Title:

Integrative Cross-species Analyses Suggest Deficits in Habituation Learning as a Widely Affected Mechanism in Intellectual Disability and Autism Spectrum Disorders

Short title:

Habituation Deficits in ID and ASD Models

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Abstract:

Background: Although habituation is one of the most ancient and fundamental forms of learning, its regulators and relevance for human disease are poorly understood.

Methods: We manipulated the orthologs of 286 genes implicated in intellectual disability (ID) with or without comorbid autism spectrum disorder (ASD) specifically in *Drosophila* neurons, and tested these models in light-off jump habituation. We dissected neuronal substrates underlying the identified habituation deficits and integrated genotype-phenotype annotations, gene ontologies and interaction networks to determine the clinical features and molecular processes that are associated with habituation deficits.

Results: We identified more than 100 genes required for habituation learning. For the vast majority of these, 93 genes, a role in habituation learning was previously unknown. These genes characterize ID disorders with macrocephaly/overgrowth and comorbid ASD. Moreover, ASD individuals from the Simons Simplex Collection carrying disruptive *de novo* mutations in these genes exhibit increased rates of specific aberrant behaviors including stereotypic speech, hyperactivity and irritability. At the molecular level, ID genes required for normal habituation are enriched in synaptic function and converge on Ras-MAPK signaling. Both increased Ras-MAPK signaling in GABAergic and decreased Ras-MAPK signaling in cholinergic neurons specifically inhibit the adaptive habituation response.

Conclusions: Our work supports the relevance of habituation learning to autism, identifies an unprecedented number of novel habituation players, supports an emerging role for inhibitory neurons in habituation and reveals an opposing, circuit-level-based mechanism for Ras-MAPK signaling. This establishes habituation as a possible, widely applicable biomarker and target for pharmacologic intervention in ID/ASD.

85 **Introduction:**

86 Habituation is one of the most ancient and fundamental forms of learning, conserved across the animal
87 kingdom (1). It causes an organism's initial response to repeated meaningless stimuli to gradually
88 decline. Learning to ignore irrelevant stimuli as a result of habituation is thought to represent a filter
89 mechanism that prevents information overload, allowing for selective attention, thereby focusing
90 cognitive resources on relevant matters. Habituation learning has been proposed to represent an
91 important element and prerequisite for higher cognitive functions (2–4). In line with this, habituation
92 in infants correlates better than other measures with later cognitive abilities (5). Habituation has also
93 emerged as a proxy for synaptic plasticity (6). However, key players and molecular mechanisms
94 underlying habituation are poorly understood (7).

95 In humans, deficits in habituation have been reported in a number of neuropsychiatric and
96 behavioral disorders. In particular, defective cortical filtering of sensory stimuli and information
97 overload, as expected to arise from habituation deficits, are thought to represent a critical mechanism
98 underlying autism spectrum disorder (ASD) (8, 9). A decreased ability to habituate has been described
99 in a fraction of ASD individuals (10–12), but has not been connected yet to specific genetic defects,
100 with a single exception. Recently, two independent studies demonstrated habituation deficits in
101 patients with Fragile X syndrome, the most common monogenic cause of intellectual disability (ID) and
102 ASD (13, 14), confirming previously reported habituation deficits in Fmr1 KO mice (15, 16). Habituation
103 deficits have also been reported in a limited number of other ID or ASD (ID/ASD) disease models (17–
104 20).

105 Because assessing human gene function in habituation is challenging, we utilized a cross-
106 species approach. We apply light-off jump habituation in *Drosophila* to increase our knowledge on the
107 genetic control of habituation and, at the same time, to address the relevance of decreased
108 habituation in ID and in comorbid ASD disorders. Since ID is present in 70% of individuals with ASD
109 (21), monogenic causes of ID provide a unique molecular windows to ASD pathology (22). *Drosophila*
110 is a powerful, well-established model for ID (23–25) and offers genome-wide resources to study gene

111 function in large scale (26, 27). Several forms of habituation have been established in *Drosophila* (28–
112 32). Deficits in light-off jump habituation have already been reported in several ID models (24, 33–37)
113 and in classical learning and memory mutants (29, 32). Moreover, this form of habituation can be
114 assessed in a high-throughput manner. In the light-off jump paradigm, the initial jump response to
115 repeated light-off stimuli gradually wanes, as has been demonstrated not due to sensory adaptation
116 (a decrease in detecting the stimulus) or motor fatigue (a decrease in the ability to execute the
117 response) but as a result of learned adaptation of the startle circuit (32). This behavior meets all
118 habituation criteria (38), including spontaneous recovery and dishabituation with a novel stimulus (32,
119 39). The rich genetic toolbox of *Drosophila* allows for dissection of neuronal substrates and
120 mechanisms of light-off jump habituation.

121 Here, we use inducible RNA interference (RNAi) in *Drosophila* to systematically assess the role
122 of *Drosophila* orthologs of 286 genes that are well-established to cause ID in humans when mutated
123 (hereinafter referred to as ID genes). 69 of them (24%) have also been implicated in ASD (40, 41)
124 (hereinafter referred to as ID plus ASD-associated genes).

125

126 **Methods and Materials**

127 **Investigated ID genes**

128 A systematic source of ID genes and their *Drosophila* orthologs is available online (SysID database,
129 sysid.cmbi.umcn.nl (42)). We investigated the *Drosophila* orthologs of 286 human ID genes from the
130 SysID category primary ID genes (**Table S1**) (containing mutations with robust published evidence for
131 causality, see **Supplemental Methods (SM)**). SysID inclusion criteria and in/exclusion criteria of
132 experimentally investigated genes are indicated in the **SM**). In brief, the vast majority of genes are
133 from the first data freeze of the SysID database (all ID genes with proven causality, status of mid 2010).
134 Genes have been included based on conservation in *Drosophila*, available tools (RNAi) from large-scale
135 resources and viability as a prerequisite for behavioral testing. No selection was performed.

136

137 **Light-off jump habituation assay**

138 3- to 7-day-old flies were subjected to the light-off jump habituation paradigm in two independent 16-
139 unit light-off jump systems. After 5 min adaption, flies were simultaneously exposed to a series of 100
140 light-off pulses (15 ms) with 1 s interval. The noise amplitude of wing vibration during jump responses
141 was recorded. An appropriate threshold (0.8 mV) was applied to filter out background noise. Data were
142 collected by a custom-made Labview Software (National Instruments). Flies were considered as
143 habituated when not jumping in five consecutive light-off trials (no-jump criterion). Habituation was
144 quantified as the number of trials required to reach the no-jump criterion (Trials To Criterion (TTC)).

145

146 Information about the identification of *Drosophila* orthologs, proposed disease mechanism, *Drosophila*
147 stocks, phenotype reproducibility, validation of jump specificity, fatigue assay, quality criteria for RNAi
148 lines, annotation of ID plus ASD associated genes, enrichment analysis, comparison of behavior and
149 cognition in ASD SSC, molecular interaction network, clustering, physical interaction enrichment (PIE),
150 data visualization and statistics are described in the **SM**.

151

152 **Results:**

153

154 **Systematic identification of habituation deficits in *Drosophila* models of ID**

155 To identify novel genes implicated in habituation, we systematically investigated the role of 278
156 *Drosophila* orthologs representing 286 human ID genes in the light-off jump habituation paradigm. We
157 induced neuron-specific knockdowns of each ID gene ortholog by RNAi (26) using 513 RNAi lines
158 fulfilling previously established quality criteria (42, 43), with two independent constructs per gene
159 whenever available. These were crossed to the panneuronal elav-Gal4 driver line (see **SM**). Knockdown
160 is a suitable approach for modeling of the here-investigated human disease conditions since (partial)
161 loss of function is considered to be the underlying mechanism in the vast majority of these disorders
162 (43) (**Table S1**). Restricting gene knockdown to neurons eliminates potential effects on viability or
163 organismal fitness originating from an essential role of genes in other tissues and establishes neuron-
164 autonomous mechanisms.

165 Knockdown and control flies of identical genetic background were subjected to a series of 100
166 light-off stimuli, hereinafter referred to as trials, in the light-off jump habituation paradigm. The
167 screening procedure and paradigm allowed us to distinguish the following parameters: viability, initial
168 jump response (percentage of flies that jumped in at least one of the first five trials), and premature
169 and reduced habituation, with the latter representing the learning-defective phenotype category of
170 main interest. Genotypes with an initial jump response ≥50% but premature habituation were
171 subjected to a secondary assay to evaluate their organismal fitness and exclude fatigue as a
172 confounder of premature habituation (see **SM** and **Figure S3**). Based on these parameters, genes were
173 assigned to at least one of four phenotype categories (**Figure 1A**): (1) "not affected": (both) tested
174 RNAi lines targeting such genes were viable, showed good initial jump response, and had no significant
175 effect on habituation (based on the FDR-corrected p-value (p_{adj}), see **SM**); (2) "reduced organismal
176 fitness": at least one RNAi line led to lethality, poor jump response (<50% initial jumpers), or premature
177 habituation because of increased fatigue; (3) "habituation deficient": at least one RNAi line showed

178 good initial jump response but failed to suppress their response with the increasing number of light-
179 off trials (based on p_{adj}); and (4) "premature habituation": at least one RNAi line showed good initial
180 jump response followed by faster decline (based on p_{adj}), without fatigue being detectable in the
181 secondary assay. Still, this latter phenotype category can result from other defects than improved
182 habituation, and will be investigated elsewhere. In this study we focus on habituation deficits (3),
183 corresponding to the phenotype that has been shown in ID and ASD (10–14).

184 We validated the experimental approach to identify genes which, if manipulated, cause
185 habituation deficits (hereinafter referred to as habituation deficient genes) by recapitulating published
186 habituation deficits of *Drosophila* ID null mutant models *G9a* (24) and *Synapsin* (44), and of the classical
187 learning and memory mutant *dunce* (29, 45, 46) (**Figure 1B,C,D)**. This demonstrated that light-off jump
188 habituation upon RNAi can efficiently identify genetic regulators of habituation learning.

189 In our screen, we found that the *Drosophila* orthologs of 98 human ID genes (35% of all
190 investigated orthologs) are required, in neurons, for habituation learning. This phenotype represents
191 a highly specific defect in behavioral adaptation to the stimulus; flies keep on jumping in response to
192 the repetitive light-off stimulus, illustrating that they do not suffer from broad neuronal transmission
193 deficits (which would disable jumping), fatigue, sensory or other deficiencies. No excessive locomotion,
194 stimulus hypersensitivity or random jumping was observed (see **Figure S1, S2** for validation of light-off
195 jump habituation assay specificity). 27% of ID gene orthologs had no effect on habituation, 41% fell
196 into the category of "reduced organismal fitness", and 8% showed "premature habituation" without
197 detectable fatigue. The complete list of habituation screen results and distribution of human ID genes
198 in phenotype categories can be found in **Table S2, S3**. The screen thus identified nearly a hundred
199 orthologs of disease genes controlling habituation learning.

200

201 **Habituation deficits characterize ID genes with synaptic function**

202 We first asked whether genes characterized by habituation deficits in *Drosophila* converge on specific
203 biological process. ID genes are known to be enriched in a number of biological processes, but which

204 are important for habituation? Performing an enrichment analysis of ID-enriched Gene Ontology-
205 based (GO) categories (see **SM**) against the background of the investigated ID genes, we found that
206 "habituation deficient" genes are significantly enriched in a sole GO-based category: processes related
207 to the synapse (22/44 ID genes, E=1.59, p=0.024, **Figure 2**, **Table S4**). No enriched GO terms were found
208 in the "not affected" category. Together, our results support synaptic processes to be crucial for
209 habituation, as previously shown for other forms of this behavior (47, 48).
210
211 ***Drosophila* habituation deficits characterize ID genes associated with macrocephaly**
212 To understand whether habituation deficits in *Drosophila* represent a proxy of specific phenotypes in
213 human individuals, we performed enrichment analysis among ID-associated clinical features (42). We
214 found that orthologs of ID genes characterized by habituation deficits in *Drosophila* are specifically
215 enriched among ID genes associated with macrocephaly/overgrowth (**Figure 3A**, E=2.19, p=0.018,
216 **Table S4**). In contrast, ID genes characterized by the severe "reduced organismal fitness" phenotype
217 category show enrichment in different, severe ID-associated features such as endocrine, limb and eye
218 anomalies, brain malformations and obesity (**Figure S4**, **Table S4**). Moreover, ID genes not giving rise
219 to habituation deficits ("not affected" category) did not show any enrichment among ID-associated
220 clinical features (**Figure 3A**, **Table S4**).
221
222 **Habituation deficits characterize ID genes associated with ASD and deficits in specific ASD-relevant**
223 **behavioral domains**
224 There is a long-known relationship between macrocephaly and autism (49). For this reason and
225 because of the potential relevance of habituation deficits to ASD (10–12), we decided to further
226 investigate the relationship of *Drosophila* habituation and human ASD. We used the Simons Simplex
227 Collection (SSC) (41), a genetically and phenotypically well-characterized cohort of sporadic ASD
228 individuals. We matched genes with likely gene-disrupting (LGD) *de novo* mutations (50) in this ASD
229 cohort to those included in our experimental *Drosophila* habituation approach. 80 ASD individuals

230 carried mutations in 54 of the investigated genes (**Table S5**). We first asked whether these ID plus ASD-
231 associated genes preferentially fall into a specific *Drosophila* phenotype category. They are
232 significantly enriched among the genes that in *Drosophila* caused habituation deficits (**Figure 4A,**
233 E=1.57, p=0.024, **Table S4**, ASD SSC). Independently, significant enrichment was obtained for high-
234 confidence ID plus ASD-associated genes identified from the SFARI database (40) (38 investigated
235 genes, **Figure 4B,** E=1.65, p=0.016, **Table S4**, ASD SFARI), suggesting a relationship between *Drosophila*
236 habituation deficits and human ASD.

237 To further characterize the relationship between *Drosophila* habituation and human
238 phenotypes, we divided the SSC individuals into two distinct clusters based on their habituation
239 phenotype in the corresponding fly models: habituation deficits (N=38 individuals, 26 genes) and no
240 habituation deficits (N=21 individuals, 14 genes) (**Table S5;** another N=21 individuals, 14 genes fall into
241 the non-informative phenotype groups "reduced organismal fitness" or "premature habituation"). We
242 compared both groups across five broad quantitative measures of behavior and cognition: cognitive
243 ability (full-scale IQ); Social Responsiveness Scale (SRS); depression and anxiety (Child Behavior
244 Checklist Internalizing Disorders, CBCL-Int); impulsivity, attention and conduct (Child Behavior
245 Checklist Externalizing Disorders, CBCL-Ext); and atypical behavior (Aberrant Behavior Checklist, ABC).
246 There was no significant difference for IQ (p=0.57), SRS (p=0.24), CBCL-Int (p=0.10) or CBCL-Ext
247 (p=0.07), but a significant difference for ABC (p=0.006; **Figure 4C**, **Table S6)** withstanding multiple
248 testing correction (p<0.01). This effect is mainly driven by the ABC subdomains of inappropriate,
249 stereotypic speech (p=0.002), with a nominal contribution from the subdomains of irritability (p=0.02)
250 and hyperactivity (p=0.03) but not lethargy (p=0.14) or stereotypy (p=0.16) (**Table S6**). In summary,
251 these data indicate that habituation deficits in *Drosophila* are relevant to ASD-implicated genes. They
252 also suggest that SSC individuals carrying LGD mutations in genes associated with habituation deficits
253 in *Drosophila* show a higher rate and/or severity of atypical behaviors associated with stereotypic
254 speech, hyperactivity and irritability.
255

256

257 **Molecular networks and modules underlying habituation**

258 With the rich repertoire of nearly a hundred genes required for habituation that moreover show
259 specificity for ASD and synapse function, we set out to determine the molecular pathways these genes
260 are operating in. ID gene products are significantly interconnected via protein-protein interactions (51,
261 52). Consistent with previously published findings (42), ID genes investigated in our screen are 1.69
262 times enriched in interactions compared to 1000 randomly chosen protein sets of the same size and
263 number of known interactions (physical interaction enrichment (PIE) score (53) =1.69; $p<0.001$). To
264 identify biologically relevant modules, we resolved this network into communities with even tighter
265 interconnectivity using unsupervised community clustering (54). This analysis resulted in 26
266 communities containing 109 proteins (**Figure 5A, Table S7**). Their proximity and specificity for ID-
267 enriched GO-based processes are depicted in **Figure S5**. Mapping "habituation deficient" genes onto
268 the communities (**Figure 5A, red circles**) highlighted modules with high incidence of habituation
269 deficits (**Figure 5A**).

270

271 **A key role for ID and ASD-associated Ras signaling in habituation**

272 Five communities form a large, interconnected module with high incidences of habituation deficits.
273 However, the tightly interconnected hub at its center is characterized by the absence of habituation
274 deficits (**Figure 5A, square)**. This hub represents the key proteins of Ras-MAPK signaling (**Figure 5B**).
275 This pathway, best known for its role in cancer, underlies a group of disorders collectively referred as
276 Rasopathies. Importantly, while 92% of the modeled ID disorders are thought to result from loss of
277 function of the underlying genes, Rasopathies are caused by gain-of-function mutations in the core
278 pathway (**Figure 5C, Table S1**). The RNAi approach used in this screen, despite addressing gene
279 function, did thus not recapitulate the molecular pathology of these specific cognitive disorders.
280 However, Rasopathies can also result from loss of function in negative regulators of the pathway. We
281 therefore asked whether exactly the same genetic mechanisms that cause Rasopathies in humans also

282 hold true for habituation deficits in *Drosophila*. In our screen, we tested habituation of two negative
283 regulators of Ras: NF1 (*Drosophila* Nf1) (55) and SPRED1 (*Drosophila* Spred) (56, 57), and of CASK
284 (*Drosophila* CASK), a scaffold protein that has been suggested to facilitate localization and function of
285 NF1 (58–60). Panneuronal knockdown of either regulator caused strong habituation deficits (**Figure**
286 **5D, in red**). We therefore tested a constitutively active *Ras* mutant, $Ras1^{R68Q}$ (61). Heterozygous
287 $Ras1^{R68Q}$ flies showed strong habituation deficits compared to the control flies with the same genetic
288 background ($p=3.56x10^{-9}$; **Figure 5D, in green**). The same was true when we overexpressed, specifically
289 in neurons, the same mutant *Ras* allele from an inducible transgenic construct ($p=1.96x10^{-6}$; **Figure 5D,**
290 **in green**). We conclude that increased activity of Ras, causing Rasopathies and associated cognitive
291 deficits in humans, causes habituation deficits in *Drosophila*.
292
293 **Habituation-inhibiting function of increased Ras-MAPK signaling maps to inhibitory/GABAergic**
294 **neurons**
295 We next aimed to identify in which type of neurons the habituation-inhibiting function of Ras-MAPK
296 signaling resides. Because the well-characterized neurons of the giant fiber circuit controlling the light-
297 off jump response are cholinergic (62), just as the majority of excitatory neurons in *Drosophila*, we first
298 tested whether increased Ras-MAPK signaling activity would induce habituation deficits when directed
299 to cholinergic neurons. For this, we adopted the knockdown of negative Ras regulators (*Nf1*, *Spred* and
300 *CASK*), expressed constitutively active *Ras1* ($Ras1^{R68Q}$), and tested expression of a gain-of-function
301 allele of *Raf* (Raf^{GOF}), a downstream mediator of Ras signaling. None of these, when driven by the
302 cholinergic Cha-Gal4 driver, recapitulated the panneuronally evoked habituation deficits (**Figure 6A**).
303 Because of the recently established role of GABAergic neurons in *Drosophila* olfactory and
304 proboscis extension reflex habituation (30, 63, 64) and the emerging importance of GABA inhibition in
305 autism (65), we next targeted GABA neurons using the Gad1-Gal4 driver and the same toolbox. This
306 consistently induced habituation deficits in all tested conditions (**Figure 6B**), recapitulating the

307 observed panneuronally evoked deficits. We conclude that the habituation-inhibiting function of
308 increased Ras-MAPK signaling maps to GABAergic neurons.
309
310 **Ras-MAPK signaling in cholinergic neurons is essential for habituation learning**
311 Impaired jump response/increased fatigue associated with *Ras, Raf* and *Mek* knockdown in the screen
312 could potentially mask an essential role for Ras signaling in habituation, in addition to the habituation-
313 inhibiting function of increased Ras-MAPK signaling. In fact, our screen also identified habituation
314 deficits upon RNAI of the positive Ras-MAPK regulators *Sos* and *Csw*We therefore downregulated Ras-
315 MAPK activity by crossing the UAS-based RNAi lines targeting *Sos* and *Csw*, but also RNAi lines targeting
316 *Ras*, *Raf* and *Mek,* to the GABAergic driver Gad1-Gal4. We did not observe any detrimental effect on
317 habituation (**Figure 6D**). In contrast, downregulating Ras-MAPK signaling in cholinergic neurons
318 consistently prevented normal habituation learning (**Figure 6C**). We conclude that Ras-MAPK signaling
319 is essential in cholinergic but not in GABAergic neurons. Thus, Ras-MAPK signaling plays a dual,
320 opposing role in inhibitory versus excitatory neurons in habituation learning.
321

322 **Discussion:**

323 ***Drosophila* screen suggests deficits in habituation learning as a widely affected mechanism in autism**

324 To systematically address the genetic basis of habituation deficits associated with neurodevelopmental
325 disorders, we investigated 286 ID genes with a clear *Drosophila* ortholog in light-off jump habituation.
326 Panneuronal knockdown of the orthologs of 98 ID genes specifically suppressed the adaptive
327 habituation response to repeated stimulation without affecting organismal health or fitness. Follow-
328 up work on the Ras-MAPK pathway raised this number to 104. 93 of these are novel regulators of
329 habituation, substantially exceeding the sum of previously known regulators of habituation across
330 species and paradigms. Stringent criteria for RNAi specificity and correction for multiple testing (see
331 **SM**) in our experiments ensured a minimal level of potential false positive discoveries. Of thirteen
332 previously identified ID genes with habituation deficits, our screen confirmed ten (**Table S8**). Our
333 approach and data, although based on experiments in another species, suggest that deficits in
334 habituation learning are a widely affected mechanism in ID. Habituation deficits might be a hallmark
335 of even more ID genes than determined here. In particular, the phenotype category of "reduced
336 organismal fitness" is likely to contain genes with promiscuous functions masking a specific role in
337 habituation learning.

338 Enrichment analysis of ID-associated clinical features revealed that "habituation deficient" ID
339 genes are preferentially characterized by macrocephaly/overgrowth, associated for long with ASD (49).
340 Strikingly, we found that mutations in genes associated with *Drosophila* habituation deficits are
341 significantly overrepresented among ID genes that are also implicated in ASD (53% (SFARI database);
342 48% (SSC cohort)). In comparison the frequency of habituation deficits among ID genes not associated
343 with ASD is 30%. SSC individuals carrying mutations in these genes show a high rate and/or severity of
344 aberrant behaviors associated with stereotypic speech, hyperactivity and irritability. Habituation
345 deficits thus represent a common phenotypic signature of ASD in *Drosophila* and highlight specific
346 behavioral subdomains affected in ASD.

347

348 **Synapse-related processes and Ras-MAPK signaling play a key role in habituation**

349 Synapse biology has been proposed to play a central role in ASD (66). Our data show that among the
350 investigated disease genes, "habituation deficient" genes are specifically enriched in genes with
351 synaptic function. This is in line with habituation representing a measurable form of synaptic plasticity
352 (8, 48).

353 Analyzing the distribution of "habituation deficient" genes in ID-specific molecular interaction
354 networks, we discovered that they accumulate in a multiple-community module and connect to the
355 Ras-MAPK pathway core proteins Ras, Raf and Mek (**Figure 5A,B**). We observed habituation deficits
356 upon panneuronal knockdown of Ras negative regulators and panneuronal expression of the
357 constitutively active *Ras* allele $Ras1^{R68Q}$ (**Figure 5C**), demonstrating that increased Ras-mediated
358 signaling causes habituation deficits. Moreover, proteins encoded by "habituation deficient" genes
359 form a significantly interconnected module (**Figure 7**). The coherence of this module further supports
360 the validity of the chosen RNAi approach to identify genes and molecular processes regulating
361 habituation learning. The module contains a number of synaptic proteins (**Figure 7**) with not yet
362 investigated roles in Ras signaling. It would be interesting to determine whether some of these enlarge
363 the spectrum of diseases caused by deregulated Ras signaling.

364

365 **Ras-MAPK signaling exerts a dual but opposing role in inhibitory versus excitatory neurons, a novel**
366 **systems-level mechanism**

367 Identification of neuronal substrates in which specific ID genes are required to warrant habituation
368 learning is important fundamental problem. Restoring the function of affected neurons might also
369 represent a suitable treatment strategy. The light-off jump startle circuit of *Drosophila* is relatively
370 simple and its cholinergic nature is well described (62). However, it is not known how habituation of
371 this circuit is regulated. The commonly accepted view regards synaptic depression in excitatory
372 neurons, induced by repetitive stimulation, as the underlying mechanism (47, 67). This has recently
373 been challenged by Ramaswami and colleagues who showed that plasticity of inhibitory, GABAergic

374 neurons drives two non-startle types of habituation (63, 64). We found that increased activity of our
375 identified key pathway, Ras-MAPK, in GABAergic but not in cholinergic neurons causes deficits in light-
376 off jump habituation. Our results thus support inhibitory circuits as crucial components of habituation
377 learning across different paradigms and sensory modalities. Further experiments are needed to
378 establish the direct involvement of GABAergic signaling. At the same time, we identified that also
379 decreased Ras-MAPK signaling activity can lead to habituation deficits. Yet, the neuronal substrates of
380 these deficits are different and map to excitatory, cholinergic neurons. Although our experiments do
381 not distinguish between developmental effects and acute circuit plasticity, the opposing role for Ras-
382 MAPK signaling on habituation may provide new insights into mechanisms of neural plasticity in health
383 and disease. It may also have crucial implications for treatment of Rasopathies. Future clinical trials, as
384 opposed to those that broadly decreased Ras activity and failed (68), may need more attention towards
385 restoring circuit function and balance.
386
387 **Translational value and application of cross-species habituation measures for diagnosis and**
388 **treatment of ID and ASD**
389 Based on our findings that habituation is widely affected in *Drosophila* models of ID, and that
390 habituation deficits are particularly common among genes also implicated in ASD, we propose that
391 disrupted habituation may contribute to ID/ASD pathology.
392 The emerging importance of inhibitory inputs for habituation ((30, 63) and this study) and
393 sensory information filtering in the cortical centers of the brain (69, 70) suggests the existence of an
394 overarching circuit-based mechanism responsible for prevention of inappropriate behavioral
395 responses (8). Though our findings that habituation deficits in *Drosophila* correlate with increased rate
396 and/or severity of atypical ASD-related behaviors compared to ID genes without habituation deficits
397 should be replicated, we speculate that disrupted habituation arising from GABAergic defects may
398 contribute to these ASD features. As such, it has potential to serve as a cross-species, mechanism-
399 specific functional readout—a pressing need for efficient personalized (pharmacological) treatment in

400 the field of neurodevelopmental disorders. Implementing suitable low-burden protocols for
401 habituation measures in clinical research and diagnostics of ID/ASD, such as those developed for
402 investigation of habituation deficits in Fragile X syndrome (13), will help to further delineate the
403 affected cognitive domains that arise from deficient habituation. In future clinical trials, these could
404 serve as objective and quantitative readouts for patient stratification in mechanism-based treatment
405 strategies and for monitoring of drug efficacy. Dissection of the underlying defective mechanisms in
406 *Drosophila* can at the same time identify novel targets for treatment, with high-throughput light-off
407 jump habituation serving as a translational pipeline for drug testing.
408

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434

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579

580

581 **Figure legends:**

582 **Figure 1. Habituation screen of intellectual disability genes, phenotype distribution and proof of**
583 **principle**

584 (A) Procedure, phenotype categories and phenotype distribution of the light-off jump habituation
585 screen. Knockdowns that resulted in lethality, no jumper phenotype (defined as less than 50% flies
586 jumping in at least one of the first five light-off trials) or premature habituation plus increased fatigue
587 were assigned to the category "reduced organismal fitness" and their habituation was not further
588 analyzed. Other phenotype categories are "habituation deficient", "not affected", and "premature
589 habituation" (the latter if no fatigue was detected in secondary assay, see example in **Figure S3**).
590 *Drosophila* orthologs of 34% of the investigated human ID genes were associated with defects in
591 habituation learning. See also **Table S2, S3**. (B, C, D) Defective habituation upon neuron-specific RNAi-
592 mediated knockdown of *G9a*, *Synapsin (syn)*, and *dunce (dnc)* (*2xGMR-wIR/+; UAS-RNAi/elav-Gal4,*
593 *UAS-Dicer-2*, in red) compared to their respective genetic background controls (*2xGMR-wIR/+; elav-*
594 *Gal4, UAS-Dicer-2/+*, in gray). Jump response curves show the average jump response (% of jumping
595 flies) over 100 light-off trials at 1 s inter-trial interval). Mean TTC: the mean number of trials that flies
596 needed to reach the no-jump criterion (see **Methods and Materials**) presented as Mean TTC ± SEM.
597 *** p_{adj}<0.001, ** p_{adj}<0.01, based on FDR-corrected lm analysis. A complete list of ID genes with
598 previously identified habituation defects is provided as **Table S8**, adding further proof of principle.

599

600 **Figure 2. Habituation defects in *Drosophila* characterize ID genes with synapse-related functions**

601 Of 25 gene ontology (GO)-based processes, "habituation deficient" genes are specifically and
602 significantly enriched in processes related to synapse (E=1.59, p=0.024). Genes with no effect on
603 habituation do not show significant enrichment in any GO process. * p<0.05, based on Fisher's exact
604 test. All enrichment scores, p-values and enriched genes are listed in **Table S4**.

605

606 **Figure 3. Habituation defects in *Drosophila* characterize ID genes associated with macrocephaly in**
607 **humans**
608 Enrichment of *Drosophila* phenotype categories across 27 ID-accompanying clinical features (42).
609 "Habituation deficient" genes show specificity for macrocephaly and/or overgrowth (E=2.19, p=0.018)
610 ** p<0.01, * p<0.05, based on Fisher's Exact test. For enrichment of "reduced organismal fitness"
611 category, see **Figure S4**. Enrichment scores, p-values and enriched genes are listed in **Table S4**.
612
613 **Figure 4. Habituation defects in *Drosophila* characterize ID genes associated with ASD and deficits in**
614 **specific behavioral domains**
615 (A,B) Enrichment of *Drosophila* phenotype categories "habituation deficient" and "not affected" in ID
616 plus ASD-associated genes identified in SFARI database (ASD SFARI, E=1.65, p=0.016, (A)) and SSC
617 cohort (ASD SSC, E=1.57, p=0.024 (B)). Circles represent total number of tested ID plus ASD-associated
618 genes. (C) Genes associated with "habituation deficient" versus "not affected" phenotype categories
619 in *Drosophila* show significantly more aberrant behaviors on the ABC (F(1,62)=8.21, p=0.006) in the
620 ASD SSC cohort. Data presented as mean score ± SEM. ** p<0.01, based on MANOVA. See also **Table**
621 **S5** (list of ASD SFARI and ASD SSC genes) and **Table S6** (complete MANOVA results).
622
623
624 **Figure 5. A central role for Ras-MAPK signaling in habituation learning**
625 (A) Highly connected communities identified by unbiased community clustering, colored by their
626 functional proximity (**Figure S5**). Red circles and gene names highlight nodes representing "habituation
627 deficient" genes. For complete list of communities and genes see **Table S7**. (B) Nodes connecting four
628 communities from the central module represent the core components of Ras-MAPK signaling. (C)
629 Schematic representation of Ras-MAPK signaling and associated mechanisms in ID disorders called
630 'Rasopathies'. (D) Increasing Ras signaling by inducing either loss of function of negative Ras regulators
631 (left side of pathway scheme) or by constitutively activating Ras (right side) disrupts habituation

632 learning. Left: Defective habituation upon neuron-specific knockdown of negative Ras regulators, *Nf1*
633 (*2xGMR-wIR/+; Nf1-RNAi*^{vdrc35877}*/elav-Gal4, UAS-Dicer-2*, N=72, in red), *Spred* (*2xGMR-wIR/+; Spred-*
634 *RNAi*^{vdrc18024}*/elav-Gal4, UAS-Dicer-2*, N=73, in red), and *CASK* (*2xGMR-wIR/+; CASK-RNAi*^{vdrc34184}*/elav-*
635 *Gal4, UAS-Dicer-2*, N=75, in red) compared to their corresponding genetic background controls
636 (*2xGMR-wIR/+; elav-Gal4, UAS-Dicer-2/+*. N: 55, 20, 25, in gray). *** p_{adj}<0.001, based on lm analysis
637 and FDR correction in the screen (see **Methods and Materials**). Right: Defects in habituation learning
638 in a heterozygous, constitutively active *Ras* mutant (*Ras1*$^{R68Q/+}$, N=55, in green) compared to its genetic
639 background control (N=43 in gray), and upon neuron-specific expression of *Ras1*^{R68Q} (*elav>Ras1*^{R68Q}:
640 *UAS-Ras1*^{R68Q}*/2xGMR-wIR; elav-Gal4, UAS-Dicer-2/+*, N=52, in green) compared to its genetic
641 background control (*2xGMR-wIR/+; elav-Gal4, UAS-Dicer-2/+*, N=34, in gray). *** p<0.001, based on
642 lm analysis. Data presented as Mean TTC ± SEM.
643
644 **Figure 6. Dual, opposing role of Ras-MAPK signaling in GABAergic and cholinergic neurons in the**
645 **regulation of habituation learning**
646 (A) No effect on habituation of *Ras1*^{R68Q} (N=51, in green), *Nf1-RNAi* (N=38, in red), *Spred1-RNAi* (N=55,
647 in red) and *CASK-RNAi* (N=39, in red) upon expression in cholinergic neurons compared to their
648 respective genetic background controls (*Cha-Gal4/+*; *2xGMR-wIR/+*, N: 54, 45, 54, 52, in gray).
649 Expression of *Raf*^{GOF} in cholinergic neurons resulted in lethality. (B) Defective habituation of *Ras1*^{R68Q}
650 (N=52, in green), *Raf*^{GOF} (N=57, in green), *Nf1-RNAi* (N=55, in red), *Spred1-RNAi* (N=37, in red) and *CASK-*
651 *RNAi* (N=54, in red) on habituation upon expression in GABAergic neurons compared to their
652 respective genetic background controls (*Gad1-Gal4/+*; *2xGMR-wIR/+*, N: 50, 50, 39, 58, 46, in gray). (C)
653 Defective habituation of *Csw-RNAi* (*UAS-Csw-RNAi*^{vdrc21756}*/Y; Cha-Gal4/+*; *2xGMR-wIR/+*, N=58), *Sos1-*
654 *RNAi* (*UAS-Sos1-RNAi*^{vdrc42848}*/Cha-Gal4; 2xGMR-wIR/+*, N=56), *Ras1-RNAi* (*UAS-Ras1-RNAi*^{vdrc106642}*/Cha-*
655 *Gal4; 2xGMR-wIR/+*, N=55), *Raf-RNAi* (*UAS-Raf-RNAi*^{vdrc20909}*/Cha-Gal4; 2xGMR-wIR/+*, N=59) and *Mek-*
656 *RNAi* (*Cha-Gal4/+; UAS-Mek-RNAi*^{vdrc40026}*/2xGMR-wIR*, N=58) in cholinergic neurons (in green)
657 compared to their respective genetic background controls (*Cha-Gal4/+*; *2xGMR-wIR/+*, N: 62, 54, 34,

658 46, 46, in gray). (D) No effect on habituation of *Csw-RNAi* (N=58), *Sos1-RNAi* (N=51), *Ras1-RNAi* (N=53),
659 *Raf-RNAi* (N=52) and *Mek-RNAi* (N=54) in GABAergic neurons (in green) compared to their respective
660 genetic background controls (*Gad1-Gal4/+*; *2xGMR-wIR/+*, N: 60, 46, 54, 39, 39, in gray). Data
661 presented as Mean TTC ± SEM. *** p<0.001, ** p<0.01, * p<0.05, based on lm analysis.
662
663 **Figure 7. Connections between "habituation deficient" genes**
664 Connections between "habituation deficient" genes, including *Ras*, identified in the reference network
665 used for community clustering (See **SM**) with significantly increased connectivity (PIE score =1.89,
666 p<0.001). Nodes are colored based on the community, to which they belong. Nodes that represent
667 "habituation deficient" genes but are not members of a community are labeled in black.

Figure 1

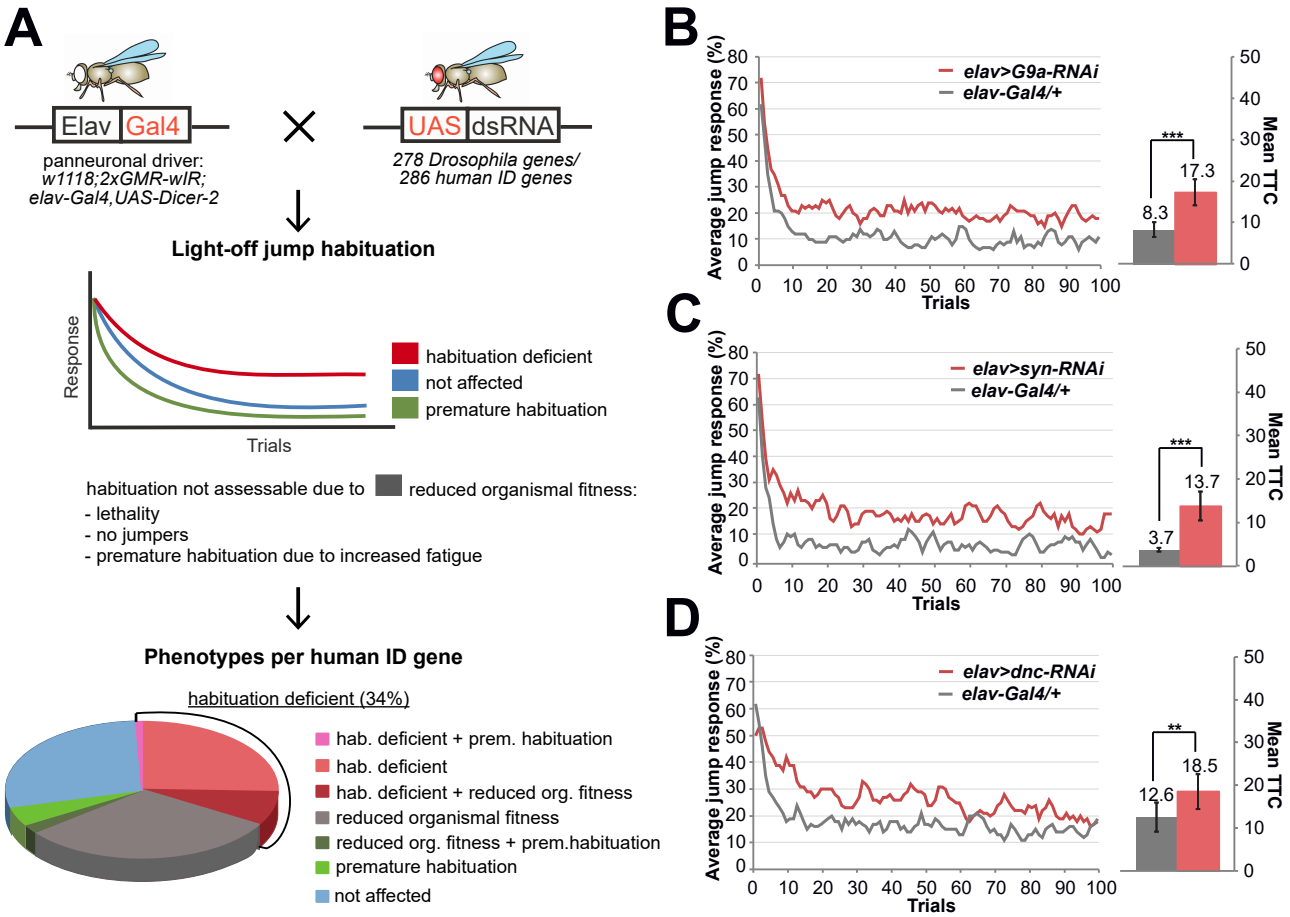

A
Elav
Gal4
panneuronal driver:
w1118;2xGMR-wIR;
elav-Gal4,UAS-Dicer-2
UAS
dsRNA
278 Drosophila genes/
286 human ID genes
Light-off jump habituation
Response
Trials
habituation deficient
not affected
premature habituation
habituation not assessable due to
reduced organismal fitness:
- lethality
- no jumpers
- premature habituation due to increased fatigue
Phenotypes per human ID gene
habituation deficient (34%)
hab. deficient + prem. habituation
hab. deficient
hab. deficient + reduced org. fitness
reduced organismal fitness
reduced org. fitness + prem.habituation
premature habituation
not affected
B
elav>G9a-RNAi
elav-Gal4/+
Average jump response (%)
Trials
Mean TTC
***
8.3
17.3
C
elav>syn-RNAi
elav-Gal4/+
***
3.7
13.7
D
elav>dnc-RNAi
elav-Gal4/+
**
12.6
18.5

Figure 2

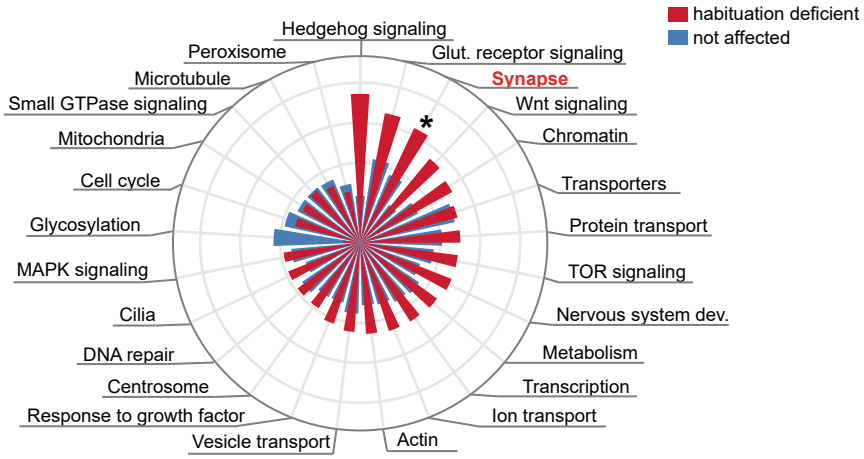

habituation deficient
not affected
Hedgehog signaling
Glut. receptor signaling
Synapse
Wnt signaling
Chromatin
Transporters
Protein transport
TOR signaling
Nervous system dev.
Metabolism
Transcription
Ion transport
Actin
Vesicle transport
Response to growth factor
Centrosome
DNA repair
Cilia
MAPK signaling
Glycosylation
Cell cycle
Mitochondria
Small GTPase signaling
Microtubule
Peroxisome
*

Figure 3

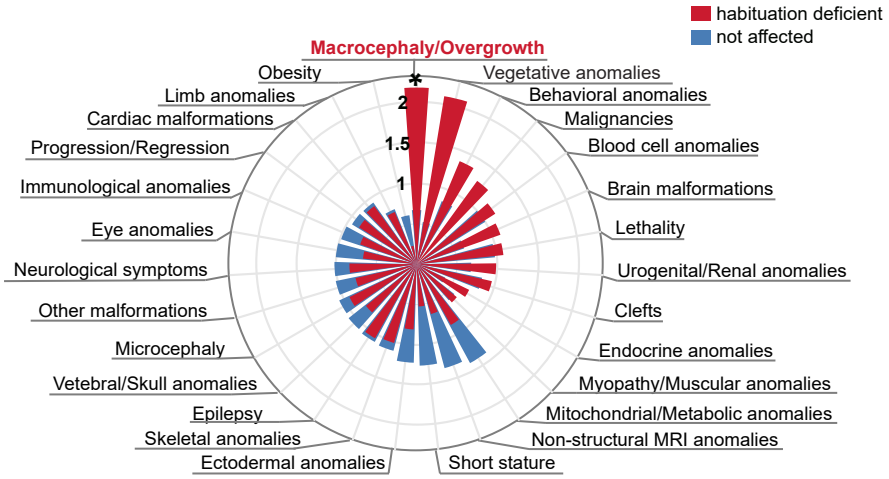

habituation deficient
not affected
Macrocephaly/Overgrowth
Vegetative anomalies
Behavioral anomalies
Malignancies
Blood cell anomalies
Brain malformations
Lethality
Urogenital/Renal anomalies
Clefts
Endocrine anomalies
Myopathy/Muscular anomalies
Mitochondrial/Metabolic anomalies
Non-structural MRI anomalies
Short stature
Ectodermal anomalies
Skeletal anomalies
Epilepsy
Vetebral/Skull anomalies
Microcephaly
Other malformations
Neurological symptoms
Eye anomalies
Immunological anomalies
Progression/Regression
Cardiac malformations
Limb anomalies
Obesity
*
2
1.5
1

Figure 4

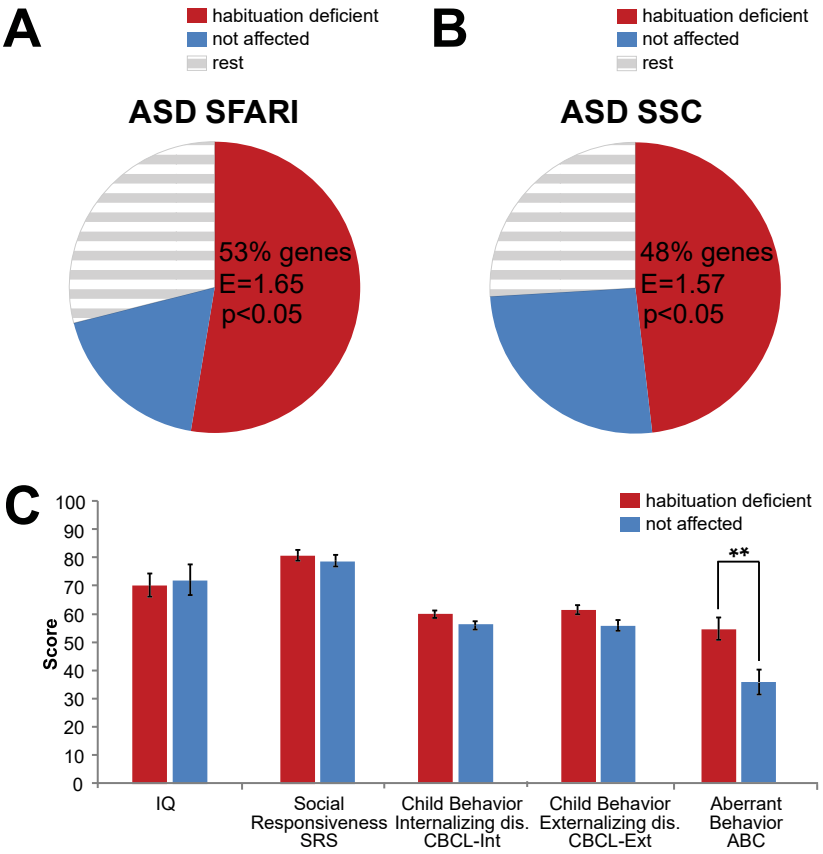

A
habituation deficient
not affected
rest
ASD SFARI
53% genes
E=1.65
p<0.05
B
habituation deficient
not affected
rest
ASD SSC
48% genes
E=1.57
p<0.05
C
habituation deficient
not affected
Score
100
90
80
70
60
50
40
30
20
10
0
**
IQ
Social Responsiveness SRS
Child Behavior Internalizing dis. CBCL-Int
Child Behavior Externalizing dis. CBCL-Ext
Aberrant Behavior ABC

Figure 5

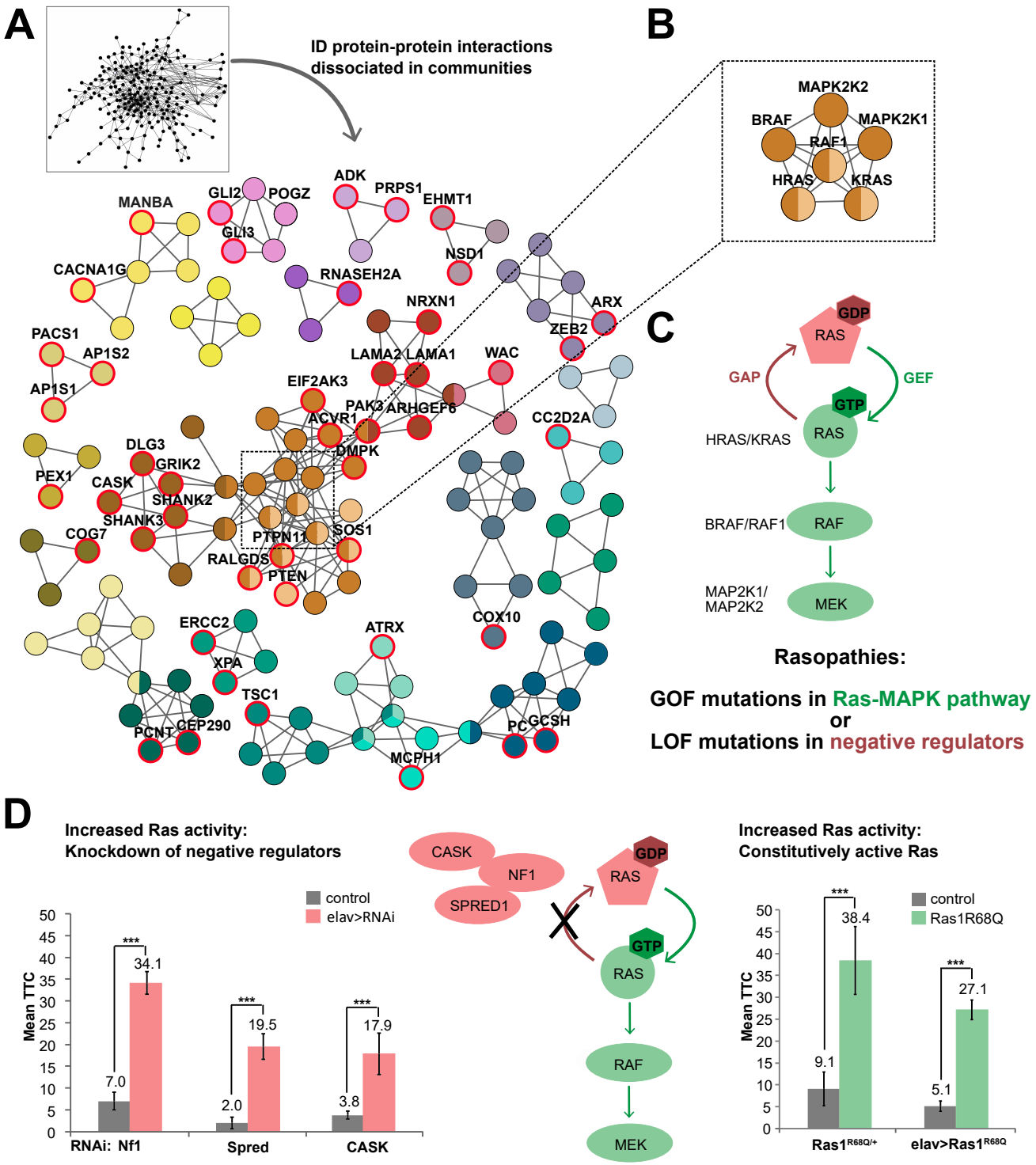

A
ID protein-protein interactions
dissociated in communities
MANBA
GLI2
POGZ
GLI3
ADK
PRPS1
EHMT1
NSD1
CACNA1G
RNASEH2A
NRXN1
ARX
ZEB2
PACS1
AP1S2
AP1S1
LAMA2
LAMA1
WAC
EIF2AK3
PAK3
ARHGEF6
ACVR1
CC2D2A
DMPK
DLG3
GRIK2
PEX1
CASK
SHANK2
SHANK3
COG7
PTPN11
SOS1
RALGDS
PTEN
COX10
ERCC2
ATRX
XPA
TSC1
PCNT
CEP290
PC
GCSH
MCPH1
B
MAPK2K2
BRAF
MAPK2K1
RAF1
HRAS
KRAS
C
GDP
RAS
GAP
GEF
GTP
HRAS/KRAS
RAS
BRAF/RAF1
RAF
MAP2K1/
MAP2K2
MEK
Rasopathies:
GOF mutations in Ras-MAPK pathway
or
LOF mutations in negative regulators
D
Increased Ras activity:
Knockdown of negative regulators
control
elav>RNAi
Mean TTC
50
45
40
35
30
25
20
15
10
5
0
***
34.1
7.0
19.5
2.0
17.9
3.8
RNAi: Nf1
Spred
CASK
CASK
NF1
SPRED1
GDP
RAS
GTP
RAS
RAF
MEK
Increased Ras activity:
Constitutively active Ras
control
Ras1R68Q
38.4
9.1
27.1
5.1
Ras1R68Q/+
elav>Ras1R68Q

Figure 6

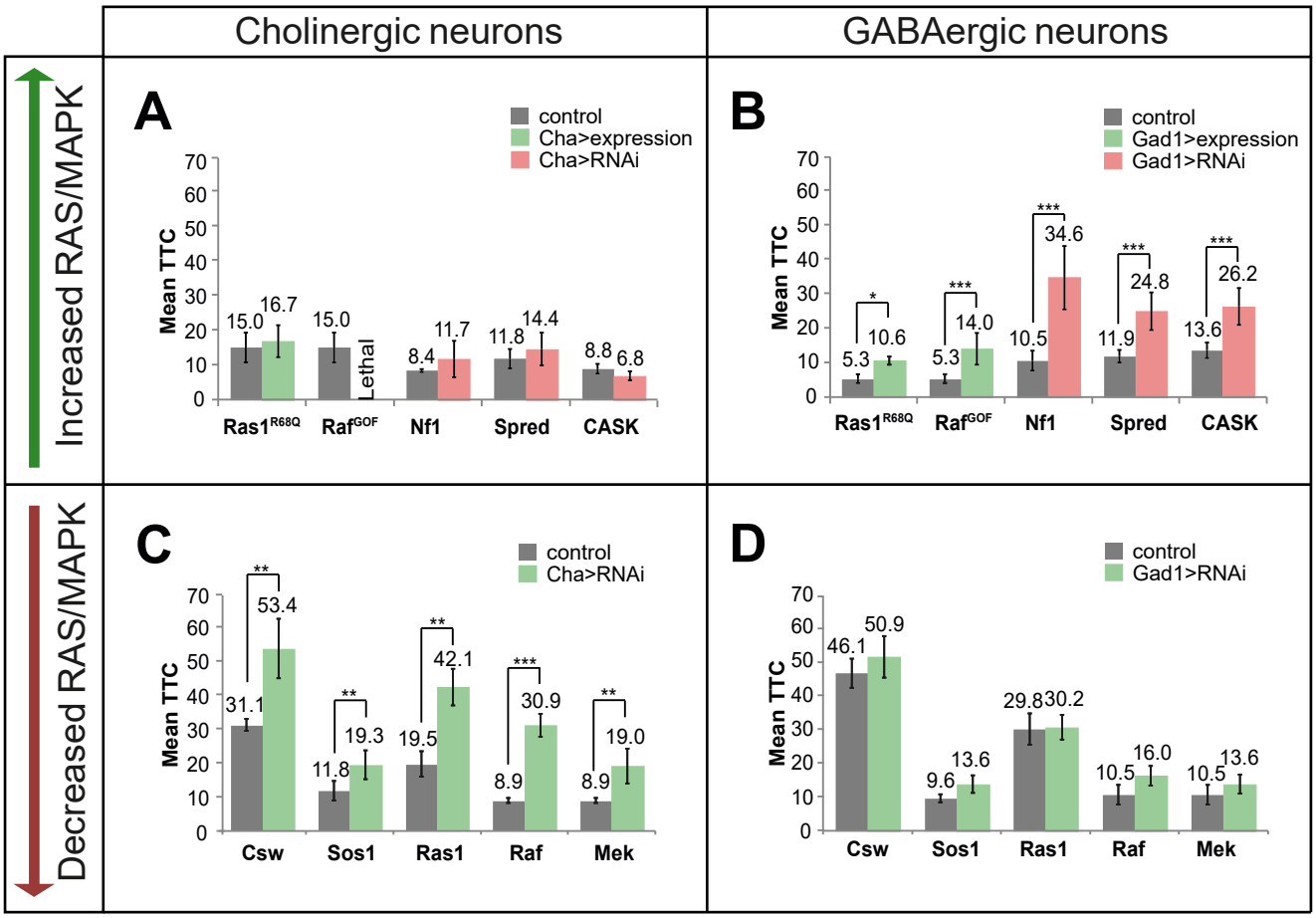
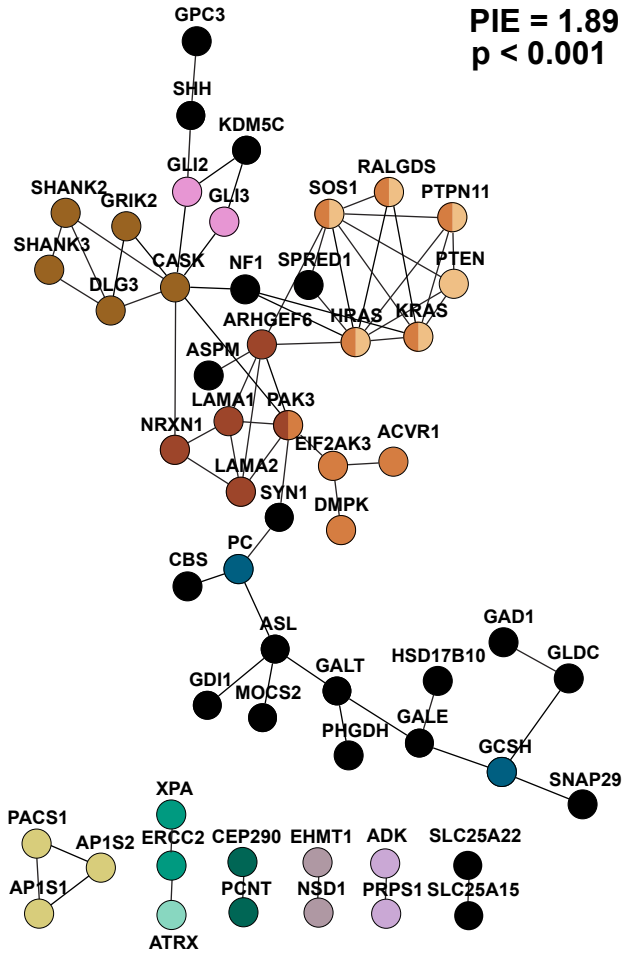

Cholinergic neurons
GABAergic neurons
Increased RAS/MAPK
Decreased RAS/MAPK
A
control
Cha>expression
Cha>RNAi
Mean TTC
15.0
16.7
15.0
Lethal
8.4
11.7
11.8
14.4
8.8
6.8
Ras1R68Q
RafGOF
Nf1
Spred
CASK
B
control
Gad1>expression
Gad1>RNAi
Mean TTC
*
5.3
10.6
***
5.3
14.0
***
10.5
34.6
***
11.9
24.8
***
13.6
26.2
Ras1R68Q
RafGOF
Nf1
Spred
CASK
C
control
Cha>RNAi
Mean TTC
**
31.1
53.4
**
11.8
19.3
**
19.5
42.1
***
8.9
30.9
**
8.9
19.0
Csw
Sos1
Ras1
Raf
Mek
D
control
Gad1>RNAi
Mean TTC
46.1
50.9
9.6
13.6
29.8
30.2
10.5
16.0
10.5
13.6
Csw
Sos1
Ras1
Raf
Mek

Figure 7


PIE = 1.89
p < 0.001
GPC3
SHH
KDM5C
GLI2
GLI3
SHANK2
GRIK2
SHANK3
DLG3
CASK
NF1
SPRED1
RALGDS
SOS1
PTPN11
PTEN
HRAS
KRAS
ARHGEF6
ASPM
LAMA1
PAK3
NRXN1
EIF2AK3
ACVR1
LAMA2
SYN1
DMPK
PC
CBS
ASL
GAD1
HSD17B10
GLDC
GALT
GDI1
MOCS2
GALE
PHGDH
GCSH
SNAP29
XPA
PACS1
AP1S2
ERCC2
CEP290
EHMT1
ADK
SLC25A22
AP1S1
PCNT
NSD1
PRPS1
SLC25A15
ATRX