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Synaptic scaffold evolution generated components of vertebrate cognitive complexity

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The origins and evolution of higher cognitive functions, including complex forms of learning, attention and executive functions, are unknown. A potential mechanism driving the evolution of vertebrate cognition early in the vertebrate lineage (550 million years ago) was genome duplication and subsequent diversification of postsynaptic genes. Here we report, to our knowledge, the first genetic analysis of a vertebrate gene family in cognitive functions measured using computerized touchscreens. Comparison of mice carrying mutations in each of the four *Dlg* paralogs showed that simple associative learning required *Dlg4*, whereas *Dlg2* and *Dlg3* diversified to have opposing functions in complex cognitive processes. Exploiting the translational utility of touchscreens in humans and mice, testing *Dlg2* mutations in both species showed that *Dlg2*'s role in complex learning, cognitive flexibility and attention has been highly conserved over 100 million years. *Dlg*-family mutations underlie psychiatric disorders, suggesting that genome evolution expanded the complexity of vertebrate cognition at the cost of susceptibility to mental illness.

Humans are thought to be different from other animals largely because of the far greater richness of their cognitive processes[1]. All animals draw upon attention, perception and simple forms of learning to adapt to changing environmental demands. Those species that have the capacity for more complex forms of associative learning and cognitive processing, such as complex visual discrimination, visuo-spatial learning and executive functioning (including cognitive flexibility and inhibitory response control), can adapt to even more complex and challenging environmental demands. These components of the cognitive repertoire are routinely assessed in humans using computerized touchscreen methods[2,3], which have proven useful in identifying specific cognitive impairments in patients with neurological and psychiatric diseases such as schizophrenia, autism, attention deficit hyperactivity disorder and Alzheimer's disease. Recent reports show it is possible to use the same touchscreen approach to measure cognition in rodents[4]. Understanding the evolution of the vertebrate cognitive repertoire and its underlying genomic mechanisms may yield fundamental insights into the origins of our behavior and perhaps identify a basis for the cognitive disorders originating from disease-associated mutations.

One approach, afforded by the touchscreen tests, is to compare the same cognitive abilities in animals and humans with similar genetic perturbations. This strategy allows the identification of cognition-essential genes in both species, which in the case of humans and mice would probe those mechanisms conserved since these species shared a common ancestor, ~100 million years ago (Mya). A related approach that probes an earlier vertebrate ancestry is the comparison of mutations in members of gene families (paralogs) that arose ~550 Mya from the two rounds of whole genome duplication (2R-WGD) at the base of the chordate evolutionary tree[5]. Genome duplications shaped the evolution of most eukaryotes, including fungi[6], plants[7] and vertebrates[8], producing phenotypic novelty[9]. Although vertebrates are widely considered to have a greater cognitive repertoire with more complex behaviors than invertebrates[10], it is unknown how this expansion in cognitive functions arose and whether the 2R-WGD that occurred in the vertebrate lineage was involved.

Here we address these issues with a focus on the role of the Discs Large homolog (Dlg) family of postsynaptic scaffold proteins, which bind neurotransmitter receptors and enzymes into signaling complexes found in the postsynaptic terminals of brain synapses[11]. Invertebrate genomes encode a single *Dlg* gene; after the 2R-WGD, most vertebrates (including 40 mammalian genomes) retained four paralogs—*Dlg1* (SAP-97, hDlg), *Dlg2* (PSD-93, Chapsyn-110), *Dlg3* (SAP-102) and *Dlg4* (PSD-95, SAP-90)—which accumulated mutations diversifying their structure (**Fig. 1a**). Using deletion mutations in the family of Dlg proteins, we have performed, to our knowledge, the first genetic dissection of the vertebrate cognitive repertoire using paralogous genes and a cross-species comparison of homologous cognitive processes in mice and humans.

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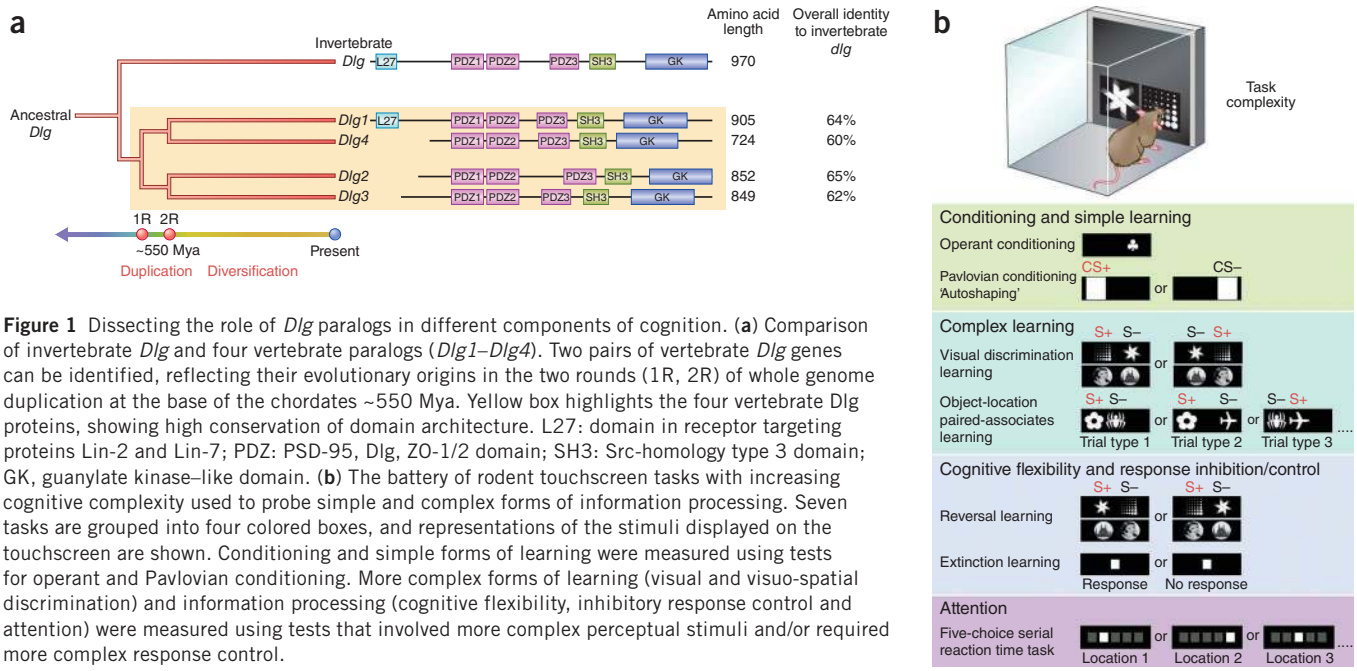

Figure 1 Dissecting the role of *Dlg* paralogs in different components of cognition. (**a**) Comparison of invertebrate *Dlg* and four vertebrate paralogs (*Dlg1*–*Dlg4*). Two pairs of vertebrate *Dlg* genes can be identified, reflecting their evolutionary origins in the two rounds (1R, 2R) of whole genome duplication at the base of the chordates ~550 Mya. Yellow box highlights the four vertebrate Dlg proteins, showing high conservation of domain architecture. L27: domain in receptor targeting proteins Lin-2 and Lin-7; PDZ: PSD-95, Dlg, ZO-1/2 domain; SH3: Src-homology type 3 domain; GK, guanylate kinase–like domain. (**b**) The battery of rodent touchscreen tasks with increasing cognitive complexity used to probe simple and complex forms of information processing. Seven tasks are grouped into four colored boxes, and representations of the stimuli displayed on the touchscreen are shown. Conditioning and simple forms of learning were measured using tests for operant and Pavlovian conditioning. More complex forms of learning (visual and visuo-spatial discrimination) and information processing (cognitive flexibility, inhibitory response control and attention) were measured using tests that involved more complex perceptual stimuli and/or required more complex response control.

RESULTS

Dlg paralogs confer differential capacities for simple forms of conditioning and associative learning

The first part of our strategy was to use mice to ask whether the duplications and divergence of the four *Dlg* genes had conferred differences in their function. Heterozygous mice of the four knockout mouse lines were bred to create homozygotes, and, as consistent with published literature, $Dlg1^{-/-}$homozygosity was embryonic lethal, in contrast to homozygous mutants for *Dlg2*, *Dlg3* or *Dlg4*, which were viable[12–14]. Homozygous mutations in $dlg^{-/-}$ in *Drosophila*[15] and *dlg-1* in *Caenorhabditis elegans*[16] are lethal, as are homozygous mutations of their mouse ortholog $Dlg1^{-/-}$ (ref. 17), suggesting that vertebrate *Dlg1* retained its ancestral functions while the functions of *Dlg2*–*Dlg4* diversified. At the level of protein sequence, the average similarity of the four paralogs is approximately 75% (in either mouse or human; **Supplementary Fig. 1a–c**). We proceeded to test the homozygous *Dlg2*, *Dlg3* and *Dlg4* mutant mice and heterozygous $Dlg1^{+/-}$ mice (as they were viable) in a battery of touchscreen tasks of increasing cognitive complexity (**Fig. 1b**). Across all the tasks, we found that the presence of a single copy of the *Dlg1* gene ($Dlg1^{+/-}$) was sufficient for normal behavior (see **Supplementary Fig. 2**), and hereafter we focus our data on the differential roles of *Dlg2*–*Dlg4*.

Two simple forms of associative learning are classical (Pavlovian) and operant (instrumental) conditioning, in which two or more events become linked or associated, such as two stimuli or a stimulus and a response. The cognitive tasks in the rodent touchscreen battery were built on the simple instrumental conditioned response of nose-poking a stimulus displayed on a touchscreen to obtain a reward. The first element of the battery was therefore the acquisition of this simple form of operant conditioning by training mice through several phases in the touchscreen (see Online Methods for details). $Dlg2^{-/-}$ and $Dlg3^{-/y}$ mice displayed rates of completing each training phase similar to those of wild-type (WT) littermate controls, indicating these genes were not essential for operant conditioning (**Fig. 2a**). In contrast, $Dlg4^{-/-}$ mice showed a marked deficit in acquisition of operant conditioning. They were able to successfully retrieve and eat reward pellets when delivery of the reward did not rely on a direct response (phases 1 and 2; see **Fig. 2** and Online Methods) but were unable to complete the required trials when the reward was contingent on an instrumental operant response (that is, touching the screen to attain a reward (phase 3; see **Fig. 2** and Online Methods). To further investigate this phenotype in $Dlg4^{-/-}$ mice, we used another simple associative learning task, a test of Pavlovian conditioned approach behavior ('autoshaping')[18]. In this task, a spatially localized conditioned stimulus reliably signals an appetitive unconditioned stimulus, a food reward. Mice were presented with a stimulus (a white vertical rectangle) displayed on either the left or the right side of the screen (**Fig. 2b**), and when the stimulus was displayed on, for example, the left side, a food reward was delivered (CS+), whereas appearance of the stimulus on the right side was never rewarded (CS–). After repeated stimulus location-reward pairings, mice normally begin to display the Pavlovian conditioned response of approaching the CS+ more often than the CS–, with the number of discriminative approaches to the CS+ and CS– serving as a measure of how well the mice have learned the association between the CS+ and the reward. Rodents show this conditioned response behavior even though there is no contingency that requires the animal to approach the stimulus to receive the food reward. WT mice robustly demonstrate associative learning and develop a strong conditioned response (making greater number of approaches to the CS+ and decreasing the number of approaches to the CS– with increased training sessions, as well as showing shorter approach latencies to the CS+ than to the CS–) (**Fig. 2b**). In contrast to WT mice, $Dlg4^{-/-}$ mice failed to demonstrate this discriminative capacity; they showed equivalent approaches to the CS+ and CS– and no differences in response latencies to either the CS+ or CS–. These data so far highlight the divergence of Dlg paralogs in their contribution to simple forms of learning and information processing: unlike *Dlg2* and *Dlg3*, *Dlg4* is essential for simple forms of associative learning. This requirement for *Dlg4* was further highlighted in the next phase of testing, where we examined all the *Dlg* mutant mice on a battery of tasks that involved more complex perceptual stimuli and/or required more complex response control. $Dlg4^{-/-}$ mice were incapable of performing the

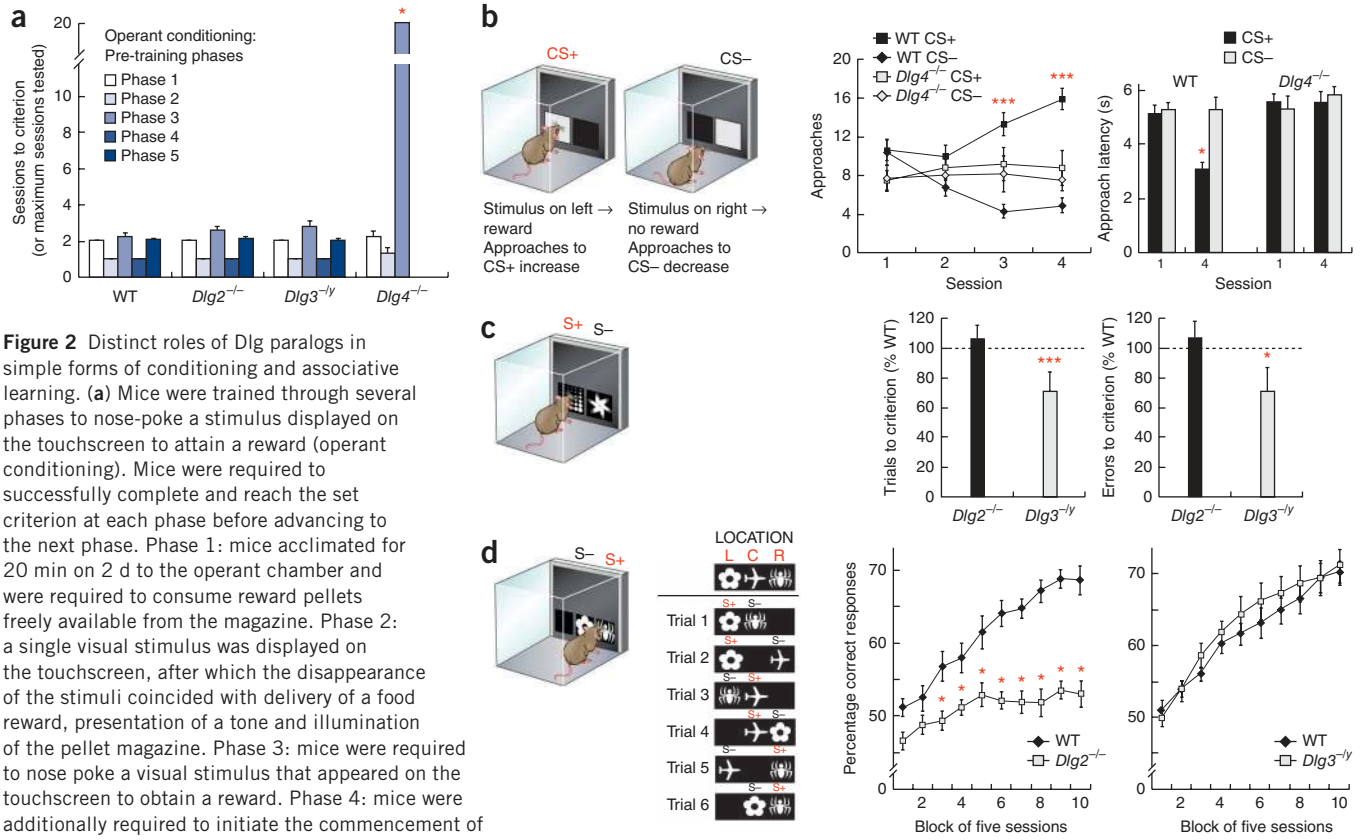

Figure 2 Distinct roles of Dlg paralogs in simple forms of conditioning and associative learning. (**a**) Mice were trained through several phases to nose-poke a stimulus displayed on the touchscreen to attain a reward (operant conditioning). Mice were required to successfully complete and reach the set criterion at each phase before advancing to the next phase. Phase 1: mice acclimated for 20 min on 2 d to the operant chamber and were required to consume reward pellets freely available from the magazine. Phase 2: a single visual stimulus was displayed on the touchscreen, after which the disappearance of the stimuli coincided with delivery of a food reward, presentation of a tone and illumination of the pellet magazine. Phase 3: mice were required to nose poke a visual stimulus that appeared on the touchscreen to obtain a reward. Phase 4: mice were additionally required to initiate the commencement of a new trial with a head entry into the pellet magazine. Phase 5: In addition to that described for previous phases, responses at a blank part of the screen during stimulus presentation produced a 5-s time-out and were not rewarded. *Dlg2*$^{-/-}$ and *Dlg3*$^{-/y}$ mice showed acquisition rates similar to those of WT littermate controls. *Dlg4*$^{-/-}$ mice were able to successfully complete phases 1 and 2 but were unable to complete phase 3, even after 20 sessions ($^{*}P < 0.05$). (**b**) Pavlovian conditioned approach. Left graph: number of approaches to CS+ and CS– by WT and *Dlg4*$^{-/-}$ mice. WT: mixed between-within subjects ANOVA, main effect of stimulus (CS+ versus CS–) $P < 0.001$, stimulus × session interaction $P < 0.001$, *post hoc* paired-samples *t*-test $^{***}P < 0.001$. Right graph: approach latency to CS+ and CS–. Independent-samples *t*-test, $^{*}P < 0.05$. (**c**) Visual discrimination learning. Total number of trials (left graph) (WT, 210.91 ± 19.76; *Dlg2*$^{-/-}$, 222.7 ± 26.18) (WT, 243.46 ± 18.25; *Dlg3*$^{-/y}$, 173.38 ± 10.06) and errors (right graph) (WT, 64.36 ± 7.9; *Dlg2*$^{-/-}$, 68.0 ± 10.13) (WT, 81.54 ± 8.60; *Dlg3*$^{-/y}$, 57.46 ± 5.27) to reach learning criterion on visual discrimination. Independent-samples *t*-tests, $^{***}P < 0.005$, $^{*}P < 0.05$. (**d**) Object-location paired-associates learning. L, left; C, center; R, right. Percentage of correct responses across training sessions for *Dlg2*$^{-/-}$ (left graph) and *Dlg3*$^{-/y}$ (right graph) mice. *Dlg2*$^{-/-}$: mixed between-within subjects ANOVA, main effect of genotype $P < 0.001$, genotype × session interaction $P < 0.001$, *post hoc* paired samples *t*-test $^{*}P < 0.05$. All values represent mean ± s.e.m.

simple operant response underlying any of the more complex tasks, consistent with the view that simple forms of associative learning are a fundamental basis and prerequisite for at least some, more complex forms of cognition.

The first of these more complex tasks was a form of learning and memory that requires a choice based on perceptual visual discrimination. Mice were presented with two stimuli simultaneously on the screen and required to learn which one was correct (that is, rewarded; the S+) and which was incorrect (that is, unrewarded; the S–; **Fig. 2c**)[19]. In this task, the learning rate of *Dlg3*$^{-/y}$ mice was significantly faster than controls, requiring fewer trials and making fewer errors to learn the task (**Fig. 2c**). In contrast, the performance of *Dlg2*$^{-/-}$ mice was indistinguishable from that of WT mice. This result is notable because it indicates not only that are there differential functions of *Dlg2* and *Dlg3* in visual discrimination learning and that neither mutation impairs basic perceptual processing abilities, but also that the *Dlg3* paralog restrains or attenuates a specific aspect of the cognitive repertoire.

We next increased the associative complexity of the task by incorporating spatial information in an object–location paired associates learning task. In this task the mice were required to learn and remember which of three objects (flower, plane, spider) was associated with one of three locations on the touchscreen (left, center, right, respectively) (**Fig. 2d**)[20,21]. This task therefore requires animals to not only discriminate the objects but also to learn the paired association between the shape and the object's location. On a given trial, only two different objects were presented: one displayed in its correct location (S+), the other in an incorrect location (S–), thereby allowing six possible trial types. Unlike the less complex visual discrimination task, on which the *Dlg3*$^{-/y}$ mutants were faster, in this task they showed normal object-location associative learning and memory. In contrast, *Dlg2*$^{-/-}$ mice were significantly impaired and continued to perform at around 50% (chance level) (**Fig. 2d**). This double genetic dissociation indicates that these two different forms of complex learning (visual and visuo-spatial learning) have distinct genetic regulation, each dependent on a different Dlg paralog.

Dlg2 and *Dlg3* have opposing cognitive actions

Complex environments confront animals not only with stable associative relationships between stimuli, responses and outcomes but also with situations in which these relationships can change. To succeed

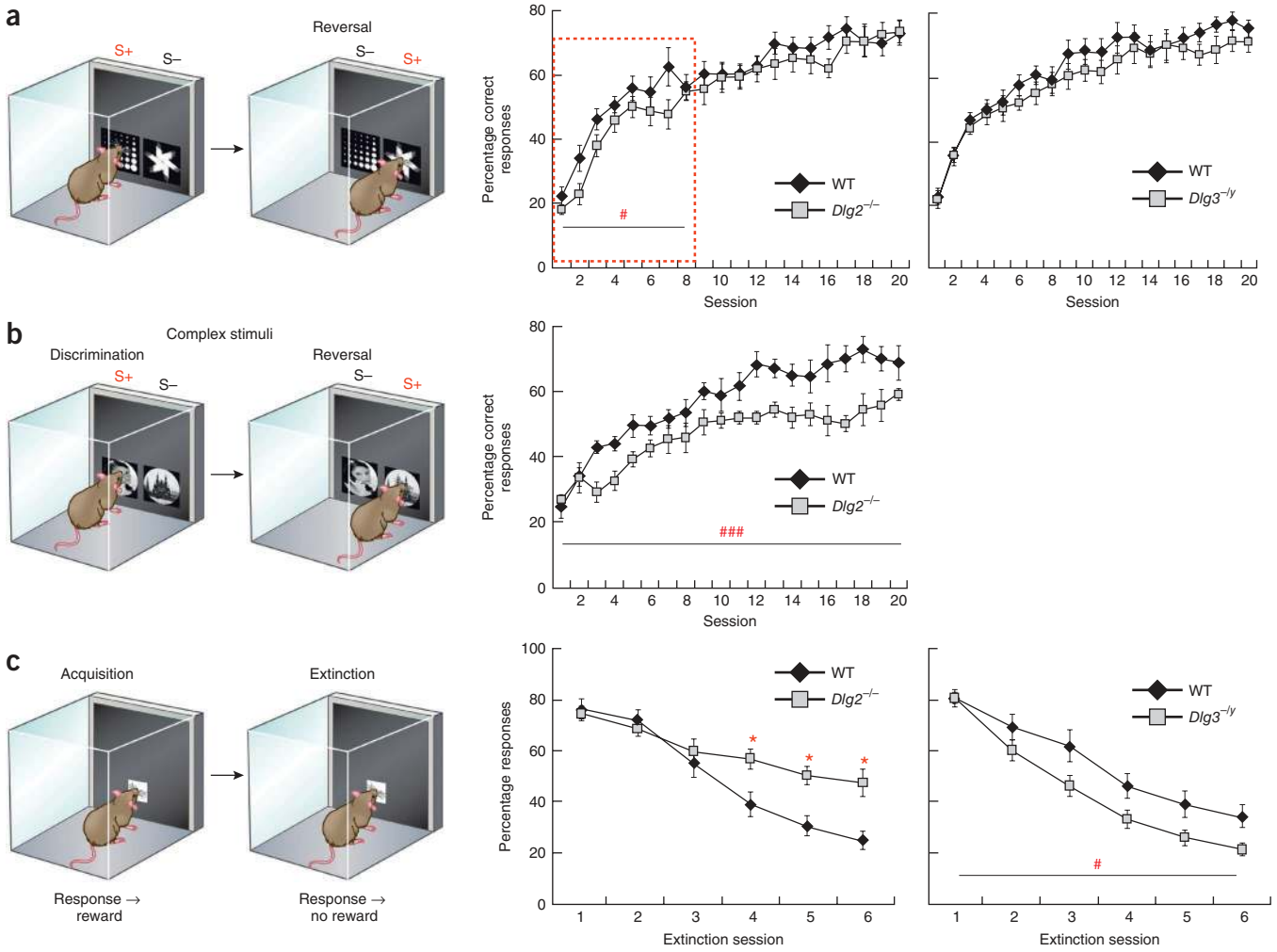

Figure 3 Dlg paralogs have distinct functions in cognitive flexibility and response inhibition. (**a**) Reversal learning. Percentage of correct responses across sessions for *Dlg2*$^{-/-}$ (left graph) and *Dlg3*$^{-/-}$ (right graph) mice. Dotted red rectangle represents the early, perseverative phase of reversal learning. *Dlg2*$^{-/-}$: mixed between-within subjects ANOVA, sessions 1–8, main effect of genotype $^{\#}P < 0.05$. (**b**) Percentage of correct responses by *Dlg2*$^{-/-}$ mice across sessions on reversal learning using complex perceptual stimuli. Mixed between-within subjects ANOVA, main effect of genotype $^{\#\#\#}P < 0.005$. (**c**) Extinction learning. Percentage of responses made across sessions by *Dlg2*$^{-/-}$ (left graph) and *Dlg3*$^{-/y}$ (right graph) mice. *Dlg2*$^{-/-}$: mixed between-within subjects ANOVA, main effect of genotype $P < 0.05$, genotype × session interaction $P < 0.005$, *post hoc* paired-samples *t*-test $^{*}P < 0.05$. *Dlg3*$^{-/y}$: mixed between-within subjects ANOVA, main effect of genotype $^{\#}P < 0.05$. All values represent mean ± s.e.m.

in such environments, animals require greater response control to be able to adapt to such changes. The genes underlying such flexible behavior are unknown. Thus, having established that *Dlg2*$^{-/-}$ and *Dlg3*$^{-/y}$ mice could learn the visual discrimination task, we reversed the reward contingences so that the previously correct option was now incorrect and vice versa (S+ and S– were switched) (**Fig. 3a**) and thereby probed their ability to inhibit the established dominant or prepotent response and acquire the new association[22]. *Dlg3*$^{-/y}$ mutants performed normally, whereas *Dlg2*$^{-/-}$ mice showed impairments. When tested with simple visual stimuli, *Dlg2*$^{-/-}$ mice showed an impairment in the early trials (the 'perseverative' phase of reversal learning, when correct responses are low because of continued responses at the previously rewarded stimulus[22,23]. However, when challenged with more complex visual stimuli with greater perceptual demands, this impairment in reversal learning was more severe and was found across all trials (**Fig. 3b**), whereas we again observed no differences in the initial discrimination learning (trials to criterion: WT, 502.36 ± 58.69; *Dlg2*$^{-/-}$, 560.43 ± 77.01). These results show a noteworthy dichotomy of function of *Dlg2* and *Dlg3* in the acquisition and reversal learning of visual discrimination: *Dlg3* regulates the acquisition of the discrimination (and the mutation amplifies the rate of learning), whereas *Dlg2* regulates the reversal or flexibility of the learned information (and the mutation attenuates the rate of reversal learning).

To examine whether another form of behavioral flexibility has the same genetic requirements as reversal learning, we assessed another task for inhibitory response control using a test for extinction learning, which measure the ability to reduce responses when that response no longer results in a favorable outcome. In the touchscreen extinction task, mice are first trained to make a response to a simple visual stimulus (white square) and obtain a food reward, after which extinction is tested by no longer rewarding the stimulus[24]. In the absence of reinforcement, WT mice rapidly decreased their responding (**Fig. 3c**). Both *Dlg2*$^{-/-}$ and *Dlg3*$^{-/y}$ mice displayed normal rates of learning during the acquisition phase of the task (as is consistent with our earlier findings that these genes are not essential for simple operant learning;

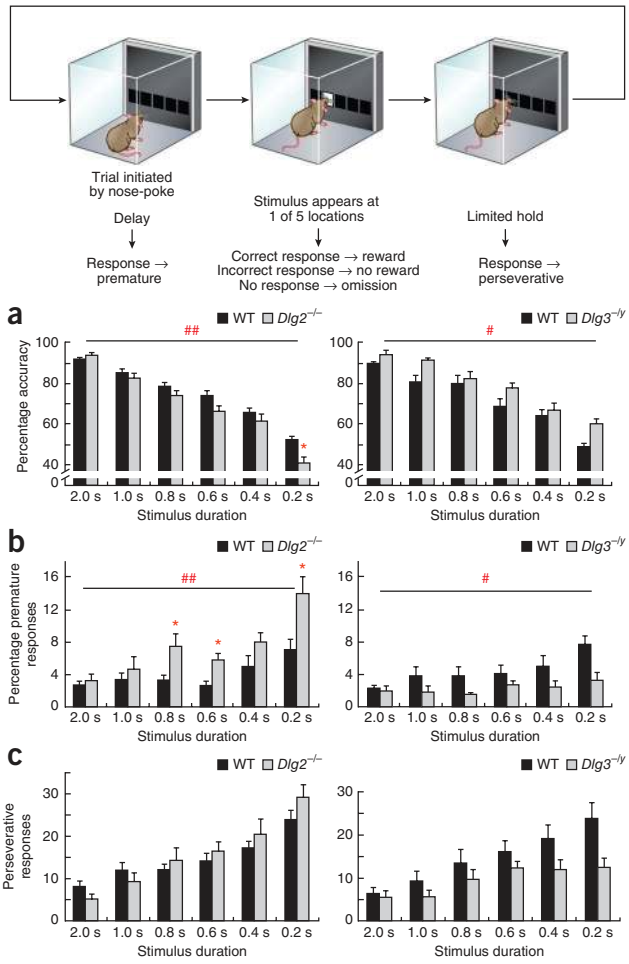

Figure 4 Dlg paralogs are differentially involved in attentional processing and response control. (**a**–**c**) Performance on the 5-CSRTT. See Online Methods for detailed description of task. *Dlg2*$^{-/-}$ mice (graphs on left) and *Dlg3*$^{-/y}$ mice (graphs on right). (**a**) Accuracy (percentage correct responses). (**b**) Percentage premature responses. (**c**) Number of perseverative responses. *Dlg2*$^{-/-}$: accuracy and premature responses, mixed between-within subjects ANOVA, main effects of genotype $^{\#\#}P < 0.01$, genotype × session interaction $P < 0.05$, *post hoc* paired samples *t*-test $^{*}P < 0.05$. *Dlg3*$^{-/y}$: accuracy and premature responses, mixed between-within subjects ANOVA, main effects of genotype $^{\#}P < 0.05$. All values represent mean ± s.e.m.

Supplementary Fig. 3). However, during the extinction phase, not only were there clear phenotypes for both *Dlg2*$^{-/-}$ and *Dlg3*$^{-/y}$ mice, but we found evidence of their opposing function: *Dlg3*$^{-/y}$ mice showed faster extinction, whereas *Dlg2*$^{-/-}$ mutants showed slower extinction (sessions 4–6) (**Fig. 3c**).

The capacity to select optimally when confronted with several alternative choices can put a high premium on divided attentional processing. Attention is not a unitary process but subsumes several processes including constructs such as selective and sustained attention and speed of processing. Attentional capacities can be critical for how well an animal is able to adapt and learn information about the environment. The five-choice serial reaction time task (5-CSRTT) measures sustained, divided attention: animals need to rapidly respond to a brief visual stimulus presented randomly in one of five spatial locations to obtain a reward (**Fig. 4a**; see Online Methods for detailed description). Accurate responding requires attention in both the temporal and spatial domains, and, moreover, the 5-CSRTT measures abnormal responding such as premature or perseverative responses, which are thought to model impulsivity and compulsivity, respectively[25]. We used a recently developed touchscreen version of this method[26] in which mice are first trained to respond to a stimulus displayed for a duration of 2 s and, after they acquire a stable performance level, the duration of the stimulus is decreased, requiring greater attention to accurately detect it. In this task, we again observed opposing functions for *Dlg2* and *Dlg3*. *Dlg3*$^{-/y}$ mice acquired the stable level of performance at the same rate as controls (**Supplementary Table 1**), and, as the stimulus duration decreased, they showed enhanced attentional selection (increased accuracy; **Fig. 4a**), diminished premature responding (**Fig. 4b**) and a trend toward decreased perseverative responses (**Fig. 4c**). In contrast, *Dlg2*$^{-/-}$ mutants took significantly longer to reach stable performance at a stimulus duration of 2 s, as well as at the less stringent condition of a 4-s stimulus duration (**Supplementary Table 1a**). With shorter stimulus durations, *Dlg2*$^{-/-}$ mice showed a significant impairment in accuracy, which was most pronounced at the shortest stimulus duration (0.2 s, with the highest attentional load; **Fig. 4a**), and they made significantly more premature responses (**Fig. 4b**); however, perseverative responding was unaffected (**Fig. 4c**). These data show a remarkable divergence of function, with each of the two closely related *Dlg2* and *Dlg3* genes having opposing influences on several measures of target detection and responding.

Genetic dissection of cognition meta-analysis

The systematic quantitative comparison of *Dlg* paralogs provides a basis for asking general questions about the organization of the behavioral measures with respect to their underlying genetic mechanisms. We can ask three questions: (i) are specific genes required for specific components of the cognitive repertoire, (ii) are there differences between simple and complex cognitive behaviors, and (iii) do any cognitive measures share the same genetic regulation? **Figure 5a** compares the results of all the touchscreen testing in the four lines of mice, with the tasks ordered from simple to complex using the organizational scheme in **Figure 1b**. This analysis shows that the Dlg family is involved in the majority (8 of 12) of the measures of simple and complex forms of cognition. The distinct pattern of gene–phenotype relationships shows that the set of Dlg paralogs confers diversity in the regulation of cognitive responses in the mouse.

In a complementary way to view these data (**Fig. 5b**), the gene-phenotype relationships can be clustered to show four groups of cognitive functions (cognitive clusters 1–4), wherein each cluster consists of the behavioral measures with the same gene dependencies. In cluster 1, simple operant conditioning is characterized by a requirement for *Dlg4*. Cluster 2 (object-location paired-associates learning, reversal learning, acquisition of 5-CSRTT) requires only *Dlg2*. In comparison, the three behaviors in cluster 3 (extinction learning, accuracy and premature responding on the 5-CSRTT) depend on both *Dlg2* and *Dlg3*, with each of these genes having opposing regulatory functions. Cluster 4 (visual discrimination) requires *Dlg3*. Thus, different *Dlg* genes either alone or in combination regulate specific sets of cognitive functions.

Conserved cognitive functions of *DLG2* in humans

Since mice and humans diverged from a common ancestor ~100 Mya, there has been strong conservation in the protein coding of Dlg orthologs (>95% similarity; **Supplementary Fig. 1a–c**) and other postsynaptic proteins[27]. To ask if there has also been conservation in

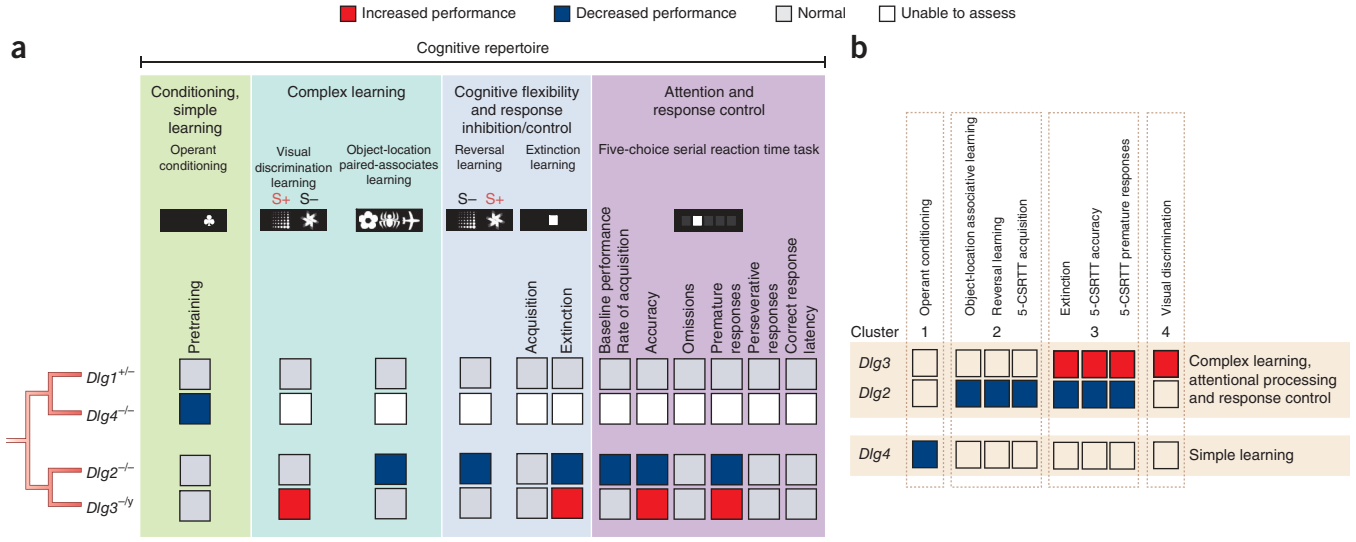

Figure 5 Dlg paralogs have diversified to play distinct roles in different cognitive functions. (**a**) Summary of *Dlg* phenotypes on twelve measures in six cognitive tests. The cognitive repertoire is grouped into four boxes according to **Figure 1b**. Invertebrate *Dlg* mutations have lethal phenotypes, as does mouse *Dlg1*$^{-/-}$; however, presence of a single copy of the *Dlg1* gene (*Dlg1*$^{+/-}$) was sufficient for these mice to perform normally across the different cognitive functions examined. *Dlg4* was essential for simple forms of associative learning. Some cognitive functions were enhanced by a mutation in one *Dlg* gene (*Dlg3*) and attenuated or suppressed by a mutation in another *Dlg* gene (*Dlg2*), revealing that *Dlg2* and *Dlg3* have opposing regulatory functions in more complex cognitive processes. (**b**) Clustering of gene-phenotype relationships shows four groups of cognitive functions (cognitive clusters 1–4).

gene expression in brain regions of mice and humans, we correlated mRNA levels of each of the four vertebrate Dlg paralogs in 17 brain regions in both species. This analysis showed that *Dlg2*, *Dlg3* and *Dlg4* were significantly correlated (Pearson's $R = 0.71$, 0.68 and 0.53, respectively; all $P < 0.05$; **Supplementary Fig. 1d**). Recent studies of the coexpression patterns of Dlg family proteins and mRNAs indicate their importance in human brain function[28–30]. Although these data show conservation in protein sequence and gene regulation, it is unknown whether the cognitive functions of *Dlg* genes are also conserved. Indeed, this has been a general problem in behavioral genetics. Although forms of learning and memory appear to be similar between humans and mice, the conservation of their genetic regulatory mechanisms has been difficult to assess, in part because assessment of cognition in mice has mostly been restricted to spatial learning and memory, and there has thus been a limitation in the comparability of the cognitive tests available for humans and rodents. Taking advantage of the ability to test many aspects of cognition in humans (and other primates) and mice (and other rodents) in the touchscreen system using analogous tasks designed to probe the same cognitive processes, we were able to ask whether the gene–phenotype relationships of the *Dlg* genes are conserved between species.

Humans carrying mutations disrupting the coding region of *DLG2* have been reported[31–34], and we assessed four of these individuals (see

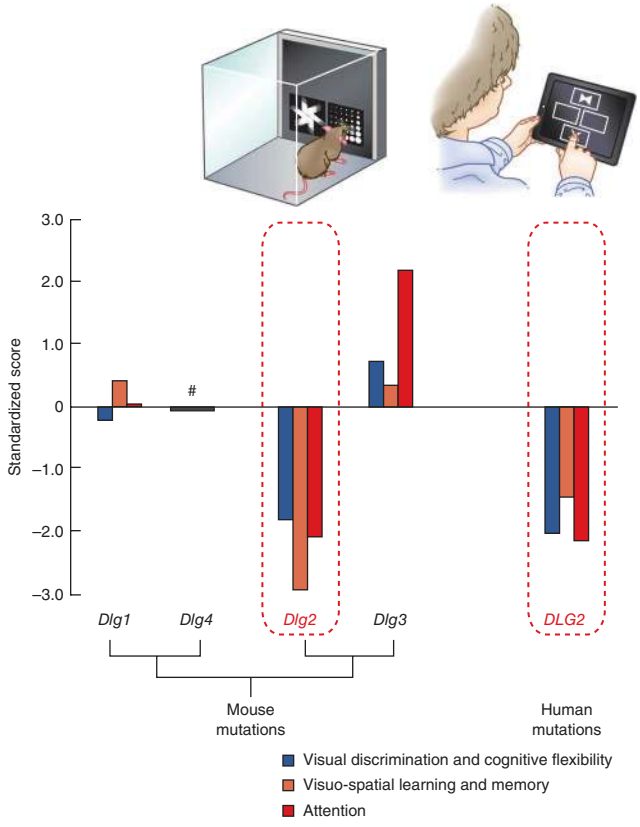

Figure 6 Conservation of *Dlg2* functions in mice and humans. Comparison of performance in touchscreen tasks for mice carrying mutations in *Dlg1*, *Dlg2*, *Dlg3* or *Dlg4* with humans carrying mutations in *DLG2* (see Online Methods). Using the Cambridge Neuropsychological Test Automated Battery (CANTAB), we assessed four people with mutations in *DLG2* on three tasks comparable to those in the rodent touchscreen battery. The intra-extradimensional set-shifting task assessed discrimination acquisition and cognitive flexibility, the paired associates learning task assessed visuo-spatial learning and memory and the rapid visual information processing task assessed sustained attention. A standardized performance score compared to WT littermates or healthy human subjects from the general population is shown, where a negative score indicates poorer than average performance. #Bar denotes the lack of data for comparison owing to the inability to test *Dlg4* mutant mice on any of the three tasks represented.

Online Methods) on a set of cognitive tasks using a touchscreen test battery, the Cambridge Neuropsychological Test Automated Battery (CANTAB). We specifically analyzed three tasks comparable to those in the rodent touchscreen battery: (i) intra-extradimensional set-shifting to assess visual discrimination acquisition and cognitive flexibility (tested using the visual discrimination and reversal learning task in mice), (ii) paired associates learning to examine visuo-spatial learning and memory (tested using the object-location paired-associates learning task in mice) and (iii) rapid visual information processing to assess sustained attention (tested with the 5-CSRTT in mice) (**Fig. 6**). Consistent with results from *Dlg2*$^{-/-}$ mice, we found that humans with mutations in *DLG2* made significantly more errors than healthy control subjects from the general population in tests of visual discrimination acquisition and cognitive flexibility (total errors in intra-extradimensional set-shifting: controls, 27.13 ± 1.52; subjects with *DLG2* mutations, 94.25 ± 51.86; $P < 0.005$) and visuo-spatial learning and memory (total errors in paired associates learning: controls, 16.68 ± 0.68; subjects with *DLG2* mutations, 38.25 ± 14.57; $P < 0.005$). In addition, humans with mutations in *DLG2* also showed decreased accuracy compared to controls in a test for sustained attention (accuracy of target detection in rapid visual information processing: controls, 0.91 ± 0.005; subjects with *DLG2* mutations, 0.8125 ± 0.02; $P < 0.005$), an effect similar to the impaired response accuracy seen in *Dlg2*$^{-/-}$ mice. Using the highly comparable performance measurements derived from the mouse and human touchscreen tests, we analyzed the same performance parameter (for example, total errors made) from each test to calculate a standardized performance score (*z*-score) compared to controls, where a negative score indicates poorer than average performance. Comparison of the profile of cognitive phenotypes observed in human *DLG2* mutations showed a notable degree of similarity to the pattern of cognitive phenotypes seen in mice with *Dlg2* mutations (**Fig. 6**). This similarity in the human-mouse *Dlg2* cognitive profile and its distinction from that of the three other *Dlg* genes is further reinforced by published and unpublished data from another 13 different genetically modified lines of mice tested in some of the same touchscreen tasks, which do not show the selective *Dlg2* phenotype profile (data not shown).

DISCUSSION

Paralog diversification, cognitive complexity and disease

Our genetic dissection in mice suggests how different components of the cognitive repertoire are related at the genetic level, and how genome evolution produced the range of vertebrate behavioral responses. Our test battery comprised seven tests (with 13 primary measures), and each of these required the function of at least one Dlg paralog, revealing that this gene family is central across all aspects of cognition tested. Notably, each vertebrate paralog had a different phenotypic profile, indicating that each gene has evolved a distinct contribution to the cognitive repertoire. One example of this divergence was the opposing direction of the phenotypes of *Dlg2* and *Dlg3* in complex cognitive behaviors. Moreover, whereas these two genes had no influence on simple conditioning, *Dlg4*, in contrast, was essential for simple forms of learning. A parsimonious model is that *Dlg4* retained an ancestral (invertebrate) function in simple forms of learning, whereas the diversification of *Dlg2* and *Dlg3* provided novel regulation of complex cognitive processes arising in vertebrates. The grouping of different behaviors (**Fig. 5b**) according to their distinct genetic underpinnings shows that it is possible to identify relationships between cognitive functions on the basis of common and distinct genetic mechanisms, which is an approach that can extend previous studies based on neuroanatomy and pharmacology[35,36].

The reciprocal effects of *Dlg2* and *Dlg3* on complex behaviors reported here suggest these two genes are essential in balancing or tuning the synaptic signaling machinery. This is supported by electrophysiological studies of synaptic long-term potentiation (LTP) in synapses in the CA1 region of the hippocampus, where *Dlg2*$^{-/-}$ mutants have reduced LTP[37] and *Dlg3*$^{-/y}$ mutants have enhanced LTP[13]. *Dlg4*$^{-/-}$ mutants show more severe LTP phenotypes[14,37] than *Dlg2*$^{-/-}$ (ref. 37) or *Dlg3*$^{-/y}$ (ref. 13) mutants, which suggests that a more severe disruption to activity-dependent synaptic strengthening is reflected in impairments in simple forms of learning. These differential roles likely reflect the distinct intracellular signaling functions mediated by Dlg proteins with their interacting proteins in the membrane-associated guanylate kinase (MAGUK)-associated signaling complexes. The accompanying article[38] reports differential association between Dlg paralogs and NMDA receptor GluN2 paralogs. Our data showing the conserved role of *Dlg2* in cognition in mice and humans, together with the conservation in expression between brain regions and protein sequence, indicates that it is the conservation at the genomic level that maintained these functions between the two species.

Human mutations in *DLG2* and *DLG3* have been reported in psychiatric disorders[31–34,39], and mouse models of psychiatric diseases rely on conservation of mechanisms with humans. Rare human *DLG2* mutations have been associated with schizophrenia in three independent studies of copy number variants[31–34], and three of the four subjects in our study have this disease (the fourth subject is the youngest and at increased risk of developing the illness). The cognitive impairments observed in *Dlg2*$^{-/-}$ mice parallel those observed in patients with schizophrenia, such as deficits in reversal learning[40,41], object-location paired-associates learning[42], extinction[43] and attention[44]. Cognitive impairments are also observed in humans with *DLG3* mutations[39], and we found that *Dlg3*$^{-/y}$ mutant mice displayed enhanced visual discrimination ability and augmented attentional function and response control. In humans, enhanced or superior performance in some cognitive domains, particularly those associated with perceptual processing, is reported in individuals with autism[45]. It is noteworthy that Dlg proteins interact with neuroligin, Shank, DLGAP2 and GluN2 proteins, which are mutated in autism[46]. Mutations in the Dlg family and their interacting proteins cause other diseases with a spectrum of cognitive and motor phenotypes[27,47].

Our data support the model that genome duplication and diversification at the base of chordate evolution around ~550 Mya was a driver of the expansion in complexity of the cognitive repertoire of vertebrates. This genomic mechanism, known to be important in generating complexity in other vertebrate biological systems[48,49], expanded the complexity of vertebrate synaptic signaling processes[50] before the anatomical diversification in many brain regions and encephalization that characterizes the tetrapod brain. Evidence that expansion of vertebrate postsynaptic signaling proteins is a general mechanism driving vertebrate behavioral complexity is supported by a study of GluN2 paralogs[38]. Notably, conservation of *Dlg2's* role in human and mouse cognition over the ~90 million years since these two mammals shared a common ancestor suggests that genomic mechanisms underpin these (disease-relevant) behaviors despite 1,000-fold differences in brain size.

Whereas on one hand these results show that genome duplication in *Dlg* and other postsynaptic gene families endowed vertebrates with an expanded and flexible set of cognitive functions, on the other hand it indicates that benefits to the behavioral repertoire came at the price

of susceptibility to mental illness because disease-causing mutations occur in these new paralogs. Our comparative touchscreen approach also demonstrates the feasibility of co-clinical trials, using humans and mice carrying the same mutations, aimed at identifying treatments for these illnesses. Together with human genome sequencing, the quantitative testing of human cognitive functions using computerized touchscreen test batteries should aid in understanding the genetic basis of cognition and its diseases.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.N., N.H.K., L.M.S., T.J.B. and S.G.N.G. conceived and designed the experiments. J.N. performed all mouse experiments and all analysis in the manuscript. A.M. administered CANTAB tests. M.J. performed *DLG2* CNV genotyping. A.M., D.H.B. and D.S.C. collected clinical data. R.D.E. provided sequence analysis and L.N.L. gene expression correlation analysis. J.N., T.J.B. and S.G.N.G. wrote the manuscript with input from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the online version of the paper.

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ONLINE METHODS

Mice. *Dlg1* heterozygous mice ($^{+/-}$) and WT littermates were generated from *Dlg1* heterozygous intercrosses and maintained on 129S5/SvEvBrd background. Homozygous knockout mice (denoted by $^{-/-}$ with the exception of male *Dlg3* null mutants, which are denoted by $^{-/y}$, as *Dlg3* is located on the X chromosome) and WT littermates were generated from heterozygous intercrosses of *Dlg2* (ref. 12), *Dlg3* (ref. 13) and *Dlg4* (ref. 14) mice and maintained on a C57BL/6J background. Male and female knockout mice from all lines developed normally to adulthood, exhibited normal body size and showed no gross abnormalities. Mice were housed in mixed groups of WT and knockouts on a 12-h light/dark cycle and all behavioral testing conducted during the light phase of the cycle. Two separate cohorts of male mice (n = 10–15 for each cohort) from each knockout line were used for cognitive testing on the touchscreen tasks. Mice were maintained on a restricted diet at or above 85% of their free-feeding body weight during behavioral testing. Water was available *ad libitum* throughout the experiment. All experimentation was conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986).

Cognitive testing in the touchscreen operant system. *Apparatus.* Testing was conducted in a touchscreen-based automated operant system consisting of an operant chamber (21.6 × 17.8 × 12.7 cm) with clear Perspex walls and stainless steel grid floor, housed in a sound- and light-attenuating box (40 × 34 × 42 cm) (Med Associates, St Albans, VT). A dispenser delivering reward pellets (14 mg, BioServ, Frenchtown, NJ) into a magazine, a house light, and a tone generator were located at one end of the chamber, and at the opposite end was a flat-screen monitor equipped with an infrared touchscreen (16 × 21.2 cm) (Craft Data Limited, Chesham, UK). A black Perspex 'mask' with windows was positioned in front of the touchscreen, allowing the presentation of stimuli to be spatially localized and prevented animals from accidentally triggering the touchscreen. Stimuli presented on the screen were controlled by custom software ("MouseCat," L.M.S. and C. Romberg), and responses made via nose-pokes at the stimuli were detected by the touchscreen and recorded by the software.

Pre-training and operant conditioning. Before testing, mice were first slowly reduced to and then maintained at or above 85% free-feeding body weight. Animals were then trained through five phases for instrumental operant conditioning similar to that previously described[24]. Mice were required to successfully complete the set criterion at each phase before advancing to the next phase. Briefly, mice were habituated to the operant chamber and to eating reward pellets from the magazine by being placed in the chamber for 20 min on 2 d and required to consume a set number of pellets that were freely available in the magazine (phase 1). In phase 2, a single visual stimulus was displayed on the screen for 30 s, after which disappearance of the stimulus coincided with delivery of a food reward, presentation of a tone and illumination of the pellet magazine (criterion: 30 trials in 60 min). For phases 2–5, a trial did not advance until the pellet was consumed. Mice then learned to nose-poke visual stimuli that appeared on the screen to obtain a reward (phase 3, criterion = 30 trials in 60 min) and to initiate each new trial with a head entry into the pellet magazine (phase 4, criterion = 30 trials in 60 min). In the last pre-training phase (phase 5), responses at a blank part of the screen during stimulus presentation produced a 5-s time-out (signaled by extinction of the house light, magazine inactive) to discourage indiscriminate screen responding (criterion = 21/30 correct responses in 60 min on 2 consecutive days). All values reported represent mean ± s.e.m.

Pavlovian conditioned approach (autoshaping). Testing was carried out in the Campden Instruments Bussey-Saksida touchscreen chamber (Campden Instruments Ltd, UK). Mice were habituated to the chamber over two daily sessions. On the first habituation session, mice were placed in the chamber for 20 min and a single delivery of reward (70 µl strawberry milkshake, Yazoo Campina Ltd) given at the beginning of the session. On the second habituation session, mice were placed in the chamber and, following a variable interval (VI, mean 40 s), delivery of a liquid reward (20 µl) coincided with illumination of the magazine light and a tone. A nose-poke into the magazine was required before the VI restarted and another trial began. Animals were observed during both habituation sessions to ensure animals successfully consumed all rewards and completed 40 trials in 60 min (on the second session) before progressing to the task.

Mice were trained to associate the presentation of a white rectangle stimulus (10 s duration) at a specific location with delivery of a reward. For example, if the stimulus was presented on the left side of the screen, it was designated the CS+ and signaled the delivery of a reward immediately after the offset of this stimulus; the other stimulus (presented on the right side of the screen) was designated the CS– and was never followed by reward delivery. Designation of the location of the CS+ (left or right) was counterbalanced between animals. Stimuli were presented on a VI (mean 40 s) schedule, and training consisted of 40 trials per session per day (20 presentations each of CS+ and CS–, presented in a pseudorandom order) for four sessions. To commence or initiate a trial, animals were required to nose-poke at the back of the chamber, ensuring that animals were centrally located at the rear of the chamber when stimuli were presented and eliminating chance approaches to the stimuli. Approaches to a stimulus were measured via an infrared beam detector. Group differences were analyzed using a mixed between-within subjects ANOVA with conditioned stimulus (CS+, CS–) as the between-subjects factor and session as the within-subjects factor. A paired-samples *t*-test was used for *post hoc* analysis to assess whether there were significant between × within-subjects interaction effects. All values reported represent mean ± s.e.m.

Visual discrimination and reversal learning. Mice were trained to discriminate between two novel, approximately equiluminescent stimuli presented in a spatially pseudorandomized manner over 30-trial sessions[24]. Responses at one stimulus (S+, correct) resulted in a reward; responses at the other stimulus (S–, incorrect) resulted in a 5-s time-out followed by a correction trial (correction error), whereby the trial was repeated until a correct choice was made. Stimuli were displayed on the screen until a response was made. Acquisition criterion for visual discrimination was attaining 80% correct responses (excluding correction trials) on 2 consecutive days, following which, mice were moved on to the reversal phase on the next session, where the designation of the same discriminated stimuli as correct versus incorrect was reversed. Reversal performance was tested over 30-trial sessions for 20 sessions.

Group differences were analyzed using an independent-samples *t*-test or a mixed between-within subjects ANOVA with genotype as the between-subjects factor and session as the within-subjects factor. A paired-samples *t*-test was used for *post hoc* analysis to assess whether there were significant between × within-subjects interaction effects. All values reported represent mean ± s.e.m.

Object-location paired-associates learning. Mice were tested for the ability to associate between three objects (flower, plane and spider) and three correct spatial locations on the touchscreen (left, center and right, respectively)[20,21]. For each trial, only two objects were presented: one object in its correct location (S+) and the other object in one of two incorrect locations (S–); therefore, there were six possible trial types. A nose-poke to the S+ resulted in delivery of a reward, and incorrect responses resulted in a 5 s time-out followed by correction trial. Nose-pokes to response windows in which no stimulus was presented were ignored. Mice were given 36 trials per session per day for 50 sessions. Group differences were analyzed using a mixed between-within subjects ANOVA with genotype as the between-subjects factor and session block as the within-subjects factor. A paired-samples *t*-test was used for *post hoc* analysis to assess whether there were significant between × within-subjects interaction effects. All values reported represent mean ± s.e.m.

Extinction. To examine acquisition and extinction of an instrumental response, mice were required to respond to a stimulus (single white square) presented in the center of the screen to obtain a reward. During acquisition, the stimulus remained on the screen until a response was made. The acquisition criterion was defined as completing 30 trials within 12.5 min on each of five consecutive sessions. Following acquisition, extinction was assessed by trials on which responses to the stimulus were no longer rewarded. The stimulus was displayed for 10 s and animals given 30 trials per session per day for six sessions. Group differences were analyzed using a mixed between-within subjects ANOVA with genotype as the between-subjects factor and session as the within-subjects factor. A paired-samples *t*-test was used for *post hoc* analysis to assess whether there were significant between × within-subjects interaction effects. All values reported represent mean ± s.e.m.

Five-choice serial reaction time task (5-CSRTT). The 5-CSRTT task procedure in the touchscreen was similar to that previously described[26,51]. Mice were trained to respond to presentations of a white square box that was pseudorandomly displayed in one of five spatial locations on the touchscreen. Each trial commenced with the illumination of the magazine light. A nose-poke to the magazine initiated the commencement of a trial and then a 5-s fixed delay during which,

if the animal prematurely touched the screen, the response was recorded as a premature response and a 5-s time-out given, followed by a 5-s inter-trial interval. The stimulus was then displayed (initially for 4 s), followed by a limited holding period. Responses during stimulus presentation were recorded either as correct (response to the stimulus window) or incorrect (response to any other window). A correct choice was signaled by a tone and delivery of reward pellet. An incorrect response was punished with a 5-s time-out. A failure to respond to any window either during stimulus display or the limited hold period was recorded as an omission. Responses made during the limited hold period were recorded as perseverative responses.

Mice were required to complete 50 trials in a 60-min session. Once an animal reached a performance criterion (completed 50 trials, >80% accuracy, <20% omissions for 3 out of 4 consecutive days) at 4-s stimulus duration, this was reduced to 2 s until animals attained the performance criterion again. Animals were then tested for 2 d at a 2-s stimulus duration to attain the baseline performance rate, following which the stimulus duration was reduced to 1.0, 0.8, 0.6, 0.4 and 0.2 s. Animals were tested 2 consecutive days at a given stimulus duration, then re-baselined at 2-s stimulus duration for at least 2 d or until the animal reattained performance criterion (>80% accuracy, <20% omissions). Percentage accuracy of responding = [correct responses/(correct responses + incorrect responses)] × 100. Percentage of omissions = (number of omissions/total number of trials) × 100. Percentage of premature responses = (number of premature responses/total number of trials) × 100. Number of perseverative responses made, latency to respond (response latency) and latency to collect rewards (reinforcer latency) were recorded. Group differences were analyzed using a mixed between-within subjects ANOVA with genotype as the between-subjects factor and stimulus duration as the within-subjects factor. A paired-samples *t*-test was used for *post hoc* analysis to assess whether there were significant between × within-subjects interaction effects. All values reported represent mean ± s.e.m.

Human *DLG2* analysis: subjects and experimental procedure. Four individuals with copy number variations (CNVs) in *DLG2* participated in this study (see **Supplementary Fig. 4**), of whom two are related (subject 1 is the mother of subject 4). Initial discovery of *DLG2* CNV carriers was made in the International Schizophrenia Consortium GWAS[33] from 1,115 Scottish schizophrenia cases (0.36%). From 978 Scottish control individuals from the general population screened, none were found to have this CNV. To further explore the GWAS results, two different multiplex amplicon quantification (MAQ) assays[52] were used: the first assay included a number of chromosomal regions previously shown to contain CNVs associated with schizophrenia[31,32] and a second assay focused specifically on *DLG2*. Twelve target amplicons comprising exons of *DLG2* and 11 reference amplicons were used (see **Supplementary Table 2**). The study was approved by the Multi-Centre Research Ethics Committee for Scotland, and subjects gave written informed consent for the collection of DNA samples for use in genetic studies.

CANTAB. Subjects were asked to perform a series of four computerized neuropsychological tests in the Cambridge Neuropsychological Test Automated Battery (CANTAB, Cambridge Cognition, Cambridge, UK). Following explanation and successful completion of a simple motor screening task (touching the center point of flashing crosses on the screen), subjects were given four tests in the following order: (i) Spatial working memory (SWM). (ii) Intra-extradimensional set-shifting task (IED): a test of rule acquisition and reversal involving several stages of visual discrimination (in which one of two stimuli is correct) and attentional set-shifting (including stages of reversal where the contingencies change such that the previously correct becomes incorrect). (iii) Rapid visual information processing (RVP): a test of sustained attention (similar to the continuous performance task) that requires individuals to monitor the continuous presentation of strings of numbers and only respond when a target sequence is displayed. (iv) Paired associate learning (PAL): a test of simple visual pattern and visuospatial associative learning. For analogous comparison with mouse data obtained from the touchscreen tasks, data from only three tests are presented here. Detailed descriptions of the three CANTAB tests used can be found on the Cambridge Cognition website http://www.cantab.com/, or see ref. 3.

Data analysis. Individual subject results were compared to the internal normative database of CANTAB (containing data from 3,000 healthy volunteers) and matched for age (a range of 9–15 years) and gender. For IED and PAL, the measure of total errors (adjusted) was used. This is a measure of the subject's efficiency in attempting the test. Thus, while a subject may pass all stages, a substantial number of errors may be made in doing so. It is crucial to note that subjects failing at any stage of the test by definition have had less opportunity to make errors. Therefore, this adjusted score is calculated to take into account each stage not attempted due to failure. For RVP, *A′* was used, which is the signal detection measure of sensitivity to errors, regardless of error tendency (range 0.00 to 1.00, bad to good). In essence, this metric is a measure of how good the subject is at detecting target sequences.

For transformation of the mouse data for comparison, mean group standard *z*-scores were calculated for each *Dlg* mutant line for each task using the following measures: visual discrimination and reversal learning (total errors made across all sessions), object-location paired-associate learning (total errors made across all sessions), five-choice serial reaction time task (average percentage accuracy for 0.2-s stimulus duration).

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