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

Simon A. Lowe, Maria M. Usowicz⁎, James J.L. Hodge⁎

School of Physiology, Pharmacology and Neuroscience, University of Bristol, University Walk, Bristol BS8 1TD, UK

ABSTRACT

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

Keywords: DYRK1A/minibrain; Alzheimer's disease; Down's syndrome; motor decline; neurodegeneration; synaptic plasticity.
Cerro et al., 2018; Martínez de Lagrán et al., 2004; Ortiz-Abalia et al., 2008; Souchet et al., 2014; Watson-Scales et al., 2018). However, the contribution of DYRK1A overexpression to the shorter life expectancy, faster age-dependent decline in cognitive and motor function, and development of AD-like pathology in DS is unclear. It is predicted to play a role as it both phosphorylates tau and alters its splicing (Shi et al., 2008; Woods et al., 2001), promoting its self-aggregation (Liu et al., 2008) into NFTs. Dyrk1A is found physically associated with NFTs in the brain to a greater level in DS-AD than non-DS associated AD (Wegiel et al., 2008; Wegiel et al., 2011). In the Ts65Dn and Ts1Cje mouse models of DS, Dyrk1A overexpression in the brain intensifies with age (Ahmed et al., 2017; Creau et al., 2016; Stringer et al., 2014; Watson-Scales et al., 2018), and this is associated with AD-DS-like histopathological changes in the aged Ts65Dn brain (García-Cerro et al., 2017; Wiseman et al., 2015). However, there is insufficient behavioural data from aged animals to directly assess the impact of DYRK1A overexpression in inducing DS-AD phenotypes.

Cognitive and motor dysfunction in individuals with DS and in mouse models of DS are associated with changes in synaptic plasticity and with changes in the number and structure of GABAergic and glutamatergic brain neurons and synapses (Battaglia et al., 2008; Contestabile et al., 2017; Duchon and Herault, 2016). Such modifications have been linked to Dyrk1a overexpression (Duchon and Herault, 2016; García-Cerro et al., 2017; García-Cerro et al., 2018; Ruiz-Meijas et al., 2016), but the effects of Dyrk1a overexpression on the basic properties of synaptic function have rarely been explored. In one study, there was no change in the frequency of miniature excitatory synaptic currents (mEPSCs) or the probability of electrically-evoked glutamate release in the prefrontal cortex of TgDyrk1a mice (Thomazeau et al., 2014). Nevertheless, since Dyrk1A controls the activity of proteins that regulate endocytosis (Murakami et al., 2012) and DYRK1A expression slows endocytosis of transmitter vesicles in hippocampal presynaptic membranes from TgDYRK1A mice (Kim et al., 2010), modulation of transmitter release at other glutamatergic synapses is likely.

To investigate the contribution of DYRK1A overexpression in the nervous system to various aspects of DS, we overexpressed minibrain (mnb), the Drosophila ortholog of DYRK1A (Duchon and Herault, 2016), in the Drosophila nervous system and implemented well-established assays in larvae and adult flies (Bykhovskaya and Vasin, 2017; Lenz et al., 2013; McGurk et al., 2015). The assays monitored motor impairment and its development with age, lifespan, age-related neurodegeneration, and synaptic dysfunction. Due to their short lifecycle, Drosophila are one of the pre-eminent models for aging and neurodegeneration (Jones and Grotewiel, 2011), both aspects of DS that are more difficult to investigate in mice. The Drosophila larval neuromuscular junction (NMJ) is a well-established model for mammalian central glutamatergic synapses and is easily accessible to electrophysiology (Bykhovskaya and Vasin, 2017). Mnb is expressed presynaptically at larval NMJs and reduces its expression changes motor nerve terminal structure and impairs recycling of transmitter vesicles, while overexpression of one isoform, mnb–F, has no effect on basal transmission but ameliorates the effects of reduced mnb expression (Chen et al., 2014). Five mnb isoforms, E-I, have been identified, all of which share a highly conserved kinase domain (Gramates et al., 2017; Hong et al., 2012). Regions of DYRK1A outside the kinase domain also appear to play important roles, but which areas exactly and how they impact function is thus far incompletely understood (Jin et al., 2015; Kelly and Rahmani, 2005; von Groote-Bidlingmaier et al., 2003). We therefore utilised mnb–H, the isoform with the longest coding region (Gramates et al., 2017; Zerbino et al., 2018). Here we report the effects of neuronal overexpression of mnb–H on motor function, the rate of motor decline with age, lifespan, age-related neurodegeneration, presynaptic structure, spontaneous transmitter release and recovery from frequency-dependent depression of electrically-evoked transmitter release.

**Fig. 1. Motor deficits in larvae, accelerated age-dependent motor decline in adult flies and shortened adult lifespan due to neuronal overexpression of mnb.** (A) Elav > mnb larvae crossed fewer lines of a 0.5 cm grid in 60 s than control larvae (Elav/+), 14.7 ± 0.44, n = 15; Elav > mnb, 12.1 ± 0.86, n = 15; mean ± SEM, *P* = .014, Student’s t-test), and with greater variance (F (14,14) = 3.768, *P* = .0184, F-test). (B) Elav > mnb larvae took longer than controls to perform a self-righting task (Elav/+), 5.5 ± 1.16 s, n = 15; Elav > mnb, 12 ± 2.56 s, n = 15; mean ± SEM, *P* = .029, Student’s t-test) and with greater variance (F (14,14) = 4.802, *P* = .0059, F-test). Each point in the plots (A, B) is from a different animal; horizontal lines indicate mean values. (C) The age-dependent decline in climbing ability in a negative geotaxis assay was steeper and showed greater variance for Elav > mnb adult flies than for controls (F (3,84) = 13.8, ***P* < .0001, repeated measures two-way ANOVA, n = 15 groups of flies); at 1 day old there was no difference in the percentage of flies that climbed successfully (Elav/+), 90.59 ± 1.82%, n = 15; Elav > mnb ± 85.94 ± 1.46%, n = 15; mean ± SEM, *P* = .8262, repeated measures two-way ANOVA and Sidak’s multiple comparison). Values plotted are mean ± SEM, n = 15 groups of 10 flies for each genotype. (D) Elav > mnb flies had a shorter lifespan relative to controls (n = 100 animals per genotype at day 0, ***P* < .0001, log-rank (Mantel-Cox) test).

**2. Results**

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

The effect of mnb overexpression in the nervous system on motor function (specifically the mnb-H splice variant) was tested using two assays of fly larval locomotion. Elav > mnb larvae, overexpressing mnb throughout the nervous system under the control of the Elav-Gal4 driver (Robinow and White, 1991), did not move as far as control larvae (Elav/+ ) in a free movement assay (Fig. 1A), which measures the ability of larvae to perform rhythmic muscle contractions necessary for gross locomotion (Kohsaka et al., 2017). They also took longer to complete a self-righting assay (Fig. 1B), which is a more complex motor task requiring larvae to enact a co-ordinated sequence of movements to right themselves after being rolled onto their backs (Picao-Osorio et al., 2015). To assess the impact of neuronal mnb overexpression on age-related decline in locomotor function, the performance of the same cohorts of adult flies was assessed in a negative geotaxis assay at different ages (Jones and Grotewiel, 2011). This showed acceleration in Elav > mnb flies of the usual age-related decline in performance (Elav/+ ). There was also evident shortening of the lifespan of Elav > mnb flies, so that the median lifespan was reduced by almost 50% (Elav/+ ,
significantly reduced in EB1 $> mnb$ flies, whereas at day 40 the number of EB neurons was EB1/+, ... the number of GFP-positive EB neurons between control (GMR/+, Elav > mnb)) and number of GFP-positive EB neurons in one brain hemisphere, with and without mnb overexpression driven by EB1-Gal4, in 1 d and 40 d old flies. Calibration bar is 10μm. (Right) The number of cells did not differ at 1 d (EB1 > mCD8-GFP; 32.59 ± 0.48, n = 15; grey) than that at 40 d in EB1 > mCD8-GFP, mnb flies; (F(1,28) = 10.56; n = 15; P = .003, repeated measures two-way ANOVA). Values plotted are mean ± SEM from 15 flies for each genotype. 2.2. Overexpression of mnb caused neurodegeneration in adult flies As DYRK1A triplication has been linked to degeneration of brain neurons in AD-DS and in Ts65Dn mice (Garcia-Cerro et al., 2017; Wegiel et al., 2008), we tested the possibility that neuronal overexpression of mnb is sufficient to cause neurotoxicity and age-related neurodegeneration using two established assays of neurodegeneration in adult flies (Lenz et al., 2013; McGurk et al., 2015). In the first, mnb was overexpressed in the eye through development and adulthood using the Glass multimer reporter driver (GMR-Gal4) (Ellis et al., 1993). The GMR $> mnb$ flies, but not control flies (GMR/+), had a reduced eye surface area and a visible “rough eye” phenotype (Fig. 2A), both of which indicate neural death and the resultant breakdown of the regularly spaced array of ommatidia making up the retina. In a second assay, the EB1 driver (EB1-Gal4) was used to overexpress mnb in the ellipsoid body (EB), a subpopulation of neurons within the central complex of the brain implicated in locomotor control (Fig. 2B) (Diaper et al., 2013). The EB cells also expressed membrane-bound GFP which enabled their visualisation. At 1 d old, there was no difference in the number of GFP-positive EB neurons between control (EB1/+) and EB1 $> mnb$ flies, whereas at day 40 the number of EB neurons was significantly reduced in EB1 $> mnb$ flies but not in control flies (Fig. 2C). Therefore, neurotoxicity caused by mnb overexpression promoted age-related neurodegeneration in a central neuron population. 2.3. Overexpression of mnb in motor neurons increased the number of synaptic boutons at the larval NMJ To investigate the effect of mnb overexpression on presynaptic morphology, mnb was overexpressed in glutamatergic motor neurons of Drosophila larvae using OK371-Gal4 (Mahr and Aberle, 2006). The neuronal membranes were labelled with horseradish peroxidase (HRP). The muscle is innervated by two motor neurons with functionally and structurally distinct presynaptic boutons; 1s (small) boutons have higher excitation thresholds, higher basal probability of release and induce larger post-synaptic potentials, while short-term and homeostatic plasticity are largely mediated by 1b (big) boutons (Atwood et al., 1997; Newman et al., 2017). These were differentiated by the stronger postsynaptic expression of Discs large (Dlg) opposite 1b boutons (Lahey et al., 1994) Analysis of the NMJ in the second abdominal larval segment, A2, showed that mnb overexpression affected the morphology of the nerve terminals of only one of the motor neurons; it increased the number of 1b boutons but did not alter the number of 1s boutons (Fig. 3A-B). The effect was not secondary to changes in muscle size, as this did not differ (surface area of muscle 6: OK371/+; 44,752 ± 1407 μm², n = 15; OK371 > mnb, 44,681 ± 3684 μm², n = 15, P = .9857).

Fig. 2. Neurotoxicity and age-related neurodegeneration caused by mnb overexpression.

(A) (Left) Representative images of the eyes of control adult flies (GMR/+ and flies with mnb overexpression driven in the eye by GMR-Gal4 (GMR $> mnb$). (Right) The surface area of the eyes in GMR $> mnb$ flies was reduced (GMR/+, 0.14 ± 0.005 mm², n = 10; GMR $> mnb$, 0.09 ± 0.005 mm², n = 10; mean ± SEM, ***p < .0001, Student’s t-test). Each point in the plot is from a different animal, horizontal lines indicate means. (B) (Left) Representative images of clusters of GFP-expressing ellipsoid body neurons in one brain hemisphere, with and without mnb overexpression driven by EB1-Gal4, in 1 d and 40 d old flies. Calibration bar is 10μm. (Right) The number of cells did not differ at 1 d (EB1 > mCD8-GFP; 32.59 ± 0.48, n = 15 (black); EB1 > mCD8-GFP, mnb; 31.47 ± 0.47, n = 15 (grey); mean ± SEM, P = .757, repeated measures two-way ANOVA and Sidak’s multiple comparison) but decreased between 1 and 40 d in EB1 > mCD8-GFP, mnb flies; (F(1,28) = 10.56; n = 15; P = .003, repeated measures two-way ANOVA). Values plotted are mean ± SEM from 15 flies for each genotype. 73 days; Elav $> mnb$, 38 days; Fig. 1D). These results indicate that neuronal overexpression of mnb alone produced a motor deficit and abnormal aging characterised by accelerated age-related locomotor impairment and a shorter lifespan.

Fig. 3. Overexpression of mnb in motor neurons increased the number of 1b boutons.

(A) Representative images of motor nerve endings at the NMJ of muscle 6/7 in the A2 segment of OK371/+ (top) and OK371 > mnb (bottom) larvae. The neuronal membrane is labelled with HRP (green); type 1b boutons but not type 1s boutons (arrowheads) are preferentially labelled with Dlg (magenta). Scale bar is 25μm. (B) OK371 > mnb NMJs had more 1b boutons (OK371/+; 44.93 ± 1.11, n = 15; OK371 > mnb, 32.80 ± 1.52, n = 15, mean ± SEM, ***p = .0003, Student’s t-test) but there was no difference in the number of 1s boutons (OK371/+; 32.6 ± 2.62, n = 15; OK371 > mnb, 38.6 ± 3.34, n = 15, mean ± SEM, P = .169, Student’s t-test). Each value plotted is from a different animal, horizontal lines indicate means. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
than 1 s-dependent mEJPs (Newman et al., 2017). In parallel with the changes in spontaneous synaptic events, there was a small (~11%) decrease in the mean amplitude of electrically-evoked excitatory junction potentials (EJPs) caused by single stimuli applied to the nerve at a low frequency (0.1 Hz) (Fig. 4C). There was no difference between EJPs in mean rise time (OK371+/+, 2.67 ± 0.178 ms, n = 8; OK371 > mnb, 3.23 ± 0.33 ms, n = 8, P = .728) or mean time constant of decay (OK371+/+, 44.9 ± 3.1 ms, n = 8; OK371 > mnb, 36.6 ± 4.6 ms, n = 8, P = .154). The relatively small fall in EJP amplitude is likely to reflect the smaller size of the 1b-dependent component of the EJP relative to that of the 1 s-dependent component (Newman et al., 2017).

2.5. Overexpression of mnb in motor neurons slowed recovery from frequency-dependent depression at the larval NMJ

To investigate the effects of neuronal mnb overexpression on recycling of synaptic vesicles during electrically-evoked transmitter release, EJPs were evoked with pairs of electrical stimuli separated by intervals of varying duration (10 ms – 10 s) or with repeated trains of 10 stimuli applied at a high frequency (10 Hz, a frequency 100 times higher than that at which the single EJPs were evoked) (Kauwe and Isacoff, 2013). At control NMJs, paired pulses separated by intervals shorter than 200 ms caused depression of the amplitude of the second EJP relative to that of the first and the depression was stronger for shorter inter-stimulus intervals (Fig. 5A). The dependence of paired-pulse depression on interval duration was unaltered in OK371 > mnb larvae (Fig. 5A), indicating that mnb overexpression did not alter release from a readily releasable pool of vesicles (Regehr, 2012). When transmitter release was evoked at control NMJs with a train of 10 stimuli at 10 Hz, there was rapid depression of the EJP amplitude by ~20% within the first 3 events (Fig. 5B). In the one-minute interval before the next train, the EJP amplitude recovered fully so that the amplitude of the first EJP in the second train was the same as in the first train (Fig. 5B). This ability to recover did not wane during the recording; the amplitude of the first EJP in each train did not differ between 8 trains (Fig. 5B). These effects are consistent with previous studies (Kauwe and Isacoff, 2013) and confirm rapid depletion and replenishment of the readily releasable pool of vesicles (Regehr, 2012). However, the same pattern of nerve stimulation produced different effects at OK371 > mnb NMJs (Fig. 5B). The percentage decrease in EJP amplitude during each train was the same as at control NMJs, but the depression was not fully reversed during the intervals between trains, so that the first EJP in each train was smaller than the first EJP in the preceding train. The depression in amplitude accumulated over the 8 trains, resulting in an overall fall of 10%. To confirm that the changes in EJP amplitude were due to presynaptic changes in transmitter release and were not postsynaptically mediated by a decrease in the unitary depolarisations comprising each EJP, we measured the amplitudes of 200 mEJPs immediately before and 200 mEJPs immediately after the series of trains at each NMJ. At both control and OK371 > mnb NMJs, the cumulative distribution of mEJP amplitudes before and after a series of trains was similar; although they were not identical, the observed slight increase in the number of larger mEJPs cannot explain the decline in EJP amplitude (Fig. 5C). These results show that mnb overexpression slowed replenishment of the readily releasable pool of vesicles, an effect consistent with the reported slowing of endocytosis of transmitter vesicles by Dyrk1a overexpression (Kim et al., 2010).

3. Discussion

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

People with DS have impaired motor skills which are evident from childhood and are caused by abnormal development of the nervous system (Malak et al., 2015; Stagni et al., 2018). Later, in middle age, they undergo faster age-dependent motor decline, which is an early marker of future dementia, comorbidities and mortality, and is likely caused by histopathological changes in the brain (Anderson-Mooney et al., 2016; Buchman and Bennett, 2011). The life expectancy of people with DS is about 28 years shorter than the general population (O’Leary et al., 2018). By taking advantage of the relatively short life cycle of Drosophila and transgenic overexpression of mnb in neurons, we have demonstrated a potential role for neuronal DYRK1A overexpression in the accelerated age-dependent decline of motor function and shortening of life expectancy in DS. The genetic basis of these aspects of DS are more difficult and costly to explore in mouse models of DS, due to the time required to study aged mice. Our finding that mnb overexpression causes age-related neurodegeneration confirms previous studies inferring a link between DYRK1A overexpression and degeneration and loss of neurons (Duchon and Herault, 2016; García-Cerro et al., 2017; Watson-Scales et al., 2018; Wegiel et al., 2008), which is associated with faster age-related decline in motor and cognitive function in DS and AD-DS. Our results also reinforce the conclusion from earlier studies with adult mice overexpressing DYRK1A or Dyrk1a, alone or as part of a chromosomal segment, that triplication of DYRK1A is likely to contribute to motor deficits in DS (Altafaj et al., 2001; Arque et al., 2013; García-Cerro et al., 2018; Martínez de Lagrán et al., 2004; Ortiz-Abalia et al., 2008; Souchet et al., 2014; Watson-Scales et al., 2018).

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

The cognitive and motor deficits in DS arise from aberrant information processing in the brain that is likely due, in part, to changes in synaptic transmission or synaptic plasticity. Individuals with DS have impaired synaptic plasticity in the motor cortex (Battaglia et al., 2008). Our finding that mbn overexpression slows replenishment of the readily releasable pool of vesicles, and also modifies basal synaptic transmission, confirms a previous suggestion that DYRK1A overexpression contributes to synaptic dysfunction and cognitive deficits associated with DS, made on the basis of the observed slowing of endocytosis of transmitter vesicles in cultured mouse hippocampal neurons over-expressing human DYRK1A (Kim et al., 2010). The effects of DYRK1A on synaptic function may be splice variant specific as we found that overexpression of the mbn-H transcript caused a decrease in mEJP and EJP amplitude, whereas overexpression of mbn-F in a previous study did not alter mEJP or EJP amplitude at the larval NMJ (Chen et al., 2014).

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

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

4. Materials and methods

4.1. Animals

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

4.2. Behaviour

mnb expression was driven throughout the nervous system using Elav-Gal4 (Robinow and White, 1991) for experiments investigating behaviour of wandering third instar larvae, the number of boutons at the larval NMJ, and synaptic transmission at the larval NMJ. All behavioural experiments took place at 25 °C. Larval locomotor experiments were conducted on a 9.5 cm petri dish containing 1.6% agarose. A single third instar wandering larva was selected, washed in a drop of distilled H2O, transferred to the agarose and allowed 30 s to acclimatise. To analyse free movement, the dish was placed over a 0.5 cm grid and the number of lines the larva crawled across in one minute was counted by eye. The self-righting assay was conducted as described elsewhere (Lowe et al., 2018; Pico-Osorio et al., 2015); the larva was gently rolled onto its back on the agarose using a fine moistened paintbrush, held for one second and released, and the time for it to right itself was recorded.

The negative geotaxis assay was performed as described previously (Ali et al., 2011). A cohort of 10 flies was transferred without anaesthesia to an empty 9.5 cm tube with a line drawn 2 cm from the top. After 1 min acclimatisation, the vial was sharply tapped 3 times to knock the flies to bottom. The number of flies to climb past the line within 10 s was recorded. 15 cohorts of 10 flies were tested for each genotype. Age-dependent changes in climbing were assessed by repeating the negative geotaxis assay at 10, 20 and 30 days post-eclosion (Gargano et al., 2005). For the survival assay, 10 cohorts of 10 once-mated females were transferred to a vial of fresh food twice weekly and the number of surviving flies recorded at each transfer.

4.3. Antibody staining and visualisation at the NMJ

Wandering third instar larvae were dissected in ice-cold, Ca2+-free HL3.1-like solution (in mM: 70 NaCl, 5 KCl, 10 NaHCO3, 115 sucrose, 5 trehalose, 5 HEPES, 10 MgCl2) to produce a larval “fillet” (Brent et al., 2009). The fillet was fixed for 30 min in 4% paraformaldehyde (Sigma Labs), washed three times in 1% Triton-X (Sigma Labs) and blocked for one hour in 5% normal goat serum (Sigma Labs) and 1% Triton-X at room temperature. It was incubated overnight in 1/500 mouse FITC-conjugated anti-horseradish peroxidase (HRP-FITC) (Jackson Immunoresearch Laboratories, 115–035-003) and 1/500 rabbit anti-Disco2 large (D1g) (Biocompare, ABIN1387516) primary antibody, then for two hours in 1/500 AlexaFlour 633-conjugated goat anti-mouse secondary antibody (ThermoFisher Scientific, A-21052) at room temperature. Each fillet was washed and mounted on a coverslip in Vectashield (Vector Laboratories). Z-series of NMJs were imaged on a Leica SP5-II confocal laser-scanning microscope using an oil immersion 40 × objective. The number of boutons at the NMJ of muscle 6/7 in segment A2 was counted manually. ImageJ (rsb.info.nih.gov/j/) was used to manually outline muscle 6 and hence calculate their area.

4.4. Neurotoxicity

Overexpression of mnb was driven in the eye using the Glass multimer reporter (GMR-Gal4). Images of the whole head of 1–2 day old flies were taken via a Zeiss AxiosCam MRm camera attached to a stereo-microscope (Zeiss SteREO Discovery.V8, up to 8 × magnification), and the surface area of the eye was calculated by manually outlining the eye in ImageJ (rsb.info.nih.gov/j/). Overexpression of mnb in GFP-tagged ellipsoid body (EB) ring neurons was achieved by crossing EBI-Gal4; UAS-mCD8-GFP flies with UAS-mnb flies. Following published methods (Williamson and Hiesinger, 2010), adult brains were dissected, fixed for
30 min in 4% paraformaldehyde and mounted on a coverslip in Vectorshield (Vector Laboratories). Slides were imaged on a Leica SP5-II confocal laser scanning microscope using an oil immersion 40 × objective. A 2-stack of 25 images at 1 μm increments was captured and combined into a 3-D projection using ImageJ (rsb.info.nih.gov/ij/); analysis was performed by scrolling through all 25 images and counting the number of cells in one brain hemisphere.

4.5. Electrophysiology

Wandering third instar larvae were dissected as for antibody staining. The motor nerves were severed just below the ventral ganglion and the brain was removed. CaCl2 (1 mM) was added to the bath so the number of cells in one brain hemisphere. Analysis was performed by scrolling through all 25 images and counting confocal laser scanning microscope using an oil immersion 40× objective. Nerves were drawn into a thin-walled glass-stimulating pipette (pulled on a Sutter Flaming/Brown P-97 micropipette puller) were filled with 3 M KCl and had resistances of 20–30 MΩ. For recording of stimulated evoked excitation junction potentials (EJPs), severed nerves were drawn into a thin-walled glass-stimulating pipette and stimulated with square-wave voltage pulses (0.1 ms, 10 V, A-M Systems Model 2100 Isolated Pulse Simulator), 10 times at 0.1 Hz. EJPs and spontaneously-occurring miniature EJPs (mEJPs) were recorded at a controlled room temperature of 22–25°C with a Geneclamp 500 amplifier (Axon Instruments) and were further amplified with a LHBF-48 × amplifier (NPI Electronic). The membrane potential was allowed to stabilise for one minute, the initial value was recorded, and then set to −70 mV by injecting current with the Geneclamp 500 amplifier. The muscle input resistance was measured by injecting current using the Axon Geneclamp 500 to bring the membrane potential to −100, −80, −60 and −40 mV, and subtracting the electrode resistance from the slope of the resulting voltage/current graph. Voltage signals were low-pass filtered at 1.67 kHz (10 kHz 4 pole Bessel on Geneclamp 500, 1.7 kHz 8-pole Bessel on LHBF-48 ×) and digitised at 25 kHz by a CED 1401 plus A/D interface (Cambridge Electronic Design, UK) using Spike2 software (v. 5.13) (CED, Cambridge, UK). Recordings were discarded if the initial resting membrane potential was more positive than −60 mV or varied by > 10% throughout the recording. Synaptic potentials were analysed off-line using Strathclyde Electrophysiology Software WinEDR (v3.5.2) and GraphPad Prism (v.6). All synaptic events were verified manually.

Amplitudes and intervals of mEJPs were compared by creating a cumulative distribution for each genotype of 1600 measurements across 8 animals, with each animal contributing 200 values. To analyse the mEJP waveform, a mean mEJP was constructed for each recording from events showing a single clear peak and a smooth decay, so as to prevent distortion of the waveform by closely occurring mEJPs. A single exponential was fitted to the decay of the mean mEJP and the 10–90% rise-time was measured. Time zero for the exponential fit was set to the time of the peak. EJPs were analysed by forming a mean of 8 animals, with each animal contributing 200 values. To analyse the cumulative amplitude distributions compared.

4.6. Statistical analysis

Statistical analysis was conducted in GraphPad Prism (v. 6, La Jolla, CA). Data were tested for normality using the Kolmogorov-Smirnov test; where appropriate means were compared using Student’s unpaired t-test, or medians were compared using a Mann-Whitney U test. EJPs evoked by pairs or trains of stimuli were compared using repeated measures 2-way ANOVA. Cumulative distributions were compared with a Kolmogorov-Smirnov test. Survival curves were compared with a Mantel-Cox test. Data are given as median or mean ± SEM. n is given per genotype. An α level of P < .05 was considered significant.

Acknowledgments

We thank Dr. Scott Waddell (University of Oxford) for Canton Special white- (CSw) flies, Dr. Kweon Yu (Korea Research Institute of Bioscience and Biotechnology) for UAS-mnb flies (mini-brain-H, CG42273) and Dr. Frank Hirth (Kings College London) for EBI-Gal4; UAS-mCD8-GFP flies.

Funding

This work was supported by a BBSRC DTP (Doctoral Training Partnership) studentship.

References

Ahmed, M.M., Block, A., Tong, S., Davison, M.T., Gardiner, K.J., 2017. Age exacerbates abnormal protein expression in a mouse model of Down syndrome. Neurobiol. Aging 57, 120–132.
Ahn, K.J., Jeong, H.K., Choi, H.S., Ryoo, S.R., Kim, Y.J., Goo, J.S., Choi, S.Y., Han, J.S., Ha, I., Song, W.J., 2006. DYRK1A BAC transgenic mice show altered synaptic plasticity with learning and memory defects. Neurobiol. Dis. 22, 463–472.
Ali, Y.O., Escala, W., Ruan, K., Zhai, R.G., 2011. Assaying Locomotor, Learning, and Memory Deficits in Drosophila Models of Neurodegeneration. J. Vis. Exp.(49), e25054.
Altaltaj, X., Dièresen, M., Baamonde, C., Marti, E., Vísa, J., Guimerà, J., Oset, M., González, J.R., Flórez, J., Fillat, C., et al., 2001. Neurodevelopmental delay, motor abnormalities and cognitive deficits in transgenic mice overexpressing Dyrk1A (mindbrain), a murine model of Down’s syndrome. Hum. Mol. Genet. 10, 1915–1923.
Altaltaj, X., Martin, E.D., Ortiz-Abalía, J., Valderrama, A., Lao-Peregrín, C., Dièresen, M., Fillat, C., 2013. Normalization of Dyrk1A expression by AAV2/1-shDyrk1A attenuates hippocampal-dependent defects in the Ts65Dn mouse model of Down syndrome. Neurobiol. Dis. 52, 117–127.
Anderson-Mooney, A.J., Schmitt, F.A., Head, E., Lott, I.T., Heilman, K.M., 2013. Gait dyspraxia as a clinical marker of cognitive decline in Down syndrome: a review of theory and proposed mechanisms. Brain Cogn. 104, 48–57.
Arque, G., Casanovas, A., Dièresen, M., 2013. Dyrk1A is dynamically expressed on subsets of motor neurons and in the neuromuscular junction: possible role in Down syn- drome. PLoS One 8, e54285.
Atwood, H.L., Karunamathi, S., Georgiou, J., Chariton, M.P., 1997. Strength of synaptic transmission at neuromuscular junctions of crustaceans and insects in relation to calcium entry. Invertebr. Neurosci. 3, 81–87.
Battaglia, F., Quartarone, A., Rizzo, V., Ghilardi, M.F., Di Rocco, A., Tortorella, G., Girlanda, P., 2008. Early impairment of synaptic plasticity in patients with down’s syndrome. Neurobiol. Aging 29, 1272–1276.
Brent, J.R., Werner, K.M., McCabe, B.D., 2009. Drosophila larval NMJ dissection. J Vis Exp 24. https://doi.org/10.3791/1107.
Buchman, A.S., Bennett, D.A., 2011. Loss of motor function in preclinical Alzheimer’s disease. Expert. Rev. Neurother. 11, 665–676.
Bukhvostova, M., Vasin, A., 2017. Electrophysiological analysis of synaptic transmission in Drosophila. Wiley Interdiscip. Rev. Dev. Biol. 6 (5). https://doi.org/10.1002/wdev.277.
Chen, C.K., Bregere, C., Paluch, J., Lu, J.F., Dickman, D.K., Chang, K.T., 2014. Activity- dependent facilitation of Synaptojanin and synaptic vesicle recycling by the Minibrain kinase. Nat. Commun. 5, 4246.
Costabel, A., Magara, S., Cancidda, L., 2017. The GABAergic hypothesis for cognitive disabilities in Down syndrome. Front. Cell. Neurosci. 11, 54.
Cramer, N.P., Xu, X., T., P.H., Galcziczi, Z., 2015. Altered intrinsic and network properties of neocortical neurons in the Ts65Dn mouse model of Down syndrome. Physiol Rep 3. Cresa, N., Cabet, E., Daubigney, F., Souchet, B., Bennai, S., Delabar, J., 2016. Specific
Malak, R., Kostiukow, A., Krawczyk-Wasielewska, A., Mojs, E., Samborski, W., 2015. The expression pattern of the Drosophila vesicular glutamate transporter: a marker protein for motorneurons and glial cells in the brain. Gene Expr. Patterns 6, 299–309.

Malak, R., Kostiukow, A., Krawczyk-Wasielewska, A., Mojs, E., Samborski, W., 2015. The expression pattern of the Drosophila vesicular glutamate transporter: a marker protein for motorneurons and glial cells in the brain. Gene Expr. Patterns 6, 299–309.

Ellin, M.C., O'Neill, E.M., Rubin, G.M., 1993. Expression of Drosophila glass protein and evidence for negative regulation of its activity in non-neuronal cells by another DNA-binding protein. Development 119, 855–865.

García-Cerro, S., Martínez, P., Vidal, V., Corrales, A., Frécher, V., Vidal, R., Rueda, N., Arbolí, M.L., Martínez-Cué, C., 2014. Expression of Dysk1A is implicated in several cognitive, electrophysiological and neuromorphological alterations found in a mouse model of Down syndrome. PLoS One 9, e106722.

García-Cerro, S., Rueda, N., Vidal, V., Lantigué, S., Martínez-Cué, C., 2017. Normalizing the gene dosage of Dysk1A in a mouse model of Down syndrome rescues several Alzheimer's disease phenotypes. Neurobiol. Dis. 106, 76–88.

Garcia-Cerro, S., Vidal, L., Lantigué, S., Berciano, M.T., Lafarga, M., Ramírez-Cabrer, P., Padro, D., Rueda, N., Martinez-Cue, C., 2018. Cerebellar alterations in a model of Down syndrome: the role of the Dysk1A gene. Neurobiol. Dis. 110, 93–101.

Liu, F., Liang, Z., Wegiel, J., Hwang, Y.W., Iqbal, K., Grundke-Iqbal, I., Ramakrishna, N., Martínez de Lagrán, M., Altafaj, X., Gallego, X., Martí, E., Estivill, X., Sahún, I., Fillat, C., 2018. Early death and causes of death of people with Down syndrome: a systematic review. J. Appl. Res. Intell. Disabil. 31, 687–709.

O’Leary, L., Hughes-McCormack, L., Dunn, K., Cooper, S.A., 2018. Early death and causes of death of people with Down syndrome: a systematic review. J. Appl. Res. Intell. Disabil. 31, 687–709.

O’Leary, L., Hughes-McCormack, L., Dunn, K., Cooper, S.A., 2018. Early death and causes of death of people with Down syndrome: a systematic review. J. Appl. Res. Intell. Disabil. 31, 687–709.