of ommatidia making up the retina. In a  
167 second assay, the *EB1* driver (*EB1-Gal4*) was used to overexpress *mnb* in the ellipsoid body  
168 (EB), a subpopulation of neurons within the central complex of the brain implicated in  
169 locomotor control (Fig. 2B) (Diaper et al., 2013). The EB cells also expressed membrane-  
170 bound GFP which enabled their visualisation. At 1 day old, there was no difference in the  
171 number of GFP-positive EB neurons between control (*EB1/+*) and *EB1>mnb* flies, whereas at  
172 day 40 the number of EB neurons was significantly reduced in *EB1>mnb* flies but not in control  
173 flies (Fig. 2C). Therefore, neurotoxicity caused by *mnb* overexpression promoted age-related  
174 neurodegeneration in a central neuron population.

175

#### 176 **Overexpression of *mnb* in motor neurons increased the number of synaptic boutons at** 177 **the larval NMJ**

178 To investigate the effect of *mnb* overexpression on presynaptic morphology, *mnb* was  
179 overexpressed in glutamatergic motor neurons of *Drosophila* larvae using *OK371-Gal4* (Mahr  
180 and Aberle, 2006). The neuronal membranes were labelled with horseradish peroxidase  
181 (HRP). The muscle is innervated by two motor neurons with functionally and structurally  
182 distinct presynaptic boutons; 1s (small) boutons have higher excitation thresholds, higher  
183 basal probability of release and induce larger post-synaptic potentials, while short-term and  
184 homeostatic plasticity are largely mediated by 1b (big) boutons (Atwood et al., 1997; Newman  
185 et al., 2017). These were differentiated by the stronger postsynaptic expression of Discs large  
186 (Dlg) opposite 1b boutons (Lahey et al., 1994) Analysis of the NMJ in the second abdominal  
187 larval segment, A2, showed that *mnb* overexpression affected the morphology of the nerve  
188 terminals of only one of the motor neurons; it increased the number of 1b boutons but did not  
189 alter the number of 1s boutons (Fig. 3A-B). The effect was not secondary to changes in muscle

190 size, as this did not differ (surface area of muscle 6: *OK371/+*,  $44752 \pm 1407 \mu\text{m}^2$ ,  $n = 15$ ;  
191 *OK371>mnb*,  $44681 \pm 3684 \mu\text{m}^2$ ,  $n = 15$ ,  $P = 0.9857$ ).

192

### 193 **Overexpression of *mnb* in motor neurons altered basal synaptic transmission at the** 194 **larval NMJ**

195 As neuronal overexpression of *mnb* increased the number of 1b boutons at the larval NMJ,  
196 and because previous studies have implicated Dyrk1a/*mnb* in the control of the recycling of  
197 neurotransmitter vesicles (Chen et al., 2014; Kim et al., 2010; Murakami et al., 2012), we  
198 investigated if spontaneous glutamate release was altered by recording spontaneously  
199 occurring miniature excitatory junction potentials (mEJPs) with intracellular microelectrodes.  
200 Since the muscle 6/7 NMJ is innervated by 1b and 1s boutons, mEJPs usually result from the  
201 release of neurotransmitter vesicles from both types of boutons (Newman et al., 2017). Our  
202 recordings revealed that mEJPs occurred more frequently but were smaller in *OK371>mnb*  
203 larvae (Fig. 4A-B). The decrease in amplitude was not secondary to a change in the electrical  
204 properties of the muscle as there was no difference in input resistance (*OK371/+*,  $3.37 \pm 0.69$   
205  $\text{M}\Omega$ ,  $n = 8$ ; *OK371>mnb* =  $3.99 \pm 0.99 \text{M}\Omega$ ,  $n = 8$ ,  $P = 0.613$ ) or resting potential (*OK371/+*, -  
206  $69.6 \pm 1.48 \text{mV}$ ,  $n = 8$ ; *OK371>mnb*,  $-67.6 \pm 1.55 \text{mV}$ ,  $n = 8$ ,  $P = 0.365$ ). Although we did not  
207 directly investigate the source of the more frequent smaller mEJPs, the notion that they are  
208 due to the observed selective increase in the number of 1b boutons is suggested by the fact  
209 that 1b-dependent mEJPs are smaller than 1s-dependent mEJPs (Newman et al., 2017). In  
210 parallel with the changes in spontaneous synaptic events, there was a small (~11 %) decrease  
211 in the mean amplitude of electrically-evoked excitatory junction potentials (EJPs) caused by  
212 single stimuli applied to the nerve at a low frequency (0.1 Hz) (Fig. 4C). There was no  
213 difference between EJPs in mean rise time (*OK371/+*,  $2.67 \pm 0.178 \text{ms}$ ,  $n = 8$ ; *OK371>mnb*,  
214  $3.23 \pm 0.33 \text{ms}$ ,  $n = 8$ ,  $P = 0.728$ ) or mean time constant of decay (*OK371/+*,  $44.9 \pm 3.1 \text{ms}$ ,  $n$   
215 = 8; *OK371>mnb*,  $36.6 \pm 4.6 \text{ms}$ ,  $n = 8$ ,  $P = 0.154$ ). The relatively small fall in EJP amplitude  
216 is likely to reflect the smaller size of the 1b-dependent component of the EJP relative to that  
217 of the 1s-dependent component (Newman et al., 2017).

218

219 **Overexpression of *mnb* in motor neurons slowed recovery from frequency-dependent**  
220 **depression at the larval NMJ**

221 To investigate the effects of neuronal *mnb* overexpression on recycling of synaptic vesicles  
222 during electrically-evoked transmitter release, EJPs were evoked with pairs of electrical stimuli  
223 separated by intervals of varying duration (10 ms – 10 s) or with repeated trains of 10 stimuli  
224 applied at a high frequency (10 Hz, a frequency 100 times higher than that at which the single  
225 EJPs were evoked) (Kauwe and Isacoff, 2013). At control NMJs, paired pulses separated by  
226 intervals shorter than 200 ms caused depression of the amplitude of the second EJP relative  
227 to that of the first and the depression was stronger for shorter inter-stimulus intervals (Fig. 5A).  
228 The dependence of paired-pulse depression on interval duration was unaltered in  
229 *OK371>mnb* larvae (Fig. 5A), indicating that *mnb* overexpression did not alter release from a  
230 readily releasable pool of vesicles (Regehr, 2012). When transmitter release was evoked at  
231 control NMJs with a train of 10 stimuli at 10 Hz, there was rapid depression of the EJP  
232 amplitude by ~20% within the first 3 events (Fig. 5B). In the one-minute interval before the  
233 next train, the EJP amplitude recovered fully so that the amplitude of the first EJP in the second  
234 train was the same as in the first train (Fig. 5B). This ability to recover did not wane during the  
235 recording; the amplitude of the first EJP in each train did not differ between 8 trains (Fig. 5B).  
236 These effects are consistent with previous studies (Kauwe and Isacoff, 2013) and confirm  
237 rapid depletion and replenishment of the readily releasable pool of vesicles (Regehr, 2012).  
238 However, the same pattern of nerve stimulation produced different effects at *OK371>mnb*  
239 NMJs (Fig. 5B). The percentage decrease in EJP amplitude during each train was the same  
240 as at control NMJs, but the depression was not fully reversed during the intervals between  
241 trains, so that the first EJP in each train was smaller than the first EJP in the preceding train.  
242 The depression in amplitude accumulated over the 8 trains, resulting in an overall fall of 10%.  
243 To confirm that the changes in EJP amplitude were due to presynaptic changes in transmitter  
244 release and were not postsynaptically mediated by a decrease in the unitary depolarisations  
245 comprising each EJP, we measured the amplitudes of 200 mEJPs immediately before and

246 200 mEJPs immediately after the series of trains at each NMJ. At both control and  
247 *OK371>mnb* NMJs, the cumulative distribution of mEJP amplitudes before and after a series  
248 of trains was similar; although they were not identical, the observed slight increase in the  
249 number of larger mEJPs cannot explain the decline in EJP amplitude (Fig. 5C). These results  
250 show that *mnb* overexpression slowed replenishment of the readily releasable pool of vesicles,  
251 an effect consistent with the reported slowing of endocytosis of transmitter vesicles by  
252 *DYRK1A* overexpression (Kim et al., 2010).

253

254

255 **Discussion**

256 This study demonstrated that neuronal overexpression of *mnb*, the *Drosophila* ortholog of  
257 DYRK1A, is sufficient to induce motor impairment, accelerate age-related decline in motor  
258 performance, shorten lifespan and cause age-dependent neurodegeneration. This study also  
259 found that neuronal *mnb* overexpression at a glutamatergic synapse alters presynaptic  
260 structure, modifies basal synaptic transmission and delays recovery from short-term synaptic  
261 depression. This provides useful information about the gene's function and the pathological  
262 effects of increased expression in a model system. However, it is important to note that this  
263 does not represent a high-fidelity recapitulation of DS or the complexity of the human *DYRK1A*  
264 locus, because *Gal4*-mediated overexpression of a specific isoform does not accurately  
265 replicate the expression level or pattern caused by triplication of a whole genomic region of  
266 human chromosome 21.

267

268 People with DS have impaired motor skills which are evident from childhood and are caused  
269 by abnormal development of the nervous system (Malak et al., 2015; Stagni et al., 2018).  
270 Later, in middle age, they undergo faster age-dependent motor decline, which is an early  
271 marker of future dementia, comorbidities and mortality, and is likely caused by  
272 histopathological changes in the brain (Anderson-Mooney et al., 2016; Buchman and Bennett,  
273 2011). The life expectancy of people with DS is about 28 years shorter than the general  
274 population (O'Leary et al., 2018). By taking advantage of the relatively short life cycle of  
275 *Drosophila* and transgenic overexpression of *mnb* in neurons, we have demonstrated a  
276 potential role for neuronal DYRK1A overexpression in the accelerated age-dependent decline  
277 of motor function and shortening of life expectancy in DS. The genetic basis of these aspects  
278 of DS are more difficult and costly to explore in mouse models of DS, due to the time required  
279 to study aged mice. Our finding that *mnb* overexpression causes age-related  
280 neurodegeneration confirms previous studies inferring a link between *DYRK1A*  
281 overexpression and degeneration and loss of neurons (Duchon and Herault, 2016; García-  
282 Cerro et al., 2017; Watson-Scales et al., 2018; Wegiel et al., 2008), which is associated with

283 faster age-related decline in motor and cognitive function in DS and AD-DS. Our results also  
284 reinforce the conclusion from earlier studies with adult mice overexpressing *DYRK1A* or  
285 *Dyrk1a*, alone or as part of a chromosomal segment, that triplication of *DYRK1A* is likely to  
286 contribute to motor deficits in DS (Altafaj et al., 2001; Arque et al., 2013; Garcia-Cerro et al.,  
287 2018; Martínez de Lagrán et al., 2004; Ortiz-Abalia et al., 2008; Souchet et al., 2014; Watson-  
288 Scales et al., 2018).

289

290 In addition to the smaller brain size and fewer brain neurons in DS and mouse models of DS,  
291 there are alterations in the structure of brain synapses that are predicted to modify synaptic  
292 function (Contestabile et al., 2017; Dierssen, 2012; Stagni et al., 2018). A previous study  
293 showed that *DYRK1A* overexpression in mice changes postsynaptic morphology in the cortex  
294 and in cultured cortical neurons by reducing the number and length of dendrites and by  
295 reducing the number of dendritic spines but elongating their shape (Martinez de Lagran et al.,  
296 2012). It also decreased the number of synapses formed. Our study shows that *mnb*  
297 overexpression changes presynaptic structure and that this happens in a neuron-specific  
298 manner; *mnb* overexpression in the two glutamatergic motoneurons innervating the larval NMJ  
299 increased the number of 1b boutons without changing the number of 1s boutons. These data  
300 are consistent with a previous study which demonstrated that reduced levels of *mnb* caused  
301 a decrease, and increased levels of the *mnb-F* transcript an increase, in the number of boutons  
302 at the NMJ (Chen et al., 2014), but did not differentiate between 1b and 1s boutons.

303

304 The cognitive and motor deficits in DS arise from aberrant information processing in the brain  
305 that is likely due, in part, to changes in synaptic transmission or synaptic plasticity. Individuals  
306 with DS have impaired synaptic plasticity in the motor cortex (Battaglia et al., 2008). Our  
307 finding that *mnb* overexpression slows replenishment of the readily releasable pool of vesicles,  
308 and also modifies basal synaptic transmission, confirms a previous suggestion that *DYRK1A*  
309 overexpression contributes to synaptic dysfunction and cognitive deficits associated with DS,  
310 made on the basis of the observed slowing of endocytosis of transmitter vesicles in cultured

311 mouse hippocampal neurons overexpressing human *DYRK1A* (Kim et al., 2010). The effects  
312 of *DYRK1A* on synaptic function may be splice variant specific as we found that  
313 overexpression of the *mnb-H* transcript caused a decrease in mEJP and EJP amplitude,  
314 whereas overexpression of *mnb-F* in a previous study did not alter mEJP or EJP amplitude  
315 at the larval NMJ (Chen et al., 2014).

316

317 The effects of neuronal *mnb* overexpression on larval NMJ function replicate some, but not  
318 all, the documented changes in glutamatergic synaptic transmission in the brain of mouse  
319 models of DS. These include a decrease in the amplitude of spontaneous excitatory  
320 postsynaptic currents (sEPSCs) in neocortical neurons of Ts65Dn mice (Cramer et al., 2015),  
321 compromised glutamate release in response to stimuli trains at hippocampal CA1 synapses  
322 of Ts1Cje mice (Siarey et al., 2005) and a decrease in EPSC amplitude in hippocampal CA3  
323 neurons of Ts65Dn mice (Hanson et al., 2007). However, in contrast to the increase in mEJP  
324 frequency caused by *mnb* overexpression at the larval NMJ, electrophysiological studies have  
325 found a decrease in the frequency of mEPSCs in hippocampal CA3 neurons of Ts65Dn mice,  
326 sEPSCs in neocortical neurons of Ts65Dn mice and sEPSCs in neurons derived from trisomy  
327 21 induced pluripotent stem cells, or no change in mEPSC frequency in the prefrontal cortex  
328 of TgDyrk1a mice or mossy fibre-CA3 synapses in Tc1 mice (Contestabile et al., 2017).

329

330 Our study further elucidates the effect of *DYRK1A* overexpression in a model system, giving  
331 insight into the contribution of increased dosage to various DS phenotypes. It supports the  
332 future development of pharmacological inhibitors of *DYRK1A* as treatments for multiple  
333 aspects of DS and DS-AD (Duchon and Herault, 2016; Stringer et al., 2017). Further work is  
334 necessary to fully understand interactions between *DYRK1A* and other triplicated Hsa21  
335 genes in DS, in specific cell types and during defined periods of development and ageing.

336

337

338

339

## 340 **Materials and Methods**

### 341 ***Animals***

342 Flies were raised with a 12 h:12 h light dark cycle with lights on at ZT 0 (Zeitgeber time) on  
343 standard *Drosophila* medium (0.7% agar, 1.0% soya flour, 8.0% polenta/maize, 1.8% yeast,  
344 8.0% malt extract, 4.0% molasses, 0.8% propionic acid, 2.3% nipagen) at 25°C. Flies were  
345 transferred to vials containing fresh medium twice weekly. The *OK371-Gal4* (Bloomington  
346 stock center numbers: 26160), *Elav-Gal4* (87060), *GMR-Gal4* (9146) flies were obtained from  
347 the Bloomington *Drosophila* Stock Centre. *Canton Special white-* (*CSw-*) flies were a gift from  
348 Dr Scott Waddell (University of Oxford), *UAS-mnb* flies (*minibrain-H*, CG42273) were kindly  
349 provided by Dr Kweon Yu (Korea Research Institute of Bioscience and Biotechnology), *EB1-*  
350 *Gal4*; *UAS-mCD8-GFP* flies were donated by Dr Frank Hirth (Kings College London).

351

### 352 ***Behaviour***

353 *mnb* expression was driven throughout the nervous system using *Elav-Gal4* (Robinow and  
354 White, 1991) for experiments investigating behaviour of wandering third instar larvae, the  
355 number of boutons at the larval NMJ, and synaptic transmission at the larval NMJ. All  
356 behavioural experiments took place at 25°C. Larval locomotor experiments were conducted  
357 on a 9.5 cm petri dish containing 1.6% agarose. A single third instar wandering larva was  
358 selected, washed in a drop of distilled H<sub>2</sub>O, transferred to the agarose and allowed 30 s to  
359 acclimatise. To analyse free movement, the dish was placed over a 0.5 cm grid and the  
360 number of lines the larva crawled across in one minute was counted by eye. The self-righting  
361 assay was conducted as described elsewhere (Lowe et al., 2018; Picao-Osorio et al., 2015);  
362 the larva was gently rolled onto its back on the agarose using a fine moistened paintbrush,  
363 held for one second and released, and the time for it to right itself was recorded.

364

365 The negative geotaxis assay was performed as described previously (Ali et al., 2011). A cohort  
366 of 10 flies was transferred without anaesthesia to an empty 9.5 cm tube with a line drawn 2



367 cm from the top. After 1 minute acclimatisation, the vial was sharply tapped 3 times to knock  
368 the flies to bottom. The number of flies to climb past the line within 10 s was recorded. 15  
369 cohorts of 10 flies were tested for each genotype. Age-dependent changes in climbing were  
370 assessed by repeating the negative geotaxis assay at 10, 20 and 30 days post-eclosure  
371 (Gargano et al., 2005). For the survival assay, 10 cohorts of 10 once-mated females were  
372 transferred to a vial of fresh food twice weekly and the number of surviving flies recorded at  
373 each transfer.

374

### 375 ***Antibody staining and visualisation at the NMJ***

376 Wandering third instar larvae were dissected in ice-cold, Ca<sup>2+</sup>-free HL3.1-like solution (in mM:  
377 70 NaCl, 5 KCl, 10 NaHCO<sub>3</sub>, 115 sucrose, 5 trehalose, 5 HEPES, 10 MgCl<sub>2</sub>) to produce a  
378 larval “fillet” (Brent et al., 2009). The fillet was fixed for 30 minutes in 4% paraformaldehyde  
379 (Sigma Labs), washed three times in 1% Triton-X (Sigma Labs) and blocked for one hour in  
380 5% normal goat serum (Fitzgerald Industries) and 1% Triton-X at room temperature. It was  
381 incubated overnight in 1/500 mouse FITC-conjugated anti-horseradish peroxidase (HRP-  
382 FITC) (Jackson Immunoresearch Laboratories, 115-035-003) and 1/500 rabbit anti-Discs  
383 large (Dlg) (Biocompare, ABIN1387516) primary antibody, then for two hours in 1/500  
384 AlexaFluor 633-conjugated goat anti-mouse secondary antibody (ThermoFisher Scientific, A-  
385 21052) at room temperature. Each fillet was washed and mounted on a coverslip in  
386 Vectashield (Vector Laboratories). Z-series of NMJs were imaged on a Leica SP5-II confocal  
387 laser-scanning microscope using an oil immersion 40 × objective. The number of boutons at  
388 the NMJ of muscle 6/7 in segment A2 was counted manually. ImageJ ([rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)) was  
389 used to manually outline muscle 6 and hence calculate their area.

390

### 391 ***Neurotoxicity***

392 Overexpression of *mnb* was driven in the eye using the *Glass multimer reporter (GMR-Gal4)*.  
393 Images of the whole head of 1-2 day old flies were taken via a Zeiss AxioCam MRm camera  
394 attached to a stereomicroscope (Zeiss SteREO Discovery.V8, up to 8× magnification), and

395 the surface area of the eye was calculated by manually outlining the eye in ImageJ  
396 (rsb.info.nih.gov/ij/). Overexpression of *mnb* in GFP-tagged ellipsoid body (EB) ring neurons  
397 was achieved by crossing *EB1-Gal4; UAS-mCD8-GFP* flies with *UAS-mnb* flies. Following  
398 published methods (Williamson and Hiesinger, 2010), adult brains were dissected, fixed for  
399 30 minutes in 4% paraformaldehyde and mounted on a coverslip in Vectashield (Vector  
400 Laboratories). Slides were imaged on a Leica SP5-II confocal laser scanning microscope  
401 using an oil immersion 40 × objective. A Z-stack of 25 images at 1 μm increments was  
402 captured and combined into a 3-D projection using ImageJ (rsb.info.nih.gov/ij/); analysis was  
403 performed by scrolling through all 25 images and counting the number of cells in one brain  
404 hemisphere.

405

#### 406 ***Electrophysiology***

407 Wandering third instar larvae were dissected as for antibody staining. The motor nerves were  
408 severed just below the ventral ganglion and the brain was removed. CaCl<sub>2</sub> (1 mM) was added  
409 to the bath solution for intracellular recording from muscle 6 of abdominal segments 2-4. Most  
410 recordings were made in the presence of thapsigargin (2 μM), which minimises contraction  
411 and hence facilitates intracellular recording, without affecting amplitudes of mEJPs or EJPs  
412 (Guerrero et al., 2005; Newman et al., 2017). Sharp microelectrodes (thick-walled borosilicate  
413 capillaries, pulled on a Sutter Flaming/Brown P-97 micropipette puller) were filled with 3M KCl  
414 and had resistances of 20-30 MΩ. For recording of stimulus evoked excitatory junction  
415 potentials (EJPs), severed nerves were drawn into a thin-walled glass-stimulating pipette and  
416 stimulated with square-wave voltage pulses (0.1 ms, 10 V, A-M Systems Model 2100 Isolated  
417 Pulse Simulator), 10 times at 0.1 Hz. EJPs and spontaneously-occurring miniature EJPs  
418 (mEJPs) were recorded at a controlled room temperature of 22-25°C with a Geneclamp 500  
419 amplifier (Axon Instruments) and were further amplified with a LHBF-48x amplifier (NPI  
420 Electronic). The membrane potential was allowed to stabilise for one minute, the initial value  
421 was recorded, and then set to -70 mV by injecting current with the Geneclamp 500 amplifier.

422 The muscle input resistance was measured by injecting current using the Axon Geneclamp  
423 500, to bring the membrane potential to -100, -80, -60 and -40 mV, and subtracting the  
424 electrode resistance from the slope of the resulting voltage/current graph. Voltage signals  
425 were low-pass filtered at 1.67 kHz (10 kHz 4 pole Bessel on Geneclamp 500, 1.7 kHz 8-pole  
426 Bessel on LHBF-48x) and digitised at 25 kHz by a CED-1401 plus A/D interface (Cambridge  
427 Electronic Design, UK) using Spike2 software (v. 5.13) (CED, Cambridge, UK). Recordings  
428 were discarded if the initial resting membrane potential was more positive than -60 mV or  
429 varied by more than 10% throughout the recording. Synaptic potentials were analysed off line  
430 using Strathclyde Electrophysiology Software WinEDR (v3.5.2) and GraphPad Prism (v.6). All  
431 synaptic events were verified manually.

432

433 Amplitudes and intervals of mEJPs were compared by creating a cumulative distribution for  
434 each genotype of 1600 measurements across 8 animals, with each animal contributing 200  
435 values. To analyse the mEJP waveform, a mean mEJP was constructed for each recording  
436 from events showing a single clear peak and a smooth decay, so as to prevent distortion of  
437 the waveform by closely occurring mEJPs. A single exponential was fitted to the decay of the  
438 mean mEJP and the 10-90% rise-time was measured. Time zero for the exponential fit was  
439 set to the time at the peak of the mEJP. EJPs were analysed by forming a mean of 10 events,  
440 measuring the 10-90% rise-time of the mean event, and fitting the decay with the sum of three  
441 exponentials (time zero was set at the time of the peak). A mean weighted time constant of  
442 decay was calculated as  $A_1 \cdot \tau_1 + A_2 \cdot \tau_2 + A_3 \cdot \tau_3$ , where  $A_1$ ,  $A_2$  and  $A_3$  are the fractional amplitudes  
443 of the three components, and  $\tau_1$ ,  $\tau_2$  and  $\tau_3$  are their time constants.

444

445 For paired pulse analysis, two EJPs were evoked, separated with intervals varying in duration  
446 between 0.01 and 10 s. The amplitude of the second event was calculated as a fraction of the  
447 amplitude of the first. Pairs of stimuli were separated by 30 s. For high-frequency stimulation,  
448 trains of 10 events evoked at 10 Hz were repeated 8 times at 1 min intervals. The amplitude

449 of each event was expressed as a fraction of a baseline value, defined as the mean amplitude  
450 of 10 single EJPs evoked at 0.1 Hz. To compare mEJP amplitude before and after the trains,  
451 200 mEJP amplitudes were measured immediately before the first train, and another 200  
452 immediately after the eighth train. The measured values were pooled from 8 NMJs for each  
453 genotype and cumulative amplitude distributions compared.

454

#### 455 ***Statistical analysis***

456 Statistical analysis was conducted in GraphPad Prism (v. 6, La Jolla, CA). Data were tested  
457 for normality using the Kolmogorov-Smirnov test; where appropriate means were compared  
458 using Student's unpaired *t*-test, or medians were compared using a Mann-Whitney *U* test.  
459 EJPs evoked by pairs or trains of stimuli were compared using repeated measures 2-way  
460 ANOVA. Cumulative distributions were compared with a Kolmogorov-Smirnov test. Survival  
461 curves were compared with a Mantel-Cox test. Data are given as median or mean  $\pm$  SEM. *n*  
462 is given per genotype. An  $\alpha$  level of  $P < 0.05$  was considered significant.

463

464

465

466

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669

## 670 **Figure Legends**

671

672 **Figure 1. Motor deficits in larvae, accelerated age-dependent motor decline in adult**  
673 **flies and shortened adult lifespan due to neuronal overexpression of *mnb***

674 (A) *Elav>mnb* larvae crossed fewer lines of a 0.5 cm grid in 60 s than control larvae (*Elav/+*,  
675  $14.7 \pm 0.44$ ,  $n = 15$ ; *Elav>mnb*,  $12.1 \pm 0.86$ ,  $n = 15$ ; mean  $\pm$  SEM,  $*P = 0.014$ , Student's *t*-test),  
676 and with greater variance ( $F(14,14) = 3.768$ ,  $P = 0.0184$ , *F*-test). (B) *Elav>mnb* larvae took



677 longer than controls to perform a self-righting task (*Elav/+*,  $5.5 \pm 1.16$  s,  $n = 15$ ; *Elav>mnb*,  $12$   
678  $\pm 2.56$  s;  $n = 15$ ; mean  $\pm$  SEM,  $*P = 0.029$ , Student's *t*-test) and with greater variance ( $F$   
679  $(14,14) = 4.802$ ,  $P = 0.0059$ ,  $F$ -test). Each point in the plots (A, B) is from a different animal;  
680 horizontal lines indicate mean values. (C) The age-dependent decline in climbing ability in a  
681 negative geotaxis assay was steeper and showed greater variance for *Elav>mnb* adult flies  
682 than for controls ( $F(3,84) = 13.8$ ;  $***P < 0.0001$ , repeated measures two-way ANOVA,  $n = 15$   
683 groups of flies); at 1 day old there was no difference in the percentage of flies that climbed  
684 successfully (*Elav/+*,  $90.59 \pm 1.82$  %,  $n = 15$ ; *Elav>mnb*  $\pm 85.94 \pm 1.46$  %,  $n = 15$ ; mean  $\pm$   
685 SEM,  $P = 0.8262$ , repeated measures two-way ANOVA and Sidak's multiple comparison).  
686 Values plotted are mean  $\pm$  SEM,  $n = 15$  groups of 10 flies for each genotype. (D) *Elav>mnb*  
687 flies had a shorter lifespan relative to controls ( $n = 100$  animals per genotype at day 0,  $***P <$   
688  $0.0001$ , log-rank (Mantel-Cox) test).

689

690 **Figure 2. Neurotoxicity and age-related neurodegeneration caused by *mnb***  
691 **overexpression**

692 (A) (Left) Representative images of the eyes of control adult flies (*GMR/+*) and flies with *mnb*  
693 overexpression driven in the eye by *GMR-Gal4* (*GMR>mnb*). (Right) The surface area of the  
694 eyes in *GMR>mnb* flies was reduced (*GMR/+*,  $0.14 \pm 0.005$  mm<sup>2</sup>,  $n = 10$ ; *GMR>mnb*,  $0.09 \pm$   
695  $0.005$  mm<sup>2</sup>,  $n = 10$ ; mean  $\pm$  SEM,  $***P < 0.0001$ , Student's *t*-test). Each point in the plot is from  
696 a different animal, horizontal lines indicate means. (B) (Left) Representative images of clusters  
697 of GFP-expressing ellipsoid body neurons in one brain hemisphere, with and without *mnb*  
698 overexpression driven by *EB1-Gal4*, in 1 d and 40 d old flies. Calibration bar is 10  $\mu$ m. (Right)  
699 The number of cells did not differ at 1 d (*EB1>mCD8-GFP*,  $32.59 \pm 0.48$ ,  $n = 15$  (black);  
700 *EB1>mCD8-GFP, mnb*;  $31.47 \pm 0.47$ ,  $n = 15$  (grey); mean  $\pm$  SEM,  $P = 0.757$ , repeated  
701 measures two-way ANOVA and Sidak's multiple comparison) but decreased between 1 and  
702 40 d in *EB1>mCD8-GFP, mnb* flies; ( $F(1,28) = 10.56$ ;  $n = 15$ ;  $P = 0.003$ , repeated measures  
703 two-way ANOVA). Values plotted are mean  $\pm$  SEM from 15 flies for each genotype.

704

705 **Figure 3. Overexpression of *mnb* in motor neurons increased the number of 1b boutons**

706 (A) Representative images of motor nerve endings at the NMJ of muscle 6/7 in the A2 segment  
707 of *OK371/+* (top) and *OK371>mnb* (bottom) larvae. The neuronal membrane is labelled with  
708 HRP (green); type 1b boutons but not type 1s boutons (arrowheads) are preferentially labelled  
709 with Dlg (magenta). Scale bar is 25  $\mu$ m. (B) *OK371>mnb* NMJs had more 1b boutons  
710 (*OK371/+*,  $24.93 \pm 1.11$ ,  $n = 15$ ; *OK371>mnb*,  $32.80 \pm 1.52$ ,  $n = 15$ , mean  $\pm$  SEM, \*\*\* $P =$   
711 0.0003, Student's *t*-test) but there was no difference in the number of 1s boutons (*OK371/+*,  
712  $32.6 \pm 2.62$ ,  $n = 15$ ; *OK371>mnb*,  $38.6 \pm 3.34$ ,  $n = 15$ , mean  $\pm$  SEM,  $P = 0.169$ , Student's *t*-  
713 test). Each value plotted is from a different animal, horizontal lines indicate means.

714

715 **Figure 4. Overexpression of *mnb* in motor neurons altered basal synaptic transmission.**

716 (A) Representative voltage recordings (3 s traces) from NMJs of *OK371/+* (black) and  
717 *OK371>mnb* (grey) larvae showing spontaneous mEJPs recorded at a membrane potential of  
718 -70 mV. (B) Cumulative frequency distributions of mEJP amplitude (left) and inter-mEJP  
719 interval (right) (1600 events, 200 from each of 8 NMJ recordings). *OK371>mnb* mEJPs were  
720 smaller (\*\* $P < 0.0001$ ) and more frequent (\*\* $P < 0.0001$ ), Kolmogorov-Smirnov test. (C)  
721 (Left) Representative image of a single electrically-evoked EJP at an *OK371/+* NMJ and an  
722 *OK371>mnb* NMJ at a membrane potential of -70 mV. (Right) The median EJP amplitude  
723 (horizontal line) was reduced in *OK371>mnb* NMJs (*OK371/+*, 51.26 mV,  $n = 8$ ; *OK371>mnb*,  
724 45.55 mV,  $n = 8$ ; mean  $\pm$  SEM, \* $P = 0.0281$ ). Each point plotted is from a different animal.

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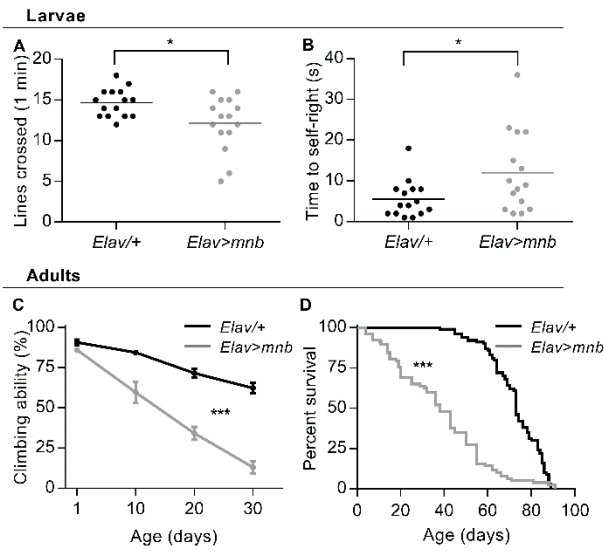
726 **Figure 5. *mnb* overexpression in motor neurons slowed recovery from frequency-**  
727 **induced depression**

728 (A) (Left) Representative pairs of stimuli evoked EJPs at *OK371/+* (black) and *OK371>mnb*  
729 (grey) NMJs. Dashed lines compare the first EJP (EJP<sub>1</sub>) and dotted lines compare the second  
730 EJP (EJP<sub>2</sub>). (Right) Plots of paired-pulse ratio (EJP<sub>2</sub>/EJP<sub>1</sub>, mean  $\pm$  SEM,  $n = 8$  for each  
731 genotype) against inter-pulse interval reveal no difference in synaptic depression ( $F(9, 126)$   
732 = 0.1343;  $n = 8$ ;  $P = 0.9987$ , repeated measures two-way ANOVA). (B) High-frequency

733 stimulation protocol. 10 EJPs were evoked at 0.1 Hz to establish a mean baseline amplitude  
734 followed by 8 trains of 10 EJPs at 10 Hz, at one minute intervals. (*Left*) Representative traces  
735 of train 1 (*upper*) and train 8 (*lower*) recorded from *OK371/+* and *OK371>mnb* NMJs. Dashed  
736 lines compare EJP<sub>1</sub> in each train. (*Right*) Plots of EJP amplitude during trains 1 and 8,  
737 expressed as a fraction of baseline amplitude (mean ± SEM,  $n = 8$  for each genotype). During  
738 the first train, the decline in EJP amplitude was unaffected by genotype ( $F(1, 14) = 0.22$ ,  $P =$   
739  $0.6486$ , repeated measures two-way ANOVA). In the eighth train, EJPs were smaller at  
740 *OK371>mnb* NMJs ( $F(1, 14) = 4.98$ ,  $P = 0.0426$ ) but the rate of decline during the 8<sup>th</sup> train was  
741 not different ( $F(9, 126) = 0.99$ ,  $P = 0.4540$ , repeated measures two-way ANOVA). (C)  
742 Cumulative frequency distributions of mEJP amplitudes ( $n = 1600$  from 8 NMJ recordings)  
743 from immediately before (solid line) and immediately after (dashed line) the trains, for *OK371/+*  
744 (*left*) and *OK371>mnb* (*right*) NMJs; after the trains, a minority of mEJPs were larger in both  
745 *OK371/+* ( $*P = 0.0117$ ) and *OK371>mnb* ( $*P = 0.018$ ) NMJs (log-rank (Mantel-Cox) test).

746

747 **Fig. 1**



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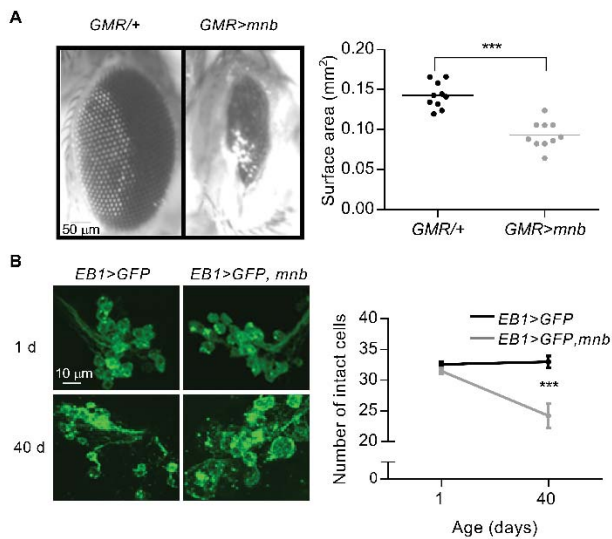
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753 **Fig. 2**

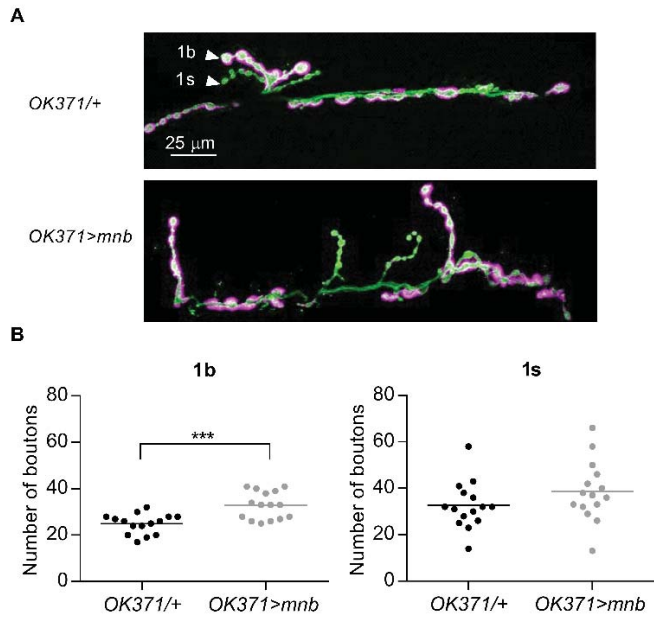


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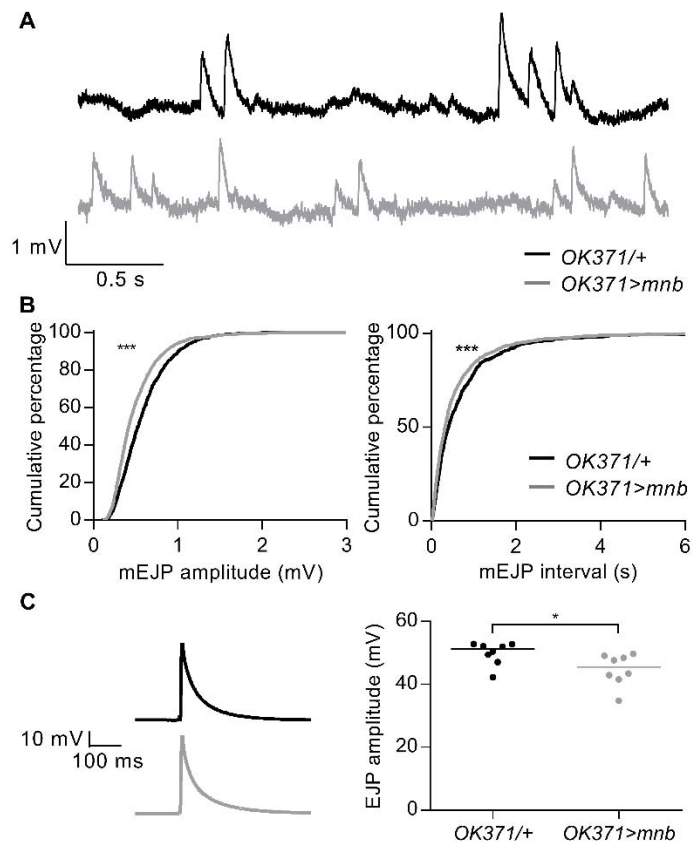
757 **Fig. 3**



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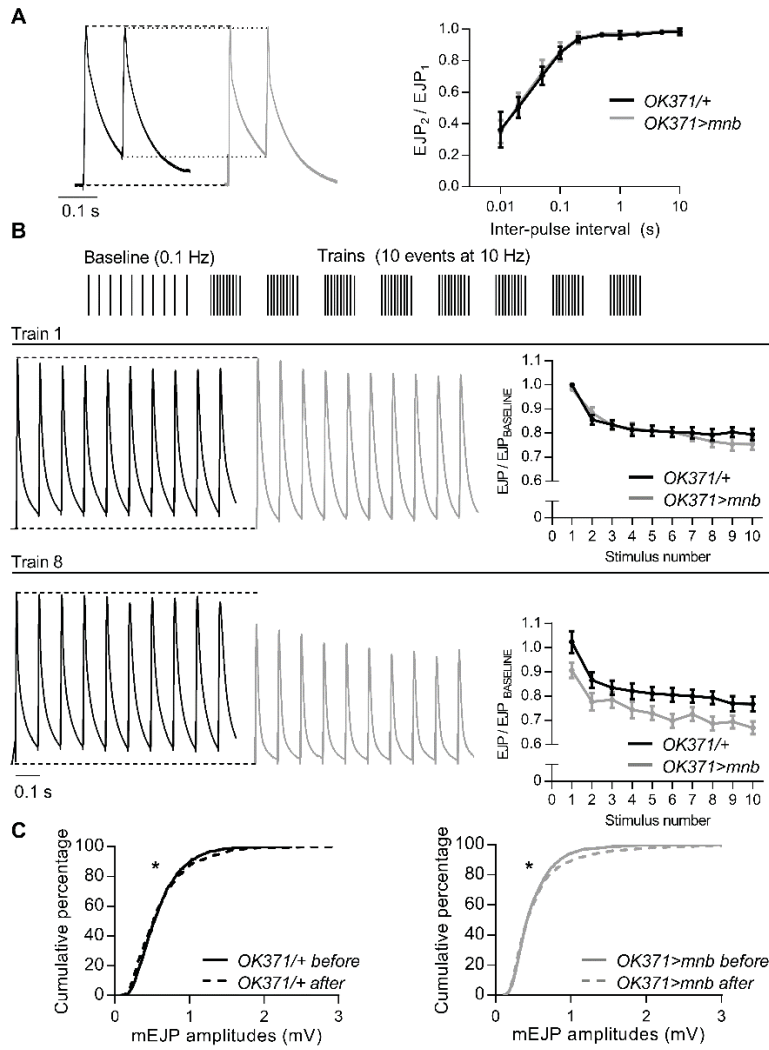
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760 **Fig. 4**



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762 **Fig. 5**



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