Investigating autism associated genes in *C. elegans* reveals candidates with a role in social behaviour

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Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterised by a triad of behavioural impairments and includes disruption in social behaviour. ASD has a clear genetic underpinning and hundreds of genes are implicated in its aetiology. However, how single penetrant genes disrupt activity of neural circuits which lead to affected behaviours is only beginning to be understood and less is known about how low penetrant genes interact to disrupt emergent behaviours. Investigations are well served by experimental approaches that allow tractable investigation of the underpinning genetic basis of circuits that control behaviours that operate in the biological domains that are neuro-atypical in autism. The model organism *C. elegans* provides an experimental platform to investigate the effect of genetic mutations on behavioural outputs including those that impact social biology. Here we use progeny-derived social cues that modulate *C. elegans* food leaving to assay genetic determinants of social behaviour. We used the SAFRI Gene database to identify *C. elegans* orthologues of human ASD associated genes. We identified a number of mutants that displayed selective deficits in response to progeny. The genetic determinants of this complex social behaviour highlight the important contribution of synaptopathy and implicates genes within cell signalling, epigenetics and phospholipid metabolism functional domains. The approach overlaps with a growing number of studies that investigate potential molecular determinants of autism in *C. elegans*. However, our use of a complex, sensory integrative, emergent behaviour provides routes to enrich new or underexplored biology with the identification of novel candidate genes with a definable role in social behaviour.

Introduction

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental behavioural disorder. ASD is characterised by a triad of behavioural impairments, these being repetitive behaviours and impairment to verbal and social communication [1]. Neuro-atypical individuals have been shown to produce altered behavioural outputs in response to a range of sensory cues [2], including chemosensory cues that drive social behaviours [3]. Impairment within the integration of sensory stimuli is thought to underlie the altered perception of such cues [4]. This highlights the importance of neural circuits in the processing of sensory information to coordinate a behavioural output in the social domain [5].

It is well established that there is a strong genetic contribution in autism [6]. The genetic architecture of ASD is complex with hundreds of genes of varying penetrance implicated in its aetiology [7]. This is complicated further by the interplay between genetic variants in the form of rare, highly penetrant, and common low penetrant variants [8, 9]. Common variants attribute polygenic risk in ASD with mutations to multiple loci having additive effects on a given phenotype [8]. The burden of common variants in an individual's genetic background can influence the degree of risk a rare variant can impose [9]. The combinatorial effect of rare and common variants contributes to the spectrum of phenotypes displayed across autism cases [10].

ASD associated genes span across a range of biological functions, for example synaptic, cell signalling and epigenetic modification [11]. Evidence suggests that although ASD genes are functionally diverse they are connected through protein interaction networks [12] and control processes such as neuronal morphology and synaptic function that modulate the activity state of neural networks [8, 13, 14]. This means that the consequence of even a

single genetic variant can be widespread through inter-connecting gene networks and have emergent effects on neural circuits [8]. For many ASD associated genes it is still unclear how they function in neural networks which underpin behavioural phenotypes [15], such as disrupted social and motor behaviour. However, investigating determinants of defined neural circuits underpinning autism associated neuro-atypical behaviour is providing traction for discreet investigation of complex traits. Study of distinct behaviours in mice has begun to unpick the effect of genetic disruption on molecular circuits and synapse function [16-18]. Additionally, the impact of genetic variation on a number of synaptic genes has been extensively studied in various animal models [19-24].

Animal models highlight the value of using orthologues to understand the function of ASD associated genes in behavioural domains associated with autism [25-28]. *C. elegans* provides a tractable system that allows for the high throughput of genetic determinants to be investigated in a simple nervous system [29]. Conservation of genes involved in synapse function and the use of integrative neurons highlights the utility of *C. elegans* neuronal function and how it co-ordinates complex sensory integrative behaviours that model the disruptions that are expressed through genetic mutations associated with autism [30, 31]. The genetic homology between the *C. elegans* and mammalian genome [32], and the fact that mutant strains are widely accessible, means that the *C. elegans* model lends itself to the systems level analysis of disease associated genes. This has led to a plethora of studies using single gene analysis to investigate the impact of genetic mutation to ASD associated gene orthologues on behavioural output [33]. As well as this, *C. elegans* have been utilised in multiple high-throughput screens which have largely used morphological and locomotory readouts to screen for behavioural deficit [34-36].

Behavioural output in response to integration of sensory cues can be assayed in *C. elegans* by way of food leaving behaviour. The propensity of a worm to leave a lawn of bacterial food can be modulated by multiple sensory cues [37]. It has been shown that in the presence of increasing numbers of progeny, adult worms will leave an otherwise replete food lawn in a dose-dependent manor. This progeny-dependent food leaving behaviour is the result of inter-organismal communication and is thought to be underpinned by a novel social circuit [38]. The utility of this social paradigm to probe autism related dysfunction was demonstrated by showing that when a penetrant mutation of human neuroligin is introduced into the worm orthologue, *nlg-1*, it results in disrupted progeny induced food leaving behaviour [39].

We have used this bona fide social paradigm to investigate genetic determinants associated with human ASD. Investigation of *C. elegans* orthologues in a subset of candidate genes identified a number that disrupt a social behavioural paradigm in the worm. Furthermore, we show that whilst a large proportion of mutants displayed behavioural deficit in the social domain, there was limited disruption to the other phenotypes investigated suggesting a selective behavioural deficit. Identification of novel candidate genes in this way has also highlighted key biological functional domains that appear to play an important role in social behaviour, therefore shedding light on the functional contribution ASD associated genes may have on the disrupted phenotypes associated with this disorder.

Materials and methods

Prioritising ASD associated genes for study in *C. elegans*

To identify genes associated with ASD we used SFARI Gene Archive (http://gene-archive.sfari.org/, version 3.0). Within this database the Human Gene Module (https://gene-

archive.sfari.org/database/human-gene/) ranks genes from 1-6 based on the evidence supporting the gene's association with ASD. Genes in category 1-High confidence and category 2-Strong candidate were selected for analysis due to the fact that data implicating those genes in ASD reach genome wide significance and there is evidence for the variant having a functional effect in humans. Orthologous genes in *C. elegans* were identified by searching the human gene name in WormBase (https://wormbase.org/, version WS264) and using the human gene Ensembl ID in OrthoList (http://www.greenwaldlab.org/ortholist/). *C. elegans* strains available for order from the *Caenorhabditis* Genetics Centre (CGC) and/or the National BioResource Project (NBRP) were prioritised for investigation. Using information gathered from WormBase, CGC, NBRP and a literature review, mutants were excluded if they were lethal, sterile or uncoordinated. Thus, we filtered for candidates best suited to investigation in the food lawn based assay. The prioritised *C. elegans* mutant strains for study can be found in Table 1. Genes were ascribed to one of five functional categories: synaptic, neuronal, cell signalling, epigenetic modifiers and phospholipid metabolism based on their function described by UniProtKB (https://www.uniprot.org/uniprot/). Genes described as having a role in synaptic transmission, structure, activity or plasticity were categorised as 'synaptic'. Genes with a role in neuronal excitability or adhesion were categorised as 'neuronal'. Genes described as having a predominant role in cell signalling pathways were categorised as 'cell signalling'. Genes with a role in transcriptional regulation or chromatin remodelling were categorised as 'epigenetic modifier'. The gene MBOAT7 is described as functioning in phospholipid metabolism and so was categorised as 'phospholipid metabolism'.

Table 1: Summary of human genes prioritised for study in *C. elegans* mutant strains

Human gene	Gene name	Protein function	*C. elegans* orthologue	Allele	Strain name	Out-crossed	Mutation	Mutation effect	Behavioural phenotype	Gene expression
Synaptic										
GRIA1	Glutamate ionotropic receptor AMPA type subunit 1	Glutamate receptor	*glr-1*	*n2461*	KP4	4	Nonsense mutation in codon 807 [40]	LOF [40]	Defective local search behaviour [41]	AVA,AVB, AVD,AVE, PVC,AIB,RMD, RIM,SMD,AVG PVQ,URY [41]
			glr-2	*tm669*	FX00669	0	Complex substitution [42]	Unpublished	Enhanced gustatory plasticity [43]	AVA,AVD,AVE PVC,RMDV, RMDD,AIA, AIB,AVG,RIG, RIA,M1 [44]
			glr-2	*ok2342*	RB1808	0	Deletion [42]	Unpublished	Unknown	
GRIN2B	Glutamate Ionotropic Receptor NMDA Type Subunit 2B	NMDA receptor	*nmr-2*	*ok3324*	VC2623	1	Deletion [42]	LOF [34]	Reduced swimming locomotion [34]	AVA,AVD,AVE, RIM,AVG, PVC [44]
			nmr-2	*tm3785*	FX03785	0	Deletion [42]	LOF [45]	Impaired learning [46]	
NLGN3	Neuroligin 3	Synaptic adhesion	*nlg-1*	*ok259*	VC228	6	Deletion to half of cholinesterase-like domain and TMD [22]	Null [22]	Reduced spontaneous reversals [22] Reduced pharyngeal pumping [47]	VA,DA,AIY, URB,URA, PVD,HSN,ADE,URX, AVJ, ALA [22, 47]
NRXN1	Neurexin 1	Synaptic adhesion	*nrx-1*	*ds1*	SG1	3	Deletion in the long *nrx-1* isoform [48]	Unpublished	Unknown	Nervous system, GABAergic neurons [49, 50]
			nrx-1	*tm1961*	FX01961	0	Deletion in the long *nrx-1* isoform [48]	Truncated protein [24]	Deficient gentle touch response [24]	
PTCHD1	Patched domain containing 1	Synaptic receptor	*ptr-5*	*gk472*	VC1067	0	Deletion [42]	Unpublished	Unknown	Unknown
SHANK 2/3	SH3 and multiple ankyrin repeat domains	Synaptic scaffold	*shn-1*	*ok1241*	RB1196	0	Deletion covering PDZ domain and	LOF [51]	None reported [51]	Widely expressed [52]

							proline rich motif [51]			
			shn-1	*gk181*	VC376	0	Deletion covering most of ANK repeat and entire PDZ domain [51]	LOF [51]	Unknown	
SLC6A1	Solute carrier family 6 member 1	GABA transporter	*snf-11*	*ok156*	RM2710	6	Deletion [53]	Putative null [53]	None reported [53]	AVL,RIBR,ALA, RIBL,GLRV, RME,AVF,EF1, EF2,EF3,EF4, Body wall muscle [54]
			snf-11	*tm625*	FX00625	0	Deletion and insertion [53]	Putative null [53]	Unknown	
SYNGAP1	Synaptic Ras GTPase activating protein 1	Ras GTPase activating protein	*gap-2*	*tm748*	JN147	0	Complex substitution [42]	LOF [55]	No effect on body bends [55]	Widely expressed [55]
			gap-2	*ok1001*	VC680	0	Complex substitution [42]	Unpublished	Unknown	
Neuronal										
CACNA1H	Calcium voltage-gated channel subunit Alpha1 H	Calcium channel	*cca-1*	*ad1650*	JD21	7	Deletion [56]	LOF [56]	Reduced pharyngeal pumping [56]	Pharyngeal muscle, neurons in pharynx and VNC [56]
CNTN4	Contactin 4	Axonal adhesion	*rig-6*	*ok1589*	VC1125	0	Deletion [57]	Hypo-morphic [57]	None reported [57]	Widely expressed in nervous system [58]
			rig-6	*gk376*	VC884	0	Deletion-knocks down expression of isoform a only [57]	Hypo-morphic [57]	Unknown	
Cell signalling										

DYRK1A	Dual specificity tyrosine phosphorylation regulated kinase 1A	Protein kinase	*hpk-1*	*pk1393*	EK273	6	Deletion to most of kinase domain [59]	Null [59]	Reduced lifespan [59]	Gonad, nervous system – not otherwise specified [59]
			mbk-1	*pk1389*	EK228	6	Deletion to most of kinase domain [60]	Putative null [60]	Reduced lifespan [60]	Somatic tissue, not otherwise specified [61]
			mbk-1	*ok402*	RB677	0	Unknown	Unknown	Reduced swimming locomotion [34]	
PTEN	Phosphatase and tensin homolog	Protein phosphatase	*daf-18*	*e1375*	CB1375	0	Insertion [62]	Reduction of function [62]	Chemotaxis deficit [63]	Widely expressed [64]
			daf-18	*ok480*	RB712	0	Deletion [65]	Putative null [65]	Abnormal mitotic arrest in dauer [66]	
Epigenetic Modifiers										
CHD8	Chromodomain helicase DNA binding protein 8	Transcription factor	*chd-7*	*gk290*	VC606	0	Deletion [42]	Unpublished	Reduced swimming locomotion [34]	Unknown
			chd-7	*gk306*	VC676	0	Deletion [42]	Unpublished	Impaired habituation [36]	
FOXP1	Forkhead box P1	Transcription factor	*fkh-7*	*gk793*	VC1646	0	Deletion [42]	Unpublished	Unknown	Widely expressed [67]
IRF2BPL	Interferon regulatory factor 2 binding protein like	Transcription factor	*tag-260*	*ok1339*	VC812	0	Insertion [42]	Putative null [68]	Unknown	Unknown
KDM6A	Lysine-specific demethylase 6A	Histone demethylase	*jmjd-3.1*	*gk387*	VC912	0	Deletion [42]	Unpublished	Unknown	PDA motor neuron and Y cell [69]
			jmjd-3.1	*gk384*	VC936	0	Insertion [42]	Null [70]	Unknown	
KMT5B	Lysine methyltransferase 5B	Transcription factor	*set-4*	*n4600*	MT14911	2	Deletion [71]	LOF [71]	Deficient dauer arrest [71]	Nervous system, not otherwise specified [71]

			set-4	*ok1481*	VC997	0	Deletion [71]	LOF [71]	Deficient dauer arrest [71]	
SETD2	Histone-lysine N-methyltransferase SETD2	Transcriptional regulation	*met-1*	*n4337*	MT16973	4	Deletion [72]	LOF [73]	Sterility at 25◦C [72]	Broadly expressed [74]
			met-1	*tm1738*	FX01738	0	Deletion [72]	Putative null [72]	Sterility at 25◦C [72]	
SETD5	Histone-lysine N-methyltransferase SETD5	Transcriptional regulation	*set-26*	*tm3526*	FX03526	0	Deletion [42]	Putative null [75]	None reported [75]	Widely expressed [76]
			set-24	*n4909*	MT16133	0	Unknown [42]	Unknown	None reported [77]	Germline specific [74]
			set-9	*n4949*	MT16426	1	Deletion [42]	Putative null [75]	None reported [77]	Germline specific [76]
Phospholipid metabolism										
MBOAT7	Membrane bound O-acyltransferase domain containing 7	Acetyl transferase	*mboa-7*	*ok1028*	RB1071	0	Deletion [78]	LOF [78]	Unknown	Muscle, vulva, intestine [79]
			mboa-7	*gk399*	VC942	0	Deletion [78]	LOF [78]	Egg laying deficit [79]	
			mboa-7	*tm3536*	FX03536	0	Deletion [78]	LOF [78]	Developmental defects [78]	
			mboa-7	*tm3645*	FX03645	0	Deletion [78]	LOF [78]	Unknown	

Human genes were used to ascribe functional domains. For each human gene the *C. elegans* orthologue used for investigation is listed and the mutant allele, known phenotypes and expression of the gene indicated. LOF stands for loss of function. AA stands for amino acids. TMD stands for transmembrane domain. LNS stands for laminin-neurexin-sex hormone-binding globulin. References are indicated.

C. elegans culturing and strains used

All *C. elegans* strains were maintained using standard conditions [80]. *C. elegans* were age synchronised by picking L4 hermaphrodites onto a new plate 18 hours prior to behavioural assays. Bristol N2 were used as wild-type control. All other strains used can be found in Table 1. Strains were obtained from either the *Caenorhabditis* Genetics Center or National BioResource Project.

Food leaving Assay

5cm NGM (nematode growth medium) plates were prepared using a standard protocol [80]. 50µl of OP50 *E.coli* at OD_{600} of 0.8 was gently spotted on the middle of an unseeded plate. Approximately 18 hours following this, seven L4+1 day old hermaphrodites were picked onto the centre of the bacterial lawn. Plates were then incubated at 20°C for 24 hours. In all food leaving assays the number of food leaving events were counted manually during a 30 minute observation period using a binocular dissecting microscope (Nikon SMZ800; X10). A food leaving event was defined as when the whole of the worm's body came off the bacterial food lawn, as previously described [39]. Following each food leaving assay the % proportion of eggs, L1 and L2 progeny on the plate was calculated. For all food leaving assays N2 and *nlg-1(ok259)* animals were analysed in parallel to other mutant cohorts. Investigators were blind to the genotypes being observed.

Pre-conditioned food leaving assay

NGM plates were prepared and seeded as described above. 18 hours after seeding assay plates, half were pre-conditioned with progeny using the protocol described previously [38] and the remaining plates were used as matched unconditioned controls. In the

149 preconditioned plates 10 gravid adults were picked onto the centre of the bacterial lawn
150 and left to lay 140-200 eggs before being picked off. 18 hours after this, for each mutant
151 under investigation, seven L4+1 day old hermaphrodites were picked onto the centre of a
152 naïve bacterial food lawn. This acts as a matched unconditioned control. Another seven
153 L4+1 day old hermaphrodites were picked onto a pre-conditioned bacterial food lawn in
154 which 140-200 eggs had developed for 18 hours. The plates were then incubated at 20˚C for
155 2 hours before food leaving events were observed for 30 minutes as described above.

156 **Pharyngeal pumping**

157 Following the measurement of food leaving at the 24 hour time point, feeding behaviour
158 was quantified by counting the pharyngeal pumping of three of the seven worms. The
159 worms selected for these measurements were on food for the observation period. One
160 pharyngeal pump was defined as one cycle of contraction-relaxation of the terminal bulb of
161 the pharyngeal muscle. This behaviour was measured for 1 minute using a binocular
162 dissecting microscope (Nikon SMZ800; X63) and the pumps per minute for each worm
163 recorded from a single observation [81].

164 **Thrashing**

165 Thrashing analysis was performed on the *C. elegans* mutants that were investigated in the
166 pre-conditioned food leaving assay. Using a 24 well plate, 6-7 N2 or mutant worms were
167 picked per well containing 500µl of M9 with 0.1% bovine serum albumin and left for 5-10
168 minutes before thrashing was observed. For each worm thrashing was counted for 30
169 seconds. Each thrash was defined as a complete movement through the midpoint of the
170 worms body and back. For each mutant under investigation N2 control worms were

171 analysed in parallel and at least two separate assays were performed. Investigators were
172 blind to the genotypes being investigated.

173 **Statistical analysis**

174 Statistical analysis was performed using GraphPad Prism 8 software. Data are expressed as
175 mean or mean ±SEM as indicated in the figure legend. Statistical tests and post-hoc analysis
176 is indicated in the figure legends. Significance level was set to $P<0.05$.

177 **Results**

178 **Selection of human ASD associated genes for study using *C. elegans* social behaviour**

179 The genetic architecture of autism is complex with over 1,000 genes currently implicated in
180 the disorder [11]. Furthermore, the functional contribution that many of these genes make
181 to the behavioural domains implicated in ASD remains unclear. We have created a pipeline
182 (Fig 1) to select *C. elegans* orthologues of human ASD associated genes and that can be
183 investigated in a paradigm of social behaviour in the worm.

184 We used SFARI Gene, a growing database which categorises ASD risk genes based on the
185 strength of evidence supporting the association. We prioritised 91 genes ranked by SFARI
186 Gene Archive (accessed October 2018) as category 1-high confidence and category 2-strong
187 candidate. Of these 91 genes, 84% (76/91) had at least one orthologue in *C. elegans.* A
188 mutant strain was available for 84% (64/76) of the orthologous genes using the criteria that
189 the mutant strain was available from the CGC and/or NBRP. Of these, 43 genes had available
190 mutants that were either lethal, sterile or uncoordinated (Fig 1). We considered that such
191 phenotypes rendered these mutants unsuitable for investigation in the social behaviour
192 assay. On this basis we selected 40 *C. elegans* mutants spanning 21 human ASD associated

genes for further investigation (Table 1). The human ASD associated genes were each assigned to a group based upon the functional description of the encoded protein in UniProtKB database. The functional groupings were: synaptic, neuronal, cell signalling, epigenetic modifiers and phospholipid metabolism. This led to a distribution of candidates highlighting 43% as synaptic genes, 33% as epigenetic modifiers, 10% as cell signalling, 9% neuronal and 5% phospholipid metabolism (Fig 1).

Screening mutants using food leaving behaviour identifies ASD associated genetic determinants of social behaviour

To investigate food leaving behaviour, mutants were picked onto the centre of a bacterial lawn and food leaving events were measured after 24 hours. During the 24 hour incubation period the adult worms lay eggs which hatch into *C. elegans* progeny. It has been previously shown that progeny-derived social cues mediate a progeny-dependent increase in adult food leaving behaviour [38]. In accordance with previous findings we observed that N2 worms left the food lawn after 24 hours at a rate of approximately 0.088 leaving events/worm/minute (Fig 2). We had previously established a blunted food leaving response for the *nlg-1(ok259)* mutant [39] and this was used as an internal measure in the current assays (Fig 2).

Against this backdrop, N2 and *nlg-1(ok259)* were investigated alongside the selected mutants we filtered through following initial selection from the SAFRI Gene data base. This comparison showed that 23 of the 39 *C. elegans* mutants showed a mean food leaving rate lower than that of *nlg-1(ok259)* suggesting food leaving impairment (Fig 2). Mutants with a reduced food leaving phenotype were distributed across the five functional categories we

defined suggesting genetic disruption within a range of molecular determinants from distinct biological domains may contribute to the emergence of *C. elegans* social behaviour.

As part of the investigation, where possible, we analysed two or more mutant alleles for a single gene (Fig 2). For some mutants, for example for *gap-2* and *rig-6* mutants, the two mutants phenocopied one another and showed a food leaving rate similar to that of N2. Interestingly, we found two loss-of-function *nmr-2* mutants which also phenocopied one another but showed significant food leaving impairment (Fig 2). In contrast, there were also instances where mutant alleles did not phenocopy each other. For example *nrx-1* and *chd-7* mutants showed one mutant allele with impaired food leaving and one with a behavioural response to progeny similar to N2 (Fig 2).

Impaired social behaviour of mutants is likely a selective response to progeny derived social cues

Previous work has identified the value of investigating additional behaviours that can be scored in the observational arena [36]. In this respect the food leaving assay allows for multi-tracking phenotypic analysis including pharyngeal pumping, development and egg laying. In the case of pharyngeal pumping and egg laying, this reflects the output of a defined neuromodulation and the possible consequence progeny exposure might have on this. In the case of development, this provides insight into whether the mutations perturb gross development, a useful consideration in a neurodevelopmental disorder.

After each food leaving assay we quantified the pharyngeal pump rate of the mutants. Pharyngeal pumping is modulated via external sensory cues such as food [82]. Therefore we wanted to test whether another sensory regulated behaviour was affected in these mutants. 87% of mutants showed no pumping phenotype (Fig 3). In fact the majority of

mutants with impaired social behaviour (Fig 2) had a pumping rate similar to N2 (Fig 3). This shows that most mutants with reduced food leaving behaviour are capable of responding to food-dependent sensory cues and co-ordinating normal feeding behaviour. In addition, the *cca-1(ad1650)* mutant which showed the most deficient pumping phenotype (Fig 3) did not show a food leaving phenotype (Fig 2), further suggesting that deficits in feeding behaviour are unlikely to explain differences in food leaving behaviour.

Next, we measured early development by quantifying the proportion of total progeny that were eggs, L1 and L2 progeny 24 hours after introducing 7 L4+1. We used % proportion to normalise for observed variation in the total number of eggs laid. 75% of mutants developed at a similar rate to N2 showing that there is no gross early developmental delay (Fig 4). Interestingly, whilst early development seems to be largely unaffected we noted a larger variation in the egg laying of distinct mutants when compared to N2 controls (S1 Fig). The number of eggs laid by a mutant is an important consideration for this assay because the density of progeny populating a food lawn is known to influence the food leaving rate of adult worms [38]. We plotted the relationship between the number of progeny produced by a mutant and the food leaving behaviour and showed that the two were correlated (Fig 5). Interestingly, this applies to the *nrx-1* and *chd-7* mutants for which the two alleles tested resulted in distinct social phenotypes (Fig 2). In each case the mutant that showed impaired social behaviour (Fig 2) also produced fewer progeny (S1 Fig). Producing fewer progeny means adult worms were exposed to fewer progeny-derived social cues and could explain the low food leaving rate seen. The correlation between social behaviour and progeny exposure, and the limited disruption seen to the other phenotypes tested, implies that progeny-derived social cues selectively effect social behaviour and therefore progeny exposure is an important consideration in this type of investigation.

Exposure to N2 progeny selectively modulates social behaviour in a number of mutants

Those strains which produced few progeny confound the assessment of the reduced food leaving behaviour as the response is dependent on the density of progeny populating the food lawn [38]. This was addressed by testing the food leaving behaviour of 30 mutants in response to an experimentally controlled number of N2 progeny. This used a pre-conditioning approach in which N2 progeny pre-populate the lawn and precondition them by mimicking the progeny population that emerge in the first food leaving assay. These assays allow the acute effect of progeny exposure on food leaving behaviour to be investigated. This secondary screen focussed on mutants that showed a mean food leaving rate lower than that of *nlg-1(ok259)* in at least one allele tested (Fig 2). Thus we directly tested the veracity of mutants that emerge from the first screen and explicitly address the potential confound of reduced progeny number. For each mutant we performed a paired experiment in which mutant food leaving was measured on a naïve, unmatched control, plate containing OP50 and a preconditioned plate that incubated 140-200 eggs for 24 hours before introducing 7 L4+1 adults. In accordance with previous findings, N2 adults showed enhanced food leaving in response to progeny and this response was blunted in the *nlg-1(ok259)* adults exposed to pre-loaded N2 progeny (Fig 6).

Analysis of mutants in response to pre-loaded N2 progeny revealed a number of mutants which left infrequently on both naïve and pre-conditioned food lawns, showing little progeny-enhanced food leaving (Fig 6). We reasoned that the low food leaving rate of these mutants could be explained by locomotory deficits. To address this we performed a thrashing assay to assess the innate movement ability of the mutants. Whilst some mutants showed minor disruption to thrashing behaviour (Fig 7) for most mutants thrashing did not

predict food leaving behaviour. For example, *nlg-1(ok259)* and *set-4* mutants showed deficits in food leaving behaviour (Fig 6) without any impairment to thrashing (Fig 7). Furthermore, four mutants of the *mboa-7* gene all showed impaired progeny-induced food leaving behaviour with only one of these mutants having reduced thrashing (Fig 7). This suggests that food leaving and thrashing behaviours are uncoupled and therefore suggests it is unlikely that simple motility deficits explain progeny-induced food leaving impairment. Therefore, deficits in progeny-enhanced food leaving behaviour may be due to an impaired ability of adult worms to respond to social cues released by progeny in order to modulate their food leaving behaviour. The social impairment we observed in mutants suggests that a variety of genes may act as molecular determinants of social behaviour. Interestingly, these genes were part of synaptic, cell signalling, epigenetic modifier and phospholipid metabolism categories. This highlights that molecular determinants from these biological domains may be important for the emergence of social behaviour.

The comparison of behaviour on naïve and pre-conditioned lawns also allowed for the analysis of egg laying behaviour in response to progeny. We quantified the number of eggs laid by each mutant on naive and pre-conditioned food lawns after each food leaving assay. Interestingly, all mutants laid the same number of eggs on naïve and pre-conditioned food lawns (S2 Fig). This shows that progeny exposure modulates food leaving behaviour and not egg laying. This therefore suggests that the circuit which integrates progeny cues to sculpt food leaving motility is independent of egg laying behaviour which is modulated by other environmental cues.

Overall, starting with 91 human ASD associated genes we used criteria based filtering to define 21 candidate genes for analysis using a *C. elegans* social paradigm. An initial screen of

social behaviour in response to progeny produced over 24 hours indicated 23 mutants with a reduced food leaving phenotype. We then confirmed the veracity of this phenotype using a progeny pre-conditioned food leaving approach and identified mutants that showed a socially impaired phenotype. The limited disruption in other phenotypes tested for these mutants suggests that reduced food leaving is a selective social impairment in response to progeny-derived social cues. Identification of these mutants highlights genetic determinants that appear to play a role in social behaviour and also suggests that a number of biological domains (synaptic, cell signalling, epigenetic modification and phospholipid metabolism) are important for the coordination of social behaviour.

Discussion

ASD is characterised by a triad of behavioural impairments including neuro-atypical behaviour in the social domain [1]. Individuals with ASD have also been shown to produce altered behavioural responses to a range of chemosensory cues such as olfactory, tactile and gustatory cues [5, 83]. Multi-sensory processing deficits identified in ASD highlights the importance of sensory integration at a circuit level [84] however, it is still unclear how disruption within neural circuits evoke a modified behavioural output. Recent experiments have highlighted the value of investigating molecular determinants of ASD in the context of defined integrative circuits to try and understand more precisely how disruption within these circuits underpins the phenotypes associated with ASD [16, 17, 36, 47]. Approaches, such as these, that better resolve the underlying mechanisms should facilitate pharmacological treatment of ASD and other neuropsychiatric disorders [85].

ASD is known to have a complex genetic architecture, with hundreds of genes with varying penetrance implicated in its aetiology [7]. Although the genetic basis is well documented the

331 functional contribution which many of the genes make to the behavioural domains
332 associated with ASD is unclear. Animal models have begun to understand genetic
333 contribution in autism [26]. The analysis of single, high penetrant, variants is becoming
334 increasingly well refined with the use of animal social behaviours. Use of social behaviours
335 underpinned by discreet neural circuits has helped establish the role of some ASD
336 associated genes in the social domain [39, 86]. However, the analysis of common, low
337 penetrant, variants is more complex. Additive effects from polygenic interaction of multiple
338 common variants contributes to wide spread disruption at distinct levels of the biological
339 system which is expressed as an emergent behaviour [8].

340 *C. elegans* have been used in targeted single gene approaches and in screens of ASD
341 associated genes to provide valuable insight into the role of some genes in sensory
342 processing, development and learning phenotypes [36, 87]. Recently we have shown the
343 utility of using a social behavioural paradigm in *C. elegans* to investigate a single ASD
344 associated gene [39]. This paradigm is based on inter-organismal signalling by use of
345 chemosensory social cues which results in a progeny-induced food leaving phenotype [38].
346 In this study we have used this social paradigm in a screen of ASD associated genes and
347 identified gene candidates with a role in *C. elegans* social behaviour.

348 We created a pipeline to prioritise human genes for investigation using *C. elegans* social
349 behaviour. From this we identified 21 human genes for investigation using 40 *C. elegans*
350 mutant orthologues. Similarities between our prioritisation strategy and those used in
351 previous *C. elegans* studies resulted in the iterative selection of some well-studied ASD
352 associated genes such as neuroligin and neurexin [34, 36]. Our study is distinct from others
353 because we biased our gene filtering approach to select for *C. elegans* mutants that were

appropriate for analysis of social behaviour using a pre-conditioned food leaving approach. We were selective in choosing mutants appropriate for our behavioural analysis, for example the exclusion of overt locomotory mutants due to their possible confounding effect on food leaving motility.

We used a single point analysis focused on progeny induced food leaving from which we also analysed pharyngeal pumping, early development and egg laying capabilities in response to progeny derived social cues. We identified a number of mutants with an altered behavioural response to progeny populating a food lawn providing evidence that *C. elegans* are capable of modelling disruption to an emergent behaviour in response to mutation to an ASD associated gene. Movement in liquid has been used in other studies to screen ASD associated genes [34]. Our analysis of thrashing in mutants identified that this type of locomotory assay does not accurately predict an impaired food leaving behaviour and does not serve as a surrogate for the more complex integrative progeny-induced social behaviour phenotype. This highlights our behavioural screen as a unique platform which is selectively tuned to identify genetic determinants with a role in social circuits, that when disrupted could appear phenotypically normal in thrashing behaviour.

The candidates that we identified as having a role in social behaviour are orthologues of human genes that range in function including synaptic, cell signalling and epigenetic modification. Genes disrupted in each of these domains are known to contribute to ASD [88, 89]. Therefore, the genes that emerge from our screen are representative of the main functional domains disrupted in autism. Our screen has therefore produced a diverse list of candidate genes that can be used to interrogate the systems level disruption that evokes modified behavioural output in ASD. Previous work has focused largely on locomotory and

morphological readouts of altered behavioural phenotypes [34-36] whereas our approach facilitates the identification of candidate genes with a role in a more complex, sensory regulated, emergent behaviour which more closely resembles the social domain disrupted in autism. Identification of candidates using this approach therefore provides a benchmark from which the social circuit can be further dissected [90].

We identified five synaptic genes, *nlg-1*, *nrx-1*, *shn-1*, *glr-1* and *nmr-2,* with a role in coordinating progeny-induced social behaviour. In the mammalian nervous system, NLGN, NRXN and SHANK's interaction at the synapse is well established and dysfunction to all three genes has been widely implicated in ASD [91]. Synaptic scaffolds including SHANK are known to interact with receptors such as AMPA and NMDA to help regulate the ion channel composition at the synapse [92]. This provides evidence that this assay for social interaction identifies behavioural disruption in orthologues of genes that function together at mammalian synapses. The role of these mammalian genes in nervous system function and/or synaptic transmission are functionally conserved in *C. elegans* [40, 49, 51, 93-95]. This means that we can resolve singular determinants with the potential to unpick genes that encode dysfunctional interactions. This raises the opportunity to model the polygenic nature of ASD [8, 36, 96-98].

Previous scaled use of assays to investigate ASD associated genes in *C. elegans* used strategies to prioritise genes before behavioural analysis [34-36]. The outcome of these studies resulted in an incomplete overlap of some genes investigated in our study. We made a comparison between *C. elegans* mutants that emerged from our study with an impaired social phenotype to mutants that have emerged from previous studies as having impaired movement and habituation phenotypes [34, 36]. However, the vast majority of mutants that

we identified with behavioural impairment are unique to this study. For example, *shn-1(gk181)* and *set-9(n4949)* show impaired social behaviour in response to progeny, whilst appearing grossly wild-type for the other phenotypes we tested. In addition these mutants do not show a behavioural phenotype in movement or habituation behaviour when investigated in previous studies [34, 36]. This highlights that the emergent behaviour we have used reveals genes that are missed when they emerge from the bioinformatic pipeline. This makes the case that applying a lower throughput observer based assay will refine previous efforts to model the functional impact of genes implicated in ASD.

The emergent behaviour that we have used is a complex, sensory integrative behaviour. Habituation learning is another complex behaviour in *C. elegans* that has been investigated in a previous screen of ASD associated genes [36]. Therefore, we wanted to identify whether there was overlap in mutants with behavioural impairment in two distinct complex behavioural phenotypes. We made a comparison of mutants that we had identified as having a social impairment to mutants that have been shown to have a habituation phenotype [36]. We identified four synaptic mutants, *nlg-1(ok259)*, *nrx-1(ds1)*, *glr-1(n2461)* and *nmr-2(ok3324)*, which have impaired social behaviour and have also been shown to have a habituation phenotype. This suggests that these genes may have a role in coordinating more than one complex sensory-regulated behaviour in *C. elegans*. This may also suggest that a key role of synaptic genes is in coordinating higher behaviours in *C. elegans*. With this in mind it would be interesting to extend the analysis of mutants with habituation impairment and screen them for social deficits. Our approach lends itself to the identification of complex behavioural deficits and so would be valuable in this analysis to further understand if there is an over-representation of synaptic genes in complex sensory integrative phenotypes.

In addition to highlighting the important contribution of synaptic, cell signalling and epigenetic genes, we identify a gene involved in phospholipid metabolism that appears to play a role in progeny-induced social behaviour. *mboa-7* is a lysoPI acetyltransferase which is important in the regulation of phospholipid membranes [99] and cell signalling [78] in *C. elegans*. In mammals, the regulation of membrane composition is important for cellular processes, signalling and nervous system function [100, 101]. Studies of the MBOAT7 ortholog in mice suggest it may function in brain development [102], however this gene is comparatively less well studied than other ASD associated genes for its functional contribution to the disorder. Therefore, the identification of this gene with a role in progeny-induced social behaviour highlights how this study enriches the understanding of the molecular determinants of social behaviour from underrepresented genes in autism.

In conclusion, investigation of ASD associated orthologues in *C. elegans* identified genes from a number of candidates implicated in ASD that disrupt social behaviour in the worm. Identification of these genes highlights how this assay might be used in quantitative approaches that can probe the single [39] and polygenic nature of ASD and its underpinning genetic architecture [36]. The robust nature of this assay provokes a better detailing of the cellular and circuit dependence of this social interaction. Guided by the cellular determinants of behaviour, investigation can extend to probe the polygenic nature of ASD and take a similar approach in other psychiatric diseases that have significant consequences for behavioural traits in the social domain [103, 104].

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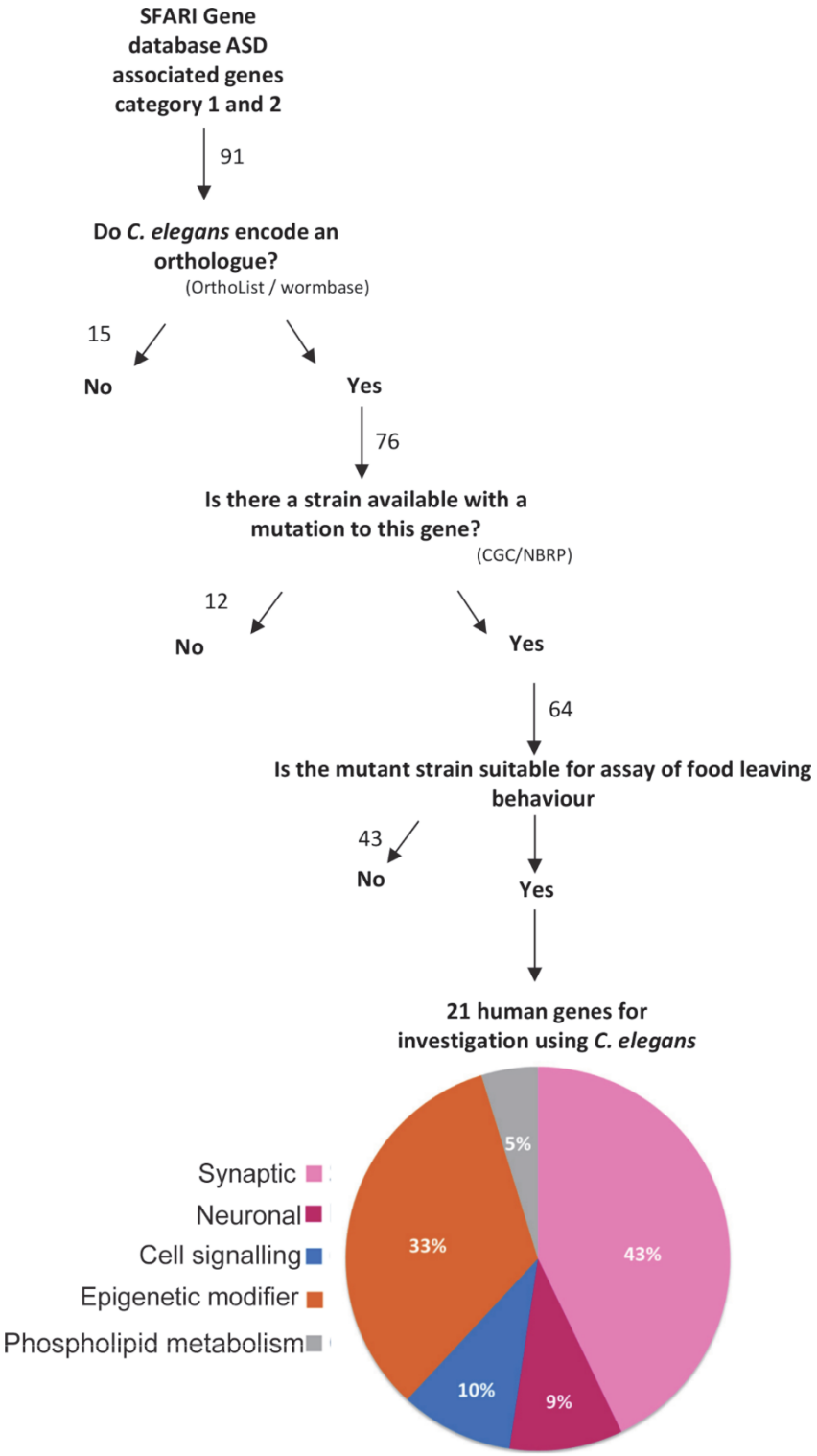


769

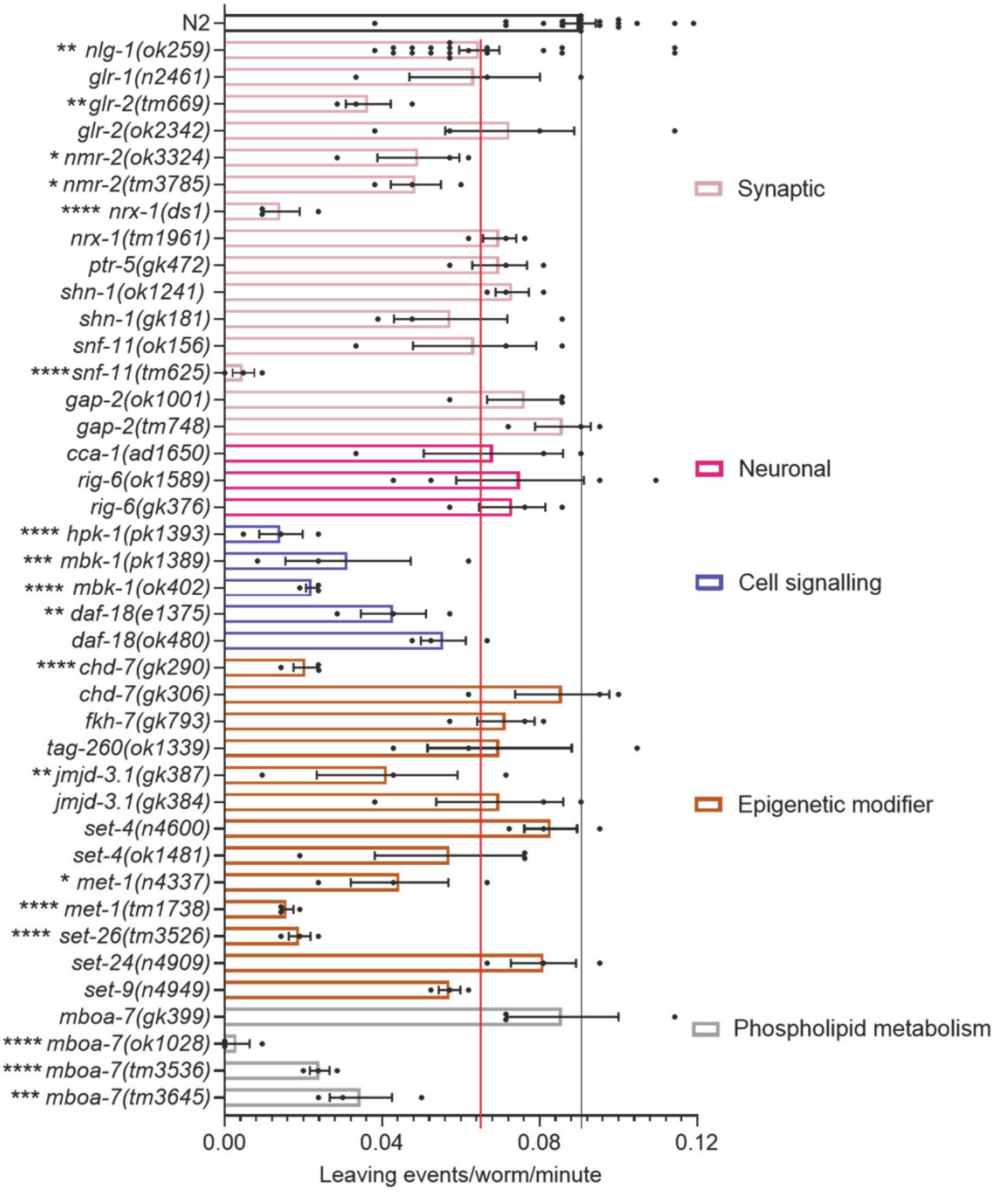
770 **Fig 1: Prioritisation and categorization of the *C. elegans* orthologues of prioritised human ASD**

771 **associated genes.** High confidence ASD associated genes in category 1 and 2 in SFARI Gene Archive

772 were input. The pipeline selects human genes which have an orthologue in *C. elegans* which can be

773 studied in an available mutant strain which is neither lethal, sterile or uncoordinated. In brackets are

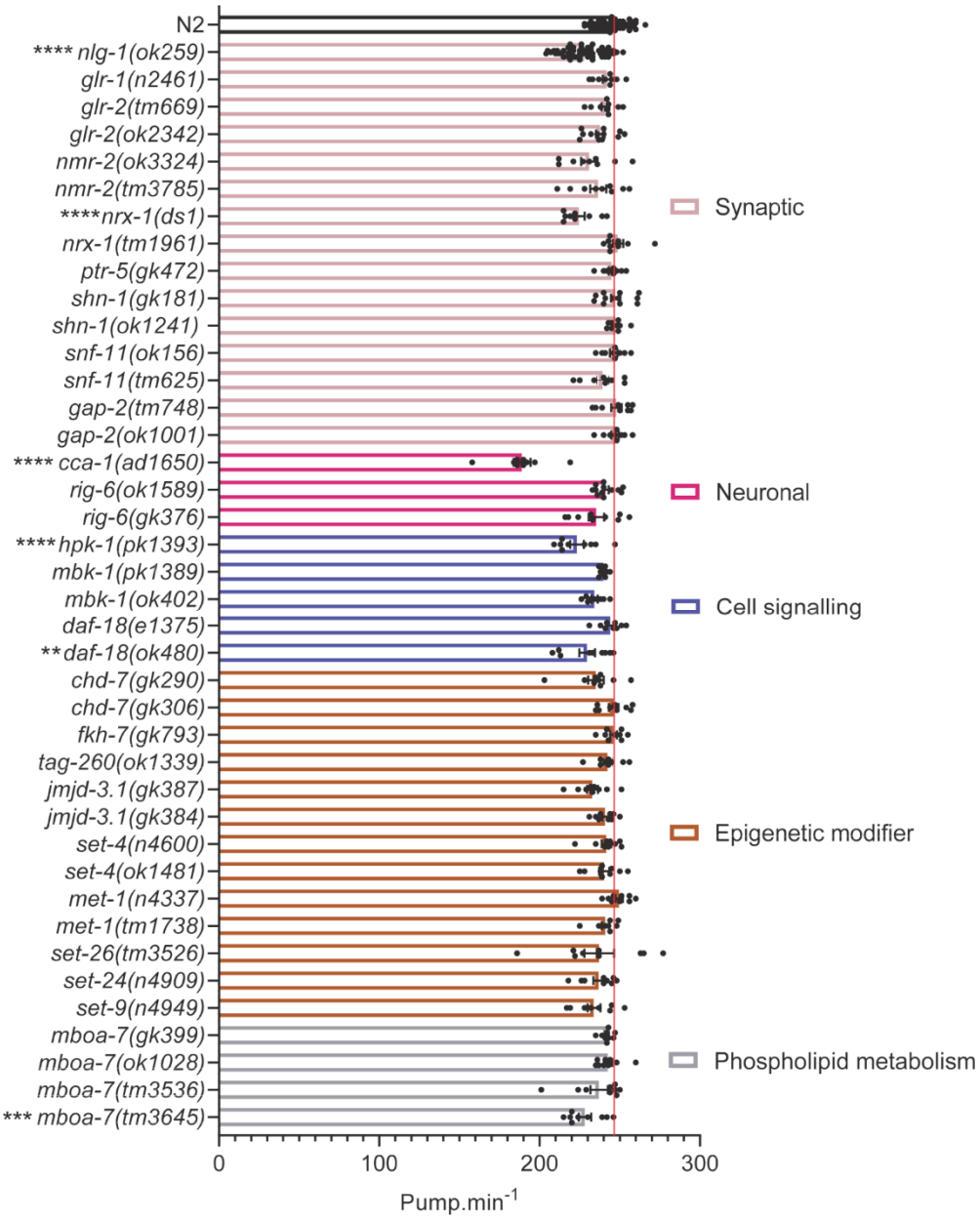
774 the resources used for analysis. CGC – *Caenorhabditis* Genetics Center. NBRP – National BioResource
775 Project. The number of genes analysed using SFARI Gene Archive (https://gene-archive.sfari.org/,
776 accessed October 2018) are stated. The pie chart indicates the percent of the 21 human genes that
777 were placed into five functional groupings.



778

779 **Fig 2: Food leaving behaviour of *C. elegans* mutants after 24 hours on food to investigate human**
780 **ASD associated genes.** A food leaving assay was performed with N2, *nlg-1(ok259)* and 39 other *C.*
781 *elegans* mutants. Genes are categorised and colour coded into different functional domains. The
782 black line indicates the number of leaving events/worm/minute for N2 control. The red line indicates

783 the food leaving rate of *nlg-1(ok259)* control. N2 and *nlg-1(ok259)* n=19. All other mutants n=3-4. All
784 data shown as mean ±SEM. Statistical analysis performed using a one-way ANOVA and Dunnetts's
785 multiple comparison test; ns, p>0.05; *, p<0.05; **, p p≤0.01; ***, p≤0.001; ****, p≤0.0001. All
786 significance relates to a comparison with N2 control.

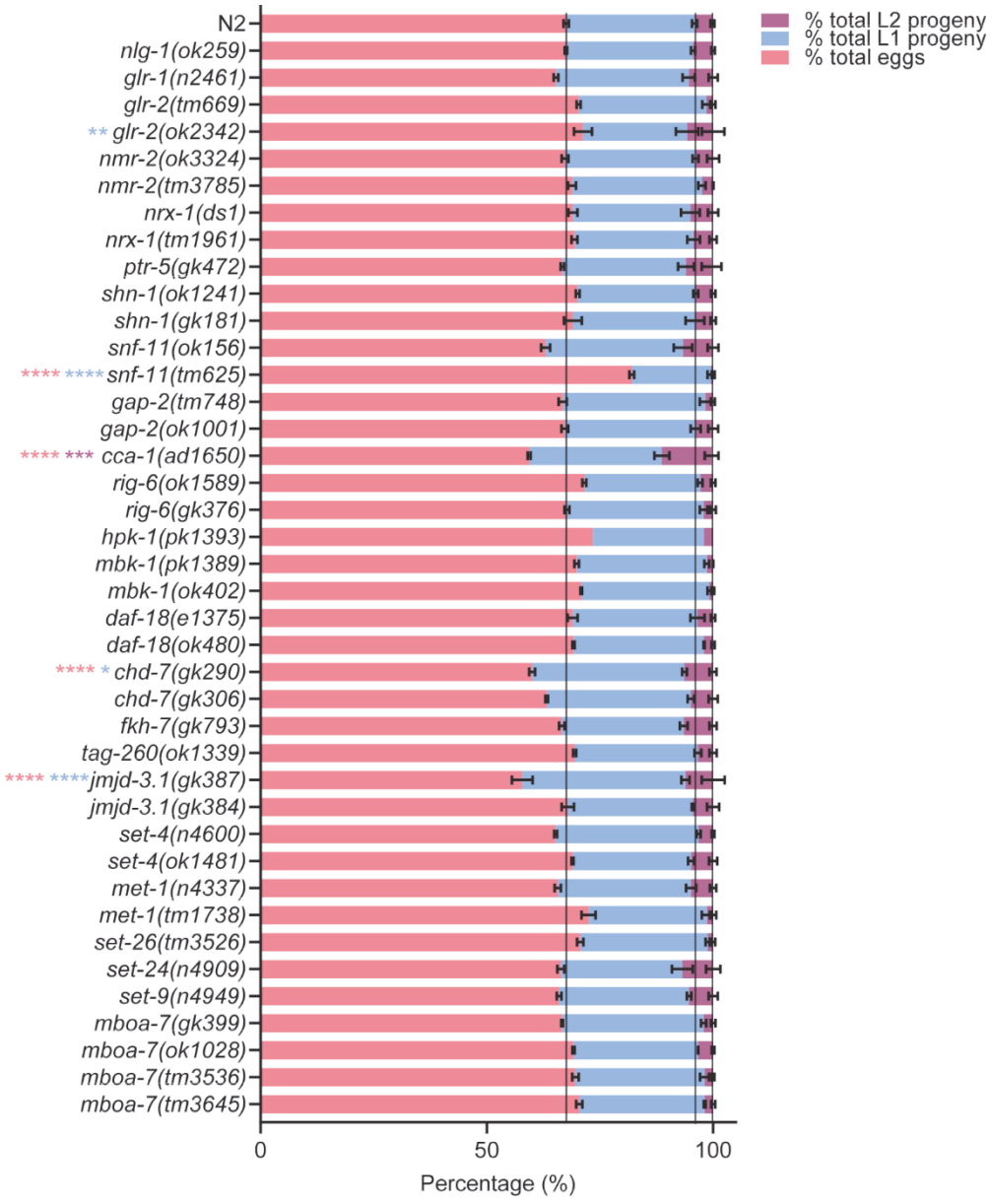


787

788 **Fig 3: Pharyngeal pump rate for *C. elegans* mutants.** After a food leaving assay at 24 hours, three
789 worms were chosen at random and their pharyngeal pump rate was counted per minute. N2 and
790 *nlg-1(ok259)* n=57. All other mutants n=9-12. The red line indicates pumps per minute for N2
791 control. All data shown as mean ±SEM. Statistical analysis performed using a one-way ANOVA and

792 Dunnetts's multiple comparison test; ns, p>0.05; *, p<0.05; **, p≤0.01; ***, p≤0.001; ****,

793 p≤0.0001. All significance relates to a comparison with N2 control.



794

795 **Fig 4: Percent total eggs and progeny produced by *C. elegans* mutants at 24 hours.** After a food

796 leaving assay from naïve food lawns occupied by 7 L4+1, the percent total offspring that were eggs,

797 L1 and L2 progeny were quantified. N2 and *nlg-1(ok259)* n=19. All other mutants n=3-4. The black

798 lines indicate % total eggs, % total L1 progeny and % total L2 progeny for N2 control. Pink asterisks

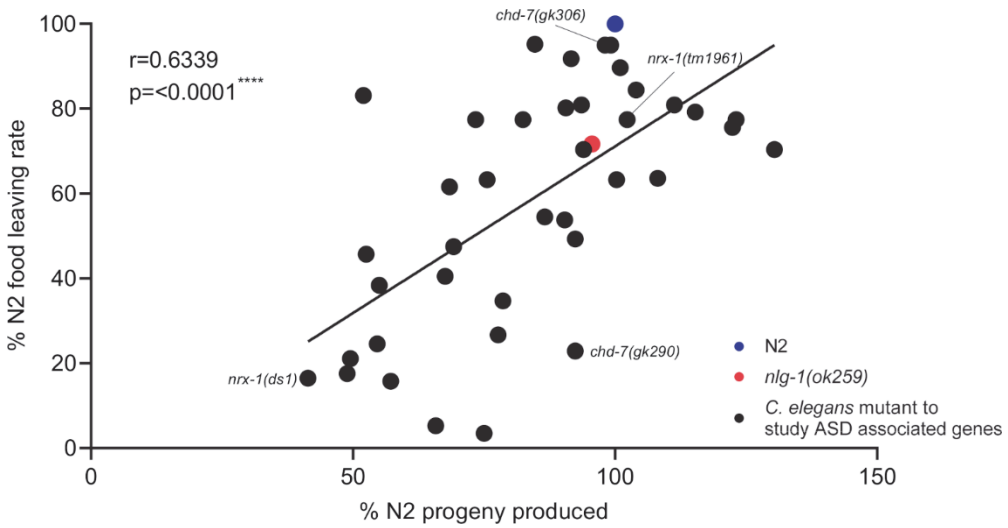
799 indicate statistical difference between mutant and N2 for % total eggs. Blue asterisks indicate

800 statistical difference between mutant and N2 for % total L1 progeny. Purple asterisks indicate

801 statistical difference between mutant and N2 for % total L2 progeny. All data shown as mean ±SEM.

802 Statistical analysis performed using a two-way ANOVA and Tukey's multiple comparison test; ns,

803 p>0.05; *, p<0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001.



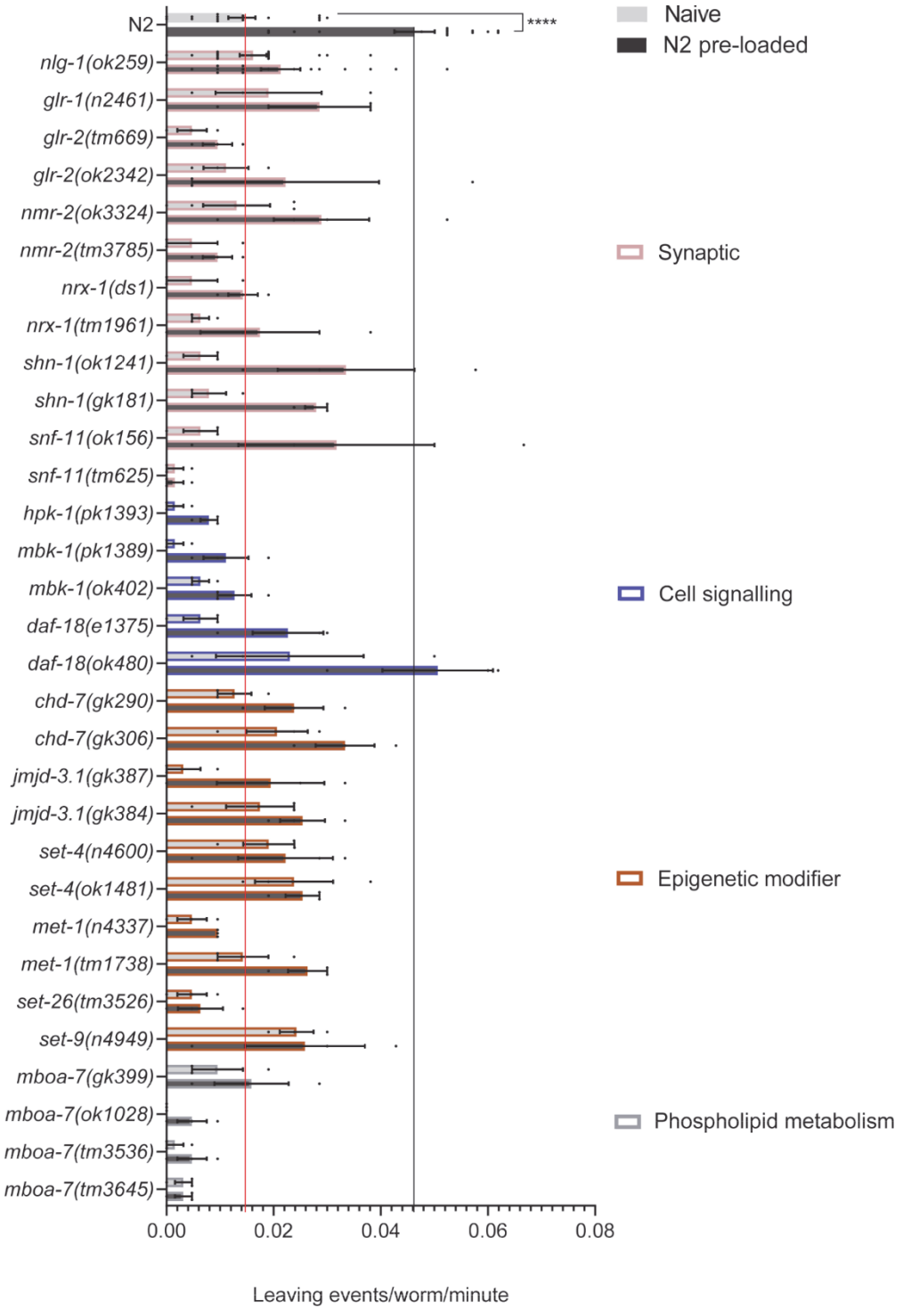
804

805 **Fig 5: Correlation between food leaving behaviour of *C. elegans* mutants and progeny production**

806 **during food leaving assay.** The percent food leaving rate and progeny produced for each *C. elegans*

807 mutant was calculated in comparison to N2. N2 and *nlg-1(ok259)* n=19. All other mutants n=3-4. All

808 data shown as mean. Statistical analysis performed using Pearson correlation coefficient.



809

810 **Fig 6: Food leaving behaviour of *C. elegans* mutants in the absence of progeny and exposure to N2**

811 **progeny.** A food leaving assay was performed with N2, *nlg-1(ok259)* and 30 other *C. elegans* mutants

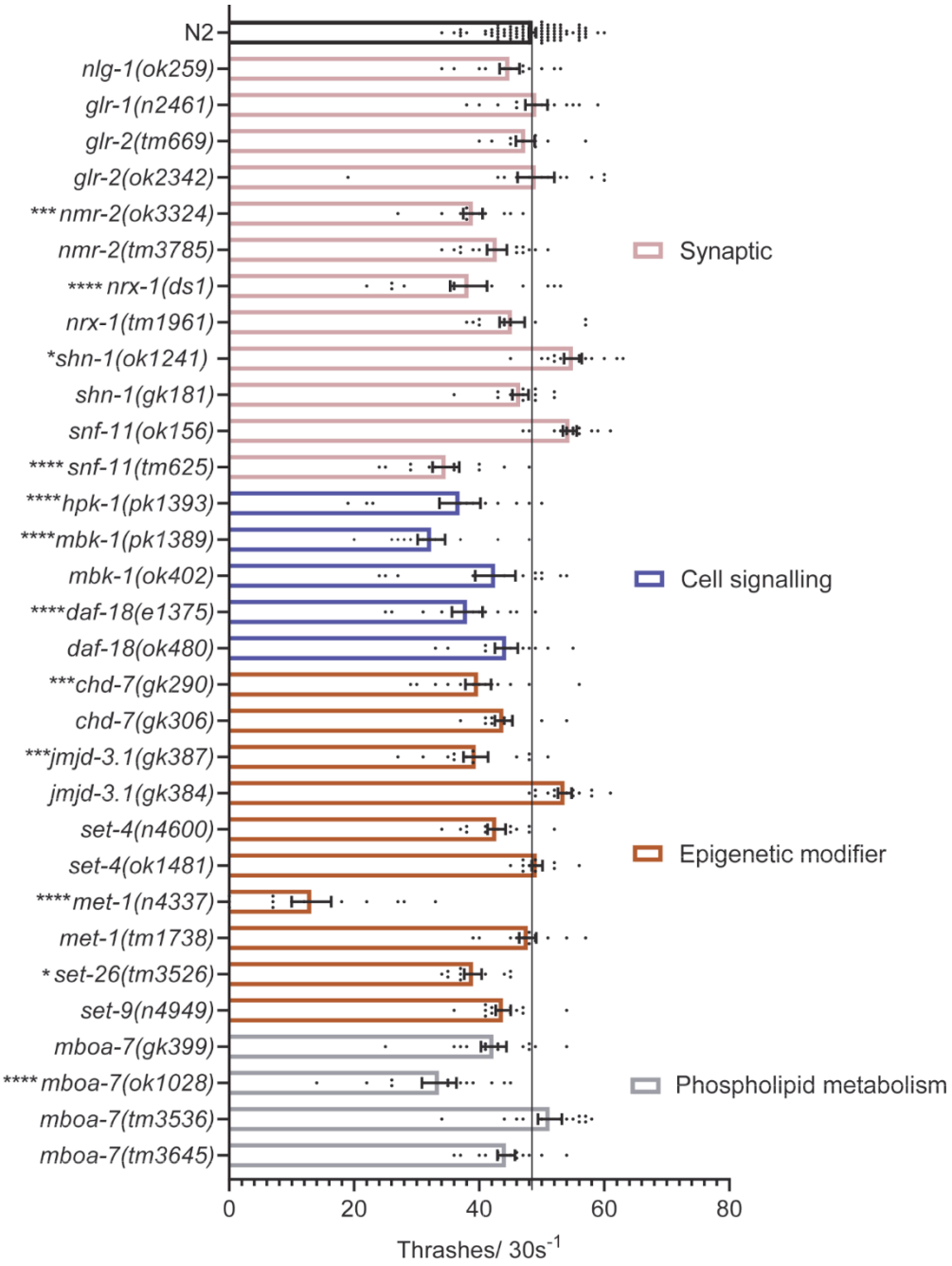
812 on naïve and pre-conditioned food lawns. A naïve lawn contains no progeny whereas a pre-

813 conditioned food lawn contains ~150-200 N2 progeny. The red line indicates the food leaving rate of

814 N2 naïve control. The black line indicates the food leaving rate of the N2 pre-loaded control. Data

815 shown as mean ±SEM. N2 and *nlg-1(ok259)* n=16. All other mutants n=3-4. Statistical analysis

816 performed using a two-way ANOVA and Sidak's multiple comparison test; ns, p>0.05; p ≤0.001****.



817

818 **Fig 7: Thrashing behaviour of *C. elegans* mutants compared to N2.** 5-10 minutes after being picked

819 into liquid medium, *C. elegans* thrashing behaviour was measured for 30 seconds per worm. The

820 black line indicates the thrashes/30s of N2 control. All data shown as mean ±SEM. N2 n=88. All other

821 mutants n=10-13. Statistical analysis performed using a one-way ANOVA and Dunnetts's multiple

822 comparison test; ns, p>0.05; *, p<0.05; ***, p≤0.001; ****, p≤0.0001. All significance relates to a

823 comparison with N2 control.

824