Title:

Integrative cross-species analyses identify deficits in habituation learning as a widely affected mechanism in Autism

Short title:

Habituation deficits in Autism 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 overgrowth/macrocephaly 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 demonstrates 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 target for pharmacologic intervention in ID/ASD.

84 **Introduction:**

85 Habituation is one of the most ancient and fundamental forms of learning, conserved across the
86 animal kingdom (1). It causes an organism's initial response to repeated not meaningful stimuli to
87 gradually decline. Learning to ignore irrelevant stimuli as a result of habituation is thought to
88 represent a filter mechanism that prevents information overload, allow for selective attention, and
89 thus to focus cognitive resources on relevant matters. Habituation learning has been proposed to
90 represent an important element and prerequisite for higher cognitive functions (2–4). In line with
91 this, habituation in infants correlates better than other measures with later cognitive abilities (5).
92 Habituation has also emerged as a good proxy for synaptic plasticity (6). However, key players and
93 molecular mechanisms underlying habituation are poorly understood (7).

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

104 We apply light-off jump habituation in *Drosophila* to increase our knowledge on the genetic
105 control of habituation and, at the same time, to assess the relevance of decreased habituation in ID
106 and in comorbid ASD disorders. *Drosophila* is a well-established model for ID (21–23) and offers
107 genome-wide resources to study gene function in large scale (24, 25). Several forms of habituation
108 have been established in *Drosophila* (26–30). Deficits in light-off jump habituation have already been
109 reported in several ID models (22, 31–35) and in classical learning and memory mutants (27, 30).

110 Moreover, this form of habituation can be assessed in a high-throughput manner. In the light-off
111 jump paradigm, the initial jump response to repeated light-off stimuli gradually wanes, as has been
112 demonstrated not due to sensory adaptation (a decrease in detecting the stimulus) or motor fatigue
113 (a decrease in the ability to execute the response) but as a result of learned adaptation of the startle
114 circuit (30). This behavior meets all habituation criteria (3), including spontaneous recovery and
115 dishabituation with a novel stimulus (30, 36). The rich genetic toolbox of *Drosophila* allows for
116 dissection of neuronal substrates and mechanisms of light-off jump habituation.

117 Here, we use inducible RNA interference (RNAi) in *Drosophila* to systematically assess the
118 role of 286 genes that are well-established to cause ID in humans when mutated (hereinafter
119 referred to as ID genes). 69 of them (24%) have also been implicated in ASD (37, 38) (hereinafter
120 referred to as ID plus ASD-associated genes). We report more than a hundred novel genes required
121 for habituation learning. This substantially exceeds the number of previously known regulators of
122 habituation across paradigms and species, and identifies habituation learning as a widely affected
123 mechanism in ID/ASD disorders. We further systematically reveal the relation of these genes to their
124 associated clinical phenotypes and molecular functions, and determine neuronal substrates of Ras-
125 MAPK signaling, which we identify as a central key pathway controlling habituation.

126

127 **Methods and Materials**

128 **Investigated ID genes**

129 A systematic source of ID genes and their *Drosophila* orthologs is available online (SysID database,
130 sysid.cmbi.umcn.nl (39)). We investigated the *Drosophila* orthologs of 286 human ID genes from the
131 SysID category primary ID genes (**Table S1**) (containing mutations with robust published evidence for
132 causality, see **Supplemental Methods (SM)**). SysID inclusion criteria and in/exclusion criteria of
133 experimentally investigated genes are indicated in the **SM**). In brief, the vast majority of genes are
134 from the first data freeze of the SysID database (all ID genes with proven causality, status of mid
135 2010). Genes have been included based on conservation in *Drosophila*, available tools (RNAi) from

136 large-scale resources and viability as a prerequisite for behavioral testing. No selection was
137 performed.
138
139 Information about the identification of *Drosophila* orthologs, proposed disease mechanism, light-off
140 habituation assay and analysis, phenotype reproducibility, *Drosophila* stocks, fatigue assay, quality
141 criteria for RNAi lines, annotation of ID plus ASD associated genes, comparison of behavior and
142 cognition in ASD SSC, molecular interaction network, clustering, physical interaction enrichment
143 (PIE), enrichment analysis, data visualization and statistics are described in the **SM**.
144

145 **Results:**

146

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

148 To identify novel genes implicated in habituation, we systematically investigated the role of 278
149 *Drosophila* orthologs representing 286 human ID genes in the light-off jump habituation paradigm.
150 We induced neuron-specific knockdowns of each ID gene ortholog by RNAi (24) using 513 RNAi lines
151 fulfilling previously established quality criteria (39, 40), with two independent constructs per gene
152 whenever available. These were crossed to the panneuronal elav-Gal4 driver line (see **SM**).
153 Knockdown is a suitable approach for modeling of the here-investigated human disease conditions
154 since (partial) loss of function is considered to be the underlying mechanism in the vast majority of
155 these disorders (40) (**Table S1**). Restricting gene knockdown to neurons eliminates potential effects
156 on viability or organismal fitness originating from an essential role of genes in other tissues and
157 establishes neuron-autonomous mechanisms.

158 Knockdown and control flies of identical genetic background were subjected to a series of
159 100 light-off stimuli, hereinafter referred to as trials, in the light-off jump habituation paradigm. The
160 screening procedure and paradigm allowed us to distinguish the following parameters: viability,
161 initial jump response (percentage of flies that jumped in at least one of the first five trials), and
162 premature and reduced habituation, with the latter representing the learning-defective phenotype
163 category of main interest. Genotypes with a good initial jump response (≥50% initial jumpers) but
164 premature habituation were subjected to a secondary assay to evaluate their organismal fitness and
165 exclude fatigue as a confounder of premature habituation (see **SM** and **Figure S1**). Based on these
166 parameters, genes were assigned to at least one of four phenotype categories (**Figure A1**): (1) "not
167 affected": (both) tested RNAi lines targeting such genes were viable, showed good initial jump
168 response, and had no significant effect on habituation (based on the FDR-corrected p-value (p_{adj}), see
169 **SM**); (2) "reduced organismal fitness": at least one RNAi line led to lethality, poor jump response
170 (<50% initial jumpers), or premature habituation because of increased fatigue; (3) "habituation

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

178 We validated the experimental approach to identify genes which, if manipulated, cause
179 habituation deficits (hereinafter referred to as habituation deficient genes) by recapitulating
180 published habituation deficits of *Drosophila* ID null mutant models *G9a* (22) and *Synapsin* (41), and of
181 the classical learning and memory mutant *dunce* (27, 42, 43) (**Figure 1B,C,D)**. This demonstrates that
182 light-off jump habituation in combination with RNAi can efficiently identify genetic regulators of
183 habituation learning.

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

194

195 ***Drosophila* habituation deficits characterize ID genes associated with macrocephaly**

196 To understand whether habituation deficits in *Drosophila* represent a proxy of specific phenotypes in
197 human individuals, we performed enrichment analysis among ID-associated clinical core features
198 (39). We found that orthologs of ID genes associated with habituation deficits in *Drosophila* are
199 specifically enriched among ID genes associated with macrocephaly/overgrowth (**Figure 2A**, E=2.21,
200 p=0.0095, **Table S4**) and vegetative anomalies (**Figure 2A**, E=2.14, p=0.0495, **Table S4**). In contrast, ID
201 genes associated with the severe "reduced organismal fitness" phenotype category show enrichment
202 in different, severe ID-associated features such as limb anomalies, brain malformations, endocrine
203 and eye anomalies (**Figure S2**, **Table S4**). Moreover, ID genes not giving rise to habituation deficits
204 ("not affected" category) did not show any enrichment among ID-associated clinical features (**Figure**
205 **2A**, **Table S4**).
206
207 **Habituation deficits characterize ID genes associated with ASD and deficits in specific ASD-relevant**
208 **behavioral domains**
209 There is a long-known relationship between macrocephaly and autism (44). For this reason and
210 because of the potential relevance of habituation deficits to ASD (10–12), we decided to further
211 investigate the relationship of *Drosophila* habituation and human ASD. We used the Simons Simplex
212 Collection (SSC) (37), a genetically and phenotypically well-characterized cohort of sporadic ASD
213 individuals. We matched genes with likely gene-disrupting (LGD) *de novo* mutations (45) in this ASD
214 cohort to those included in our experimental *Drosophila* habituation approach. 86 ASD individuals
215 carried mutations in 59 of the investigated genes (**Table S5**). We first asked whether these ID plus
216 ASD-associated genes preferentially fall into a specific *Drosophila* phenotype category. Strikingly,
217 they are significantly enriched among the genes that in *Drosophila* caused habituation deficits (**Figure**
218 **3A,** E=1.57, p=0.014, **Table S4**, ASD SSC). Independently, significant enrichment was obtained for
219 high-confidence ID plus ASD-associated genes identified from the SFARI database (38) (41
220 investigated genes, **Figure 3B,** E=1.58, p=0.023, **Table S4**, ASD SFARI), confirming a relationship
221 between *Drosophila* habituation deficits and human ASD.

222 To further characterize the relationship between *Drosophila* habituation and human
223 phenotypes, we divided the SSC individuals into two distinct clusters based on their habituation
224 phenotype in the corresponding fly models: habituation deficits (N=41 individuals, 29 genes) and no
225 habituation deficits (N=23 individuals, 16 genes) (**Table S5**). We compared both groups across five
226 broad quantitative measures of behavior and cognition: cognitive ability (full-scale IQ); Social
227 Responsiveness Scale (SRS); depression and anxiety (Child Behavior Checklist Internalizing Disorders,
228 CBCL-Int); impulsivity, attention and conduct (Child Behavior Checklist Externalizing Disorders, CBCL-
229 Ext); and atypical behavior (Aberrant Behavior Checklist, ABC). There was no significant difference for
230 IQ (p=0.80), SRS (p=0.49), or CBCL-Int (p=0.07), but a significant difference for CBCL-Ext (p=0.04) and
231 ABC (p=0.004; **Figure 3C**, **Table S6)**. The latter persists even in case of multiple testing correction
232 (p<0.01). This effect is mainly driven by the ABC subdomains of inappropriate, stereotypic speech
233 (p=0.003), with a nominal contribution from the subdomains of hyperactivity (p=0.016) and
234 irritability (p=0.03) but not by lethargy (p=0.11) or stereotypy (p=0.16) (**Table S6**). In summary, these
235 data provide a direct indication that habituation deficits in *Drosophila* are relevant to ASD-implicated
236 genes. They also suggest that SSC individuals carrying LGD mutations in genes associated with
237 habituation deficits in *Drosophila* show a higher rate and/or severity of atypical behaviors associated
238 with stereotypic speech, hyperactivity and irritability.
239
240 **Habituation deficits characterize ID genes with synaptic function**
241 Specificity of "habituation deficient" genes in distinct human disease phenotypes suggests that these
242 may converge on specific mechanisms also at the molecular level. ID genes are known to be enriched
243 in a number of biological processes, but which are important for habituation? Performing an
244 enrichment analysis of ID-enriched Gene Ontology-based (GO) categories (see **SM**) against the
245 stringent background of the investigated ID genes, we found that "habituation deficient" genes are
246 significantly enriched in a sole GO-based category: processes related to the synapse (22/44 ID genes,
247 E=1.59, p=0.024, **Figure 4**, **Table S4**). No enriched GO terms were found in the "not affected"

248 category. Together, our results support synaptic processes to be crucial for habituation, as previously
249 shown for other forms of this behavior (46, 47).
250
251 **Molecular networks and modules underlying habituation**
252 With the rich repertoire of nearly a hundred genes required for habituation that moreover show
253 specificity for ASD and synapse function, we set out to determine the molecular pathways these
254 genes are operating in. ID gene products are significantly interconnected via protein-protein
255 interactions (48, 49). Consistent with previously published findings (39), ID genes investigated in our
256 screen are 1.69 times enriched in interactions compared to 1000 randomly chosen protein sets of the
257 same size and number of known interactions (physical interaction enrichment (PIE) score (50) =1.69;
258 $p<0.001$). To identify biologically relevant modules, we resolved this network into communities with
259 even tighter interconnectivity using unsupervised community clustering (51). This analysis resulted in
260 26 communities containing 109 proteins (**Figure 5A, Table S7**). Their proximity and specificity for ID-
261 enriched GO-based processes are depicted in **Figure S3**. Mapping "habituation deficient" genes onto
262 the communities (**Figure 5A, red circles**) highlighted modules with high incidence of habituation
263 deficits (**Figure 5A**).
264
265 **A key role for ID and ASD-associated Ras signaling in habituation**
266 Five communities form a large, interconnected module with high incidences of habituation deficits.
267 However, the tightly interconnected hub at its center is characterized by the absence of habituation
268 deficits (**Figure 5A, square)**. This hub represents the key proteins of Ras-MAPK signaling (**Figure 5B**).
269 This pathway, best known for its role in cancer, underlies a group of disorders collectively referred as
270 Rasopathies. Importantly, while 92% of the modeled ID disorders are thought to result from loss of
271 function of the underlying genes, Rasopathies are caused by gain-of-function mutations in the core
272 pathway (**Figure 5C, Table S1**). The RNAi approach used in this screen, despite addressing gene
273 function, did thus not recapitulate the molecular pathology of these specific cognitive disorders.

274 However, Rasopathies can also result from loss of function in negative regulators of the pathway. We
275 therefore asked whether exactly the same genetic mechanisms that cause Rasopathies in humans
276 also hold true for habituation deficits in *Drosophila*. In our screen, we tested habituation of three
277 negative regulators of Ras: NF1 (*Drosophila* Nf1), which can directly inactivate Ras through the GAP
278 enzymatic activity (52), SPRED1 (*Drosophila* Spred) (53, 54), and CASK (*Drosophila* CASK) (55).
279 Panneuronal knockdown of either negative regulator caused strong habituation deficits (**Figure 5D, in**
280 **red**). We therefore tested a constitutively active *Ras* mutant, $Ras1^{R68Q}$ (56). Heterozygous $Ras1^{R68Q}$
281 flies showed strong habituation deficits compared to the control flies with the same genetic
282 background ($p=3.56x10^{-9}$; **Figure 5D, in green**). Moreover, the same was true when we
283 overexpressed, specifically in neurons, the same mutant *Ras* allele from an inducible transgenic
284 construct ($p=1.96x10^{-6}$; **Figure 5D, in green**). We conclude that increased activity of Ras, causing
285 Rasopathies and associated cognitive deficits in humans, causes habituation deficits in *Drosophila*.
286
287 **Habituation-inhibiting function of increased Ras-MAPK signaling maps to inhibitory/GABAergic**
288 **neurons**
289 We next aimed to identify in which type of neurons the habituation-inhibiting function of Ras-MAPK
290 signaling resides. Because the well-characterized neurons of the giant fiber circuit controlling the
291 light-off jump response in *Drosophila* are cholinergic (57), just as the majority of excitatory neurons
292 in *Drosophila*, we first tested whether increased Ras-MAPK signaling activity would induce
293 habituation deficits when directed to cholinergic neurons. For this, we adopted the knockdown of
294 negative Ras regulators (*Nf1*, *Spred* and *CASK*) and expressed constitutively active *Ras1* ($Ras1^{R68Q}$). In
295 addition, we tested expression of a gain-of-function allele of *Raf* (Raf^{GOF}), a downstream mediator of
296 Ras signaling. We crossed these inducible UAS alleles to the cholinergic Cha-Gal4 driver and assessed
297 habituation. None of these conditions recapitulated the panneuronally evoked habituation deficits
298 (**Figure 6A**).

299 Because of the recently established role of GABAergic neurons in *Drosophila* olfactory and
300 proboscis extension reflex habituation (28, 58, 59) and the emerging importance of GABA inhibition
301 in autism (60), we next targeted GABA neurons using the Gad1-Gal4 driver and the same toolbox to
302 increase Ras-mediated signaling activity. Notably, this consistently induced habituation deficits in all
303 tested conditions (**Figure 6B**), recapitulating the observed panneuronally evoked deficits. We
304 conclude that the habituation-inhibiting function of increased Ras-MAPK signaling maps to
305 GABAergic neurons.
306
307 **Ras-MAPK signaling in cholinergic neurons is essential for habituation learning**
308 In the panneuronal screen, RNAi-mediated knockdown of the Ras pathway core components *Ras, Raf*
309 and *Mek* resulted, likely due to their promiscuous role and fundamental importance, in impaired
310 jump response or increased fatigue ("reduced organismal fitness" category). This could potentially
311 mask an essential role for Ras signaling in habituation, in addition to the habituation-inhibiting
312 function of increased Ras-MAPK signaling. In fact, in our panneuronal RNAi screen not only
313 knockdown of negative regulators but also of two established positive Ras-MAPK regulators, *Sos* and
314 *Csw*, resulted in habituation deficits. We therefore addressed whether decreased Ras-MAPK signaling
315 could also be critical for habituation learning. We downregulated Ras-MAPK activity by crossing the
316 UAS-based RNAi lines targeting *Sos* and *Csw*, but also RNAi lines targeting *Ras*, *Raf* and *Mek,* to the
317 GABAergic driver Gad1-Gal4. We did not observe any detrimental effect on habituation (**Figure 6D**).
318 In contrast, downregulating Ras-MAPK signaling using the same toolset in cholinergic neurons
319 consistently prevented normal habituation learning (**Figure 6C**). We conclude that Ras-MAPK
320 signaling is essential in cholinergic but not in GABAergic neurons. Thus, Ras-MAPK signaling plays a
321 dual, opposing role in inhibitory versus excitatory neurons in habituation learning.
322

323 **Discussion:**

324 ***Drosophila* screen identifies deficits in habituation learning as a widely affected mechanism in**
325 **autism**

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

339 Enrichment analysis of ID-associated clinical features revealed that "habituation deficient" ID
340 genes are preferentially characterized by macrocephaly/overgrowth. Macrocephaly has been since
341 long associated with ASD (44). Strikingly, we found that mutations in genes associated with
342 *Drosophila* habituation deficits are significantly overrepresented among ASD individuals (SFARI
343 database, 50%; SSC cohort: 48% of genes). SSC individuals carrying mutations in these genes show a
344 high rate and/or severity of aberrant behaviors associated with stereotypic speech, hyperactivity and
345 irritability, suggesting that habituation deficits may contribute to specific behavioral anomalies in
346 ASD. Habituation deficits thus represent a common phenotypic signature of ASD in *Drosophila* and
347 highlight specific behavioral subdomains affected in ASD.

348

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

350 Synapse biology has been proposed to play a central role in ASD (61). Our data show that among the
351 investigated disease genes, "habituation deficient" genes are specifically enriched in genes with
352 synaptic function. This is in line with habituation representing a measurable form of synaptic
353 plasticity (8, 47).

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

365

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

368 Identification of neuronal substrates in which specific ID genes are required to warrant habituation
369 learning is important for understanding of the neuronal mechanisms underlying habituation in flies
370 and cognitive dysfunction in individuals with ID/ASD. Restoring the function of affected neurons
371 might represent a suitable treatment strategy. The light-off jump startle circuit of *Drosophila* is
372 relatively simple and well described. The chemical component is *Drosophila*'s major excitatory
373 neurotransmitter, acetylcholine (57). However, it is not known how habituation of this circuit is
374 regulated. The commonly accepted view regards synaptic depression in excitatory neurons, induced

375 by repetitive stimulation, as the underlying mechanism (46, 62). This has recently been challenged by
376 Ramaswami and colleagues who showed that plasticity of inhibitory, GABAergic neurons drives two
377 non-startle types of habituation (58, 59). We found that increased activity of our identified key
378 pathway, Ras-MAPK, in GABAergic but not in cholinergic neurons causes deficits in light-off jump
379 habituation. Our results thus support inhibitory circuits as crucial components of habituation learning
380 across different paradigms and sensory modalities. Further experiments are needed to establish the
381 direct involvement of GABAergic signaling. At the same time, we identified that also decreased Ras-
382 MAPK signaling activity can lead to habituation deficits. Yet, the neuronal substrates of these deficits
383 are different and map to excitatory, cholinergic neurons. Although our experiments do not
384 distinguish between developmental effects and acute circuit plasticity, the opposing role for Ras-
385 MAPK signaling on habituation may provide new insights into mechanisms of neural plasticity in
386 health and disease. It may also have crucial implications for treatment of Rasopathies. Future clinical
387 trials, as opposed to those that broadly decreased Ras activity and failed (63), may need more
388 attention towards restoring circuit function and balance.
389
390 **Translational value and application of cross-species habituation measures for diagnosis and**
391 **treatment of ID and ASD**
392 Based on our findings that habituation is widely affected in *Drosophila* models of ID, and that
393 habituation deficits are particularly common among genes also implicated in ASD, we propose that
394 disrupted habituation may contribute to ID/ASD pathology.
395 The emerging importance of inhibitory inputs for habituation ((28, 58) and this study) and
396 sensory information filtering in the cortical centers of the brain (64, 65) suggests the existence of an
397 overarching circuit-based mechanism responsible for prevention of inappropriate behavioral
398 responses (8). Though our findings that habituation deficits in *Drosophila* correlate with increased
399 rate and/or severity of atypical behaviors compared to ID/ASD genes without habituation deficits
400 should be replicated, we speculate that disrupted habituation arising from GABAergic defects

401 represents a mechanism that contributes to these core ID/ASD features. As such, it has potential to
402 serve as a cross-species, mechanism-specific functional readout—a pressing need for efficient
403 personalized (pharmacological) treatment in the field of neurodevelopmental disorders.
404 Implementing suitable low-burden protocols for habituation measures in clinical research and
405 diagnostics of ID/ASD, such as those developed for investigation of habituation deficits in Fragile X
406 syndrome (13), will help to further delineate the affected cognitive domains that arise from deficient
407 habituation. In future clinical trials, these could serve as objective and quantitative readouts for
408 patient stratification in mechanism-based treatment strategies and for monitoring of drug efficacy.
409 Dissection of the underlying defective mechanisms in *Drosophila* can at the same time identify novel
410 targets for treatment, with high-throughput light-off jump habituation serving as a translational
411 pipeline for drug testing.
412

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574 **Supplemental information**

575 **Document S1.**

576 **Figures S1-S3, Tables S5, S8, Supplemental Methods**

577

578 **Document S2.**

579 **Table S1**

580 **List of investigated human ID genes**

581

582 **Table S2. List of investigated human ID genes, corresponding Drosophila orthologs, RNAi lines, and**

583 **habituation results**

584

585 **Table S3**

586 **Distribution of human ID genes in four phenotype categories identified in Drosophila light-off jump**

587 **habituation screen**

588

589 **Table S4. Enrichments**

590

591 **Table S5. List of investigated SSC and SFARI genes**

592

593 **Table S7. Interaction communities**

594

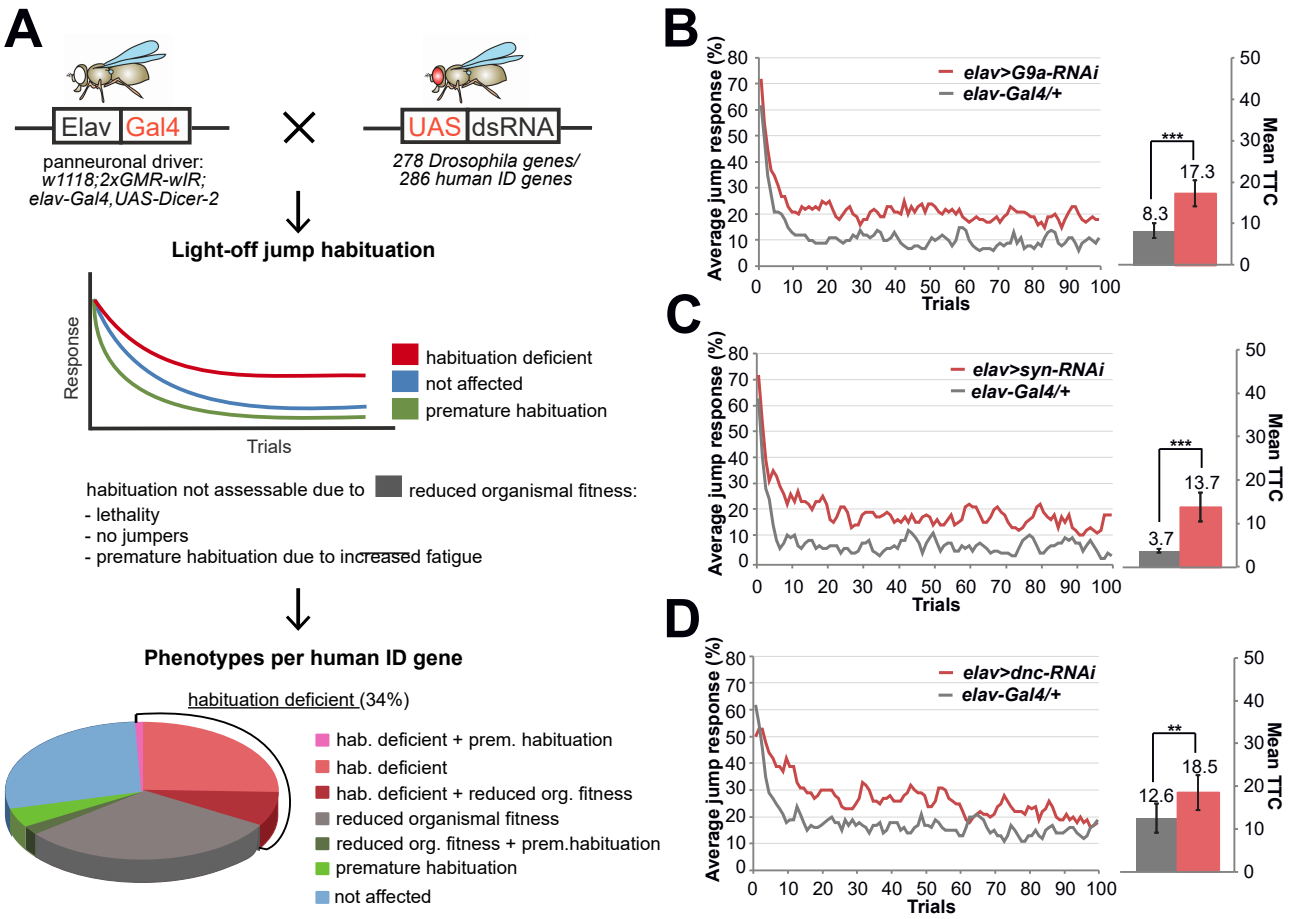

Figure 1. Habituation screen of intellectual disability genes, phenotype distribution and proof of principle
(A) Procedure, phenotype categories and phenotype distribution of the light-off jump habituation screen. Knockdowns that resulted in lethality, no jumper phenotype (defined as less than 50% flies jumping in at least one of the first five light-off trials) or premature habituation plus increased fatigue were assigned to the category "reduced organismal fitness" and their habituation was not further analyzed. Other phenotype categories are "habituation deficient", "not affected", and "premature habituation" (the latter if no fatigue was detected in secondary assay, see example in **Figure S1**). Drosophila orthologs of 34% of the investigated human ID genes were associated with defects in habituation learning. See also T**able S2, S3**. (B, C, D) Defective habituation upon neuron-specific RNAi-mediated knockdown of G9a, Synapsin (syn), and dunce (dnc) (2xGMR-wIR/+; UAS-RNAi/elav-Gal4, UAS-Dicer-2, in red) compared to their respective genetic background controls (2xGMR-wIR/+; elav-Gal4, UAS-Dicer-2/+, in gray). Jump response curves show the average jump response (% of jumping flies) over 100 light-off trials at 1 s inter-trial interval). Mean TTC: the mean number of trials that flies needed to reach the no-jump criterion (see Methods and Materials) presented as Mean TTC ± SEM. *** padj<0.001, ** padj<0.01, based on FDR-corrected lm analysis. A complete list of ID/ASD genes with previously identified habituation defects is provided as **Table S8**, adding further proof of principle.

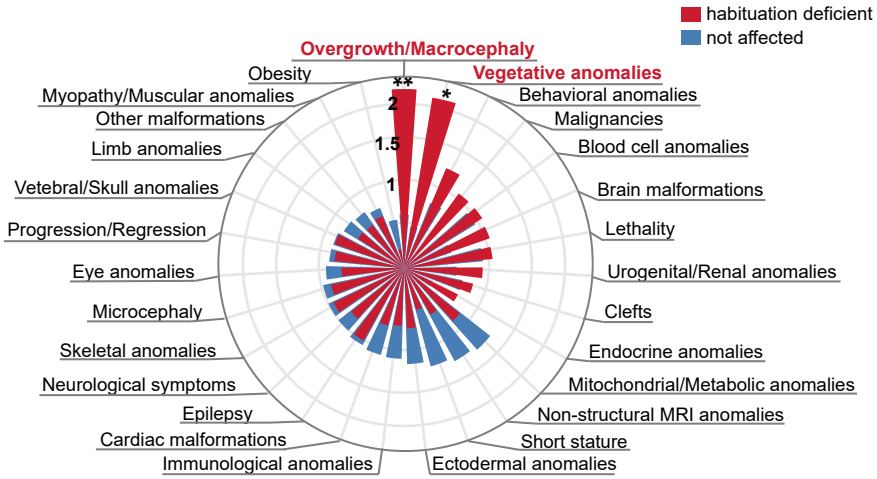

Figure 2. Habituation defects in Drosophila characterize ID genes associated with macrocephaly in humans
Enrichment of Drosophila phenotype categories across 27 ID-accompanying clinical features (39). "Habituation deficient" genes show specificity for macrocephaly and/or overgrowth (E=2.21, p=0.0095) and vegetative anomalies (E=2.14, p=0.049) ** p<0.01, * p<0.05, based on Fisher's Exact test. For enrichment of "reduced organismal fitness" category, see Figure S2. Enrichment scores, p-values and enriched genes are listed in **Table S4**.

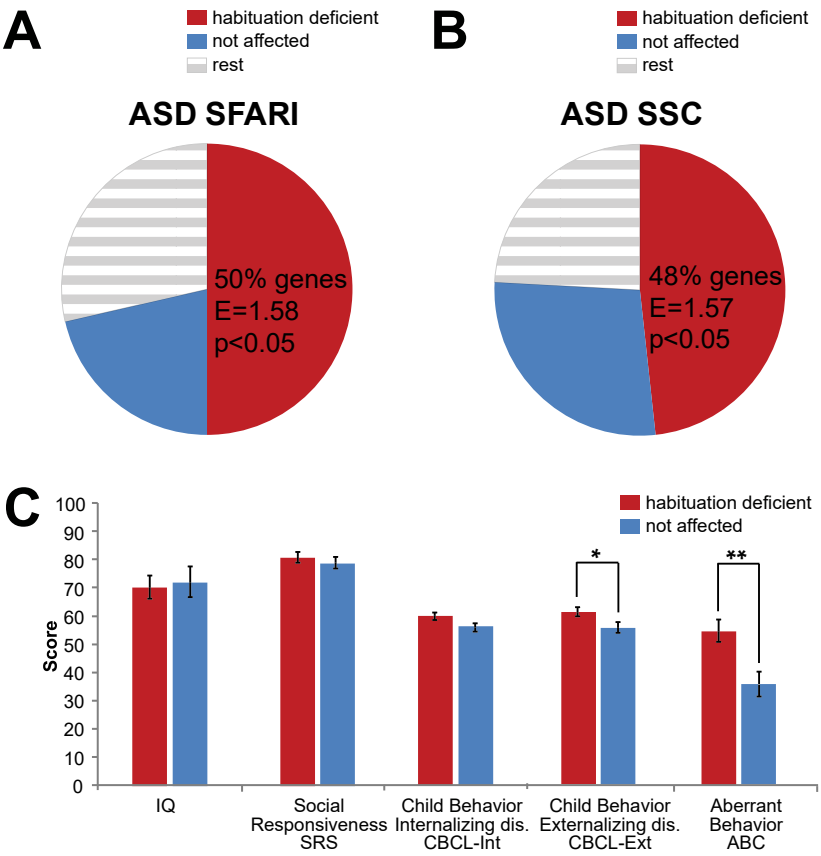

Figure 3. Habituation defects in Drosophila characterize ID genes associated with ASD and deficits in specific behavioral domains

(A,B) Enrichment of Drosophila phenotype categories "habituation deficient" and "not affected" in ID plus ASD-associated genes identified in SFARI database (ASD SFARI, E=1.58, p=0.023, (A)) and SSC cohort (ASD SSC, E=1.57, p=0.014 (B)). Circles represent total number of tested ID plus ASD-associated genes. (C) Genes associated with "habituation deficient" versus "not affected" phenotype categories in Drosophila show significantly more aberrant behaviors on the ABC ($F(1,62)=8.9$, $p=0.004$) and more CBCL externalizing symptoms ($F(1,62)=4.3$, with suggestive p-value, $p=0.04$) in the ASD SSC cohort. Data presented as mean score ± SEM. ** $p<0.01$, * $p<0.05$, based on MANOVA. See also **Table S5** (list of ASD SFARI and ASD SSC genes) and **Table S6** (complete MANOVA results).

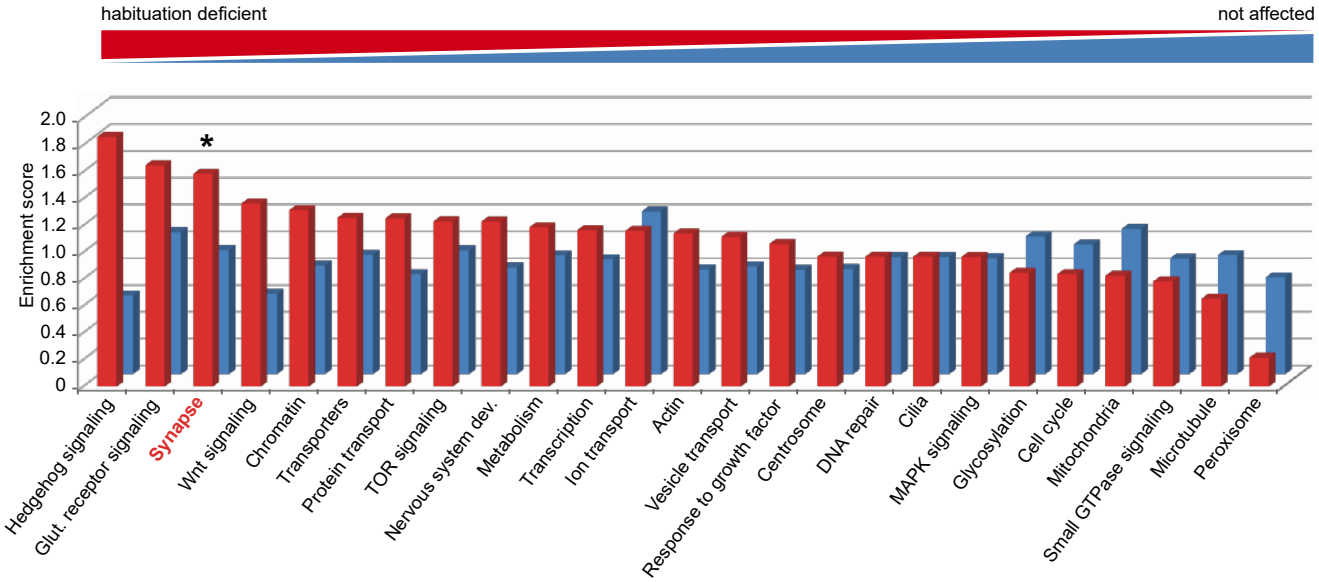

Figure 4. Habituation defects in Drosophila characterize ID genes with synapse-related functions
Of 25 gene ontology (GO)-based processes, "habituation deficient" genes are specifically and significantly enriched in processes related to synapse (E=1.59, p=0.024). Genes with no effect on habituation do not show significant enrichment in any GO process. * $p<0.05$, based on Fisher's exact test. All enrichment scores, p-values and enriched genes are listed in **Table S4**.

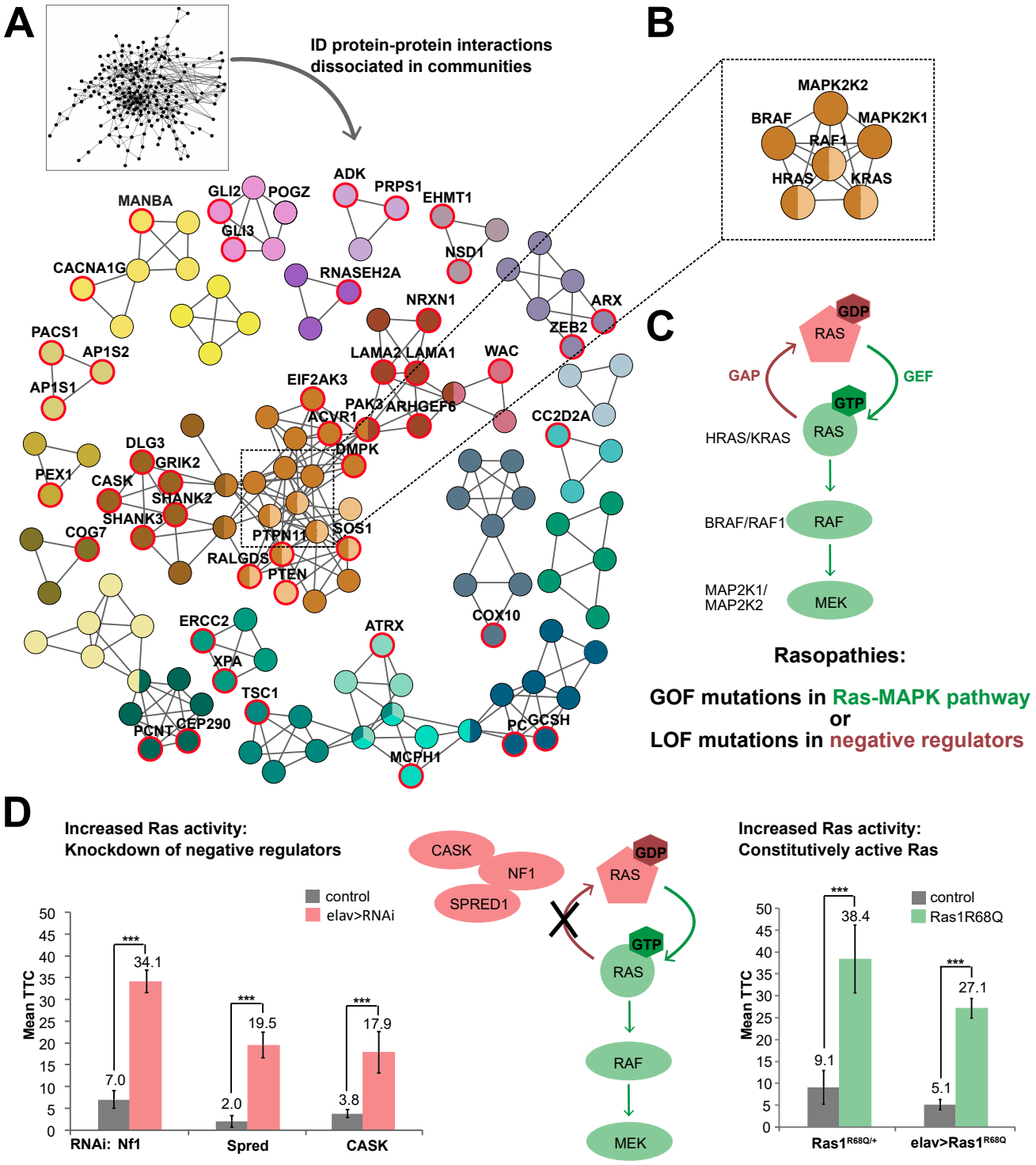

Figure 5. A central role for Ras-MAPK signaling in habituation learning

(A) Highly connected communities identified by unbiased community clustering, colored by their functional proximity (**Figure S3**). Red circles and gene names highlight nodes representing "habituation deficient" genes. For complete list of communities and genes see **Table S7**. (B) Nodes connecting four communities from the central module represent the core components of Ras-MAPK signaling. (C) Schematic representation of Ras-MAPK signaling and associated mechanisms in ID disorders called 'Rasopathies'.

(D) Increasing Ras signaling by inducing either loss of function of negative Ras regulators (left side of pathway scheme) or by constitutively activating Ras (right side) disrupts habituation learning. Left: Defective habituation upon neuron-specific knockdown of negative Ras regulators, Nf1 (2xGMR-wIR/+; Nf1-RNAivdrc35877/elav-Gal4, UAS-Dicer-2, N=72, in red), Spred (2xGMR-wIR/+; Spred-RNAivdrc18024/elav-Gal4, UAS-Dicer-2, N=73, in red), and CASK (2xGMR-wIR/+; CASK-RNAivdrc34184/elav-Gal4, UAS-Dicer-2, N=75, in red) compared to their corresponding genetic background controls (2xGMR-wIR/+; elav-Gal4, UAS-Dicer-2/+. N: 55, 20, 25, in gray). *** padj<0.001, based on lm analysis and FDR correction in the screen (see **Methods and Materials**). Mean TTC ± SEM.Right: Defects in habituation learning in a heterozygous, constitutively active Ras mutant (Ras1R68Q/+, N=55, in green) compared to its genetic background control (N=43 in gray), and upon neuron-specific expression of Ras1R68Q (elav>Ras1R68Q: UAS-Ras1R68Q/2xGMR-wIR; elav-Gal4, UAS-Dicer-2/+, N=52, in green) compared to its genetic background control (2xGMR-wIR/+; elav-Gal4, UAS-Dicer-2/+, N=34, in gray). *** p<0.001, based on lm analysis. Data presented as Mean TTC ± SEM

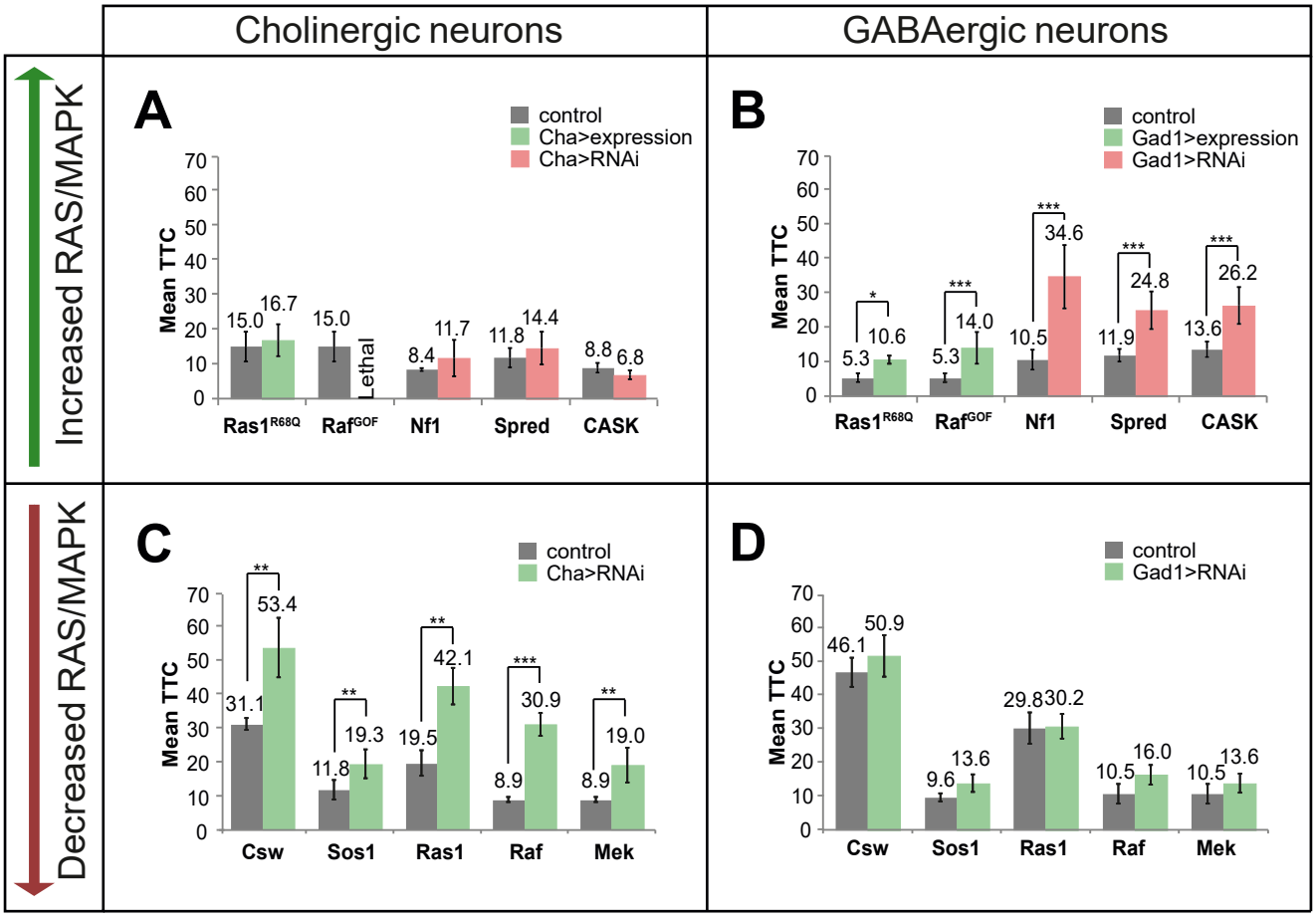

Figure 6. Dual, opposing role of Ras-MAPK signaling in GABAergic and cholinergic neurons in the regulation of habituation learning

(A) No effect on habituation of Ras1R68Q (N=51, in green), Nf1-RNAi (N=38, in red), Spred1-RNAi (N=55, in red) and CASK-RNAi (N=39, in red) upon expression in cholinergic neurons compared to their respective genetic background controls (Cha-Gal4/+; 2xGMR-wIR/+, N: 54, 45, 54, 52, in gray). Expression of RafGOF in cholinergic neurons resulted in lethality. (B) Defective habituation of Ras1R68Q (N=52, in green), RafGOF (N=57, in green), Nf1-RNAi (N=55, in red), Spred1-RNAi (N=37, in red) and CASK-RNAi (N=54, in red) on habituation upon expression in GABAergic neurons compared to their respective genetic background controls (Gad1-Gal4/+; 2xGMR-wIR/+, N: 50, 50, 39, 58, 46, in gray). (C) Defective habituation of Csw-RNAi (UAS-Csw-RNAivdrc21756/Y; Cha-Gal4/+; 2xGMR-wIR/+, N=58), Sos1-RNAi (UAS-Sos1-RNAivdrc42848/Cha-Gal4; 2xGMR-wIR/+, N=56), Ras1-RNAi (UAS-Ras1-RNAivdrc106642/Cha-Gal4; 2xGMR-wIR/+, N=55), Raf-RNAi (UAS-Raf-RNAivdrc20909/Cha-Gal4; 2xGMR-wIR/+, N=59) and Mek-RNAi (Cha-Gal4/+; UAS-Mek-RNAivdrc40026/2xGMR-wIR, N=58) in cholinergic neurons (in green) compared to their respective genetic background controls (Cha-Gal4/+; 2xGMR-wIR/+, N: 62, 54, 34, 46, 46, in gray). (D) No effect on habituation of Csw-RNAi (N=58), Sos1-RNAi (N=51), Ras1-RNAi (N=53), Raf-RNAi (N=52) and Mek-RNAi (N=54) in GABAergic neurons (in green) compared to their respective genetic background controls (Gad1-Gal4/+; 2xGMR-wIR/+, N: 60, 46, 54, 39, 39, in gray). Data presented as Mean TTC ± SEM. *** $p<0.001$, ** $p<0.01$, * $p<0.05$, based on lm analysis.

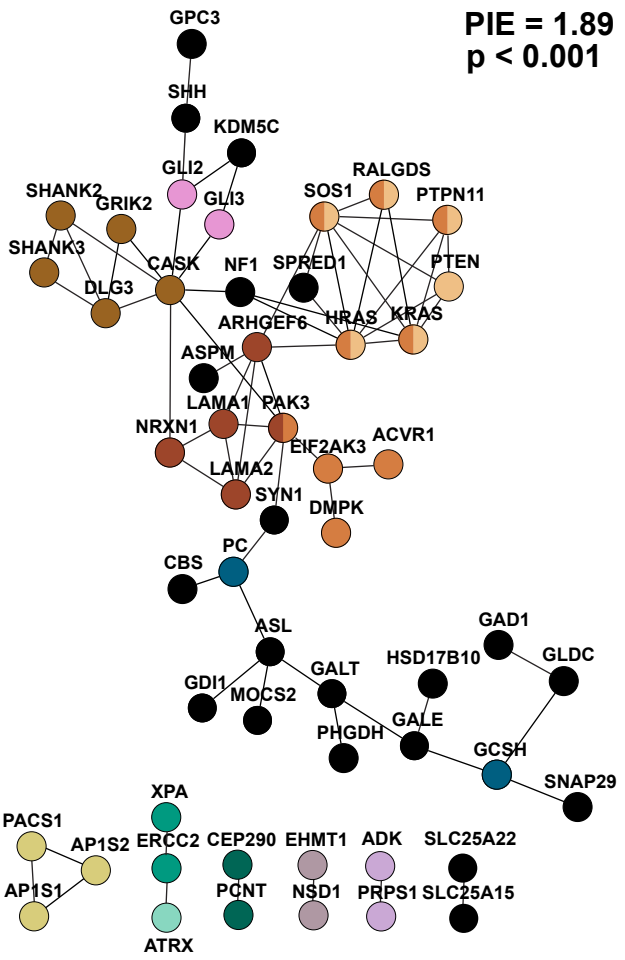

Figure 7. Connections between "habituation deficient" genes

Connections between "habituation deficient" genes, including Ras, identified in the reference network used for community clustering (See **SM)** with significantly increased connectivity (PIE score =1.89, p<0.001). Nodes are colored based on the community, to which they belong. Nodes that represent "habituation deficient" genes but are not members of a community are labeled in black.