Tequila, a Neurotrypsin Ortholog, Regulates Long-Term Memory Formation in *Drosophila*

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Tequila, a Neurotrypsin Ortholog, Regulates Long-Term Memory Formation in *Drosophila*

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Mutations in the human neurotrypsin gene are associated with autosomal recessive mental retardation. To further understand the pathophysiological consequences of the lack of this serine protease, we studied Tequila (Teq), the *Drosophila* neurotrypsin ortholog, using associative memory as a behavioral readout. We found that *teq* inactivation resulted in a long-term memory (LTM)–specific defect. After LTM conditioning of wild-type flies, *teq* expression transiently increased in the mushroom bodies. Moreover, specific inhibition of *teq* expression in adult mushroom bodies resulted in a reversible LTM defect. Hence, the Teq pathway is essential for information processing in *Drosophila*.

Mental retardation (MR) is the most common handicap in children and young adults, affecting 1 to 3% of the population. The causes of MR are diverse, and genetic and metabolic diseases account for about one-third of cases. Understanding the mechanisms of MR has long been hampered by both the complexity and the heterogeneity of these conditions. This is particularly true for nonsyndromic MRs (i.e., MR with apparently normal brain development and no other clinical features). A mutation in the human neurotrypsin gene (*PRSS12*) has been reported in nonsyndromic MR (*1*). Neurotrypsin is a multidomain neuronal trypsin-like serine protease predominantly expressed in the developing and adult nervous system (*2*). Neurotrypsin might be involved in synaptic development (*2*). However, its exact function remains elusive.

Progress in *Drosophila melanogaster* genetics and similarities between human and fly genomes have made comparative approaches feasible (*3*). MR-associated molecules are remarkably well conserved across the two species; 87% of the genes involved in MR have a fly ortholog. Moreover, in 76% of the cases, the extent and type of amino acid sequence similarities suggest similar functions (*3*). Thus, neurotrypsin and the only *Drosophila* ortholog, Teq, show a high degree of amino acid conservation, particularly in the region of the functional domains (fig. S1).

Whether the cognitive disorders caused by neurotrypsin mutations are due to improper brain maturation or to a primary plasticity defect during information processing is uncertain. To address this issue, we studied the involvement of *teq* in long-term memory (LTM). We used classical conditioning of an odor-avoidance response. In this paradigm, the flies are exposed

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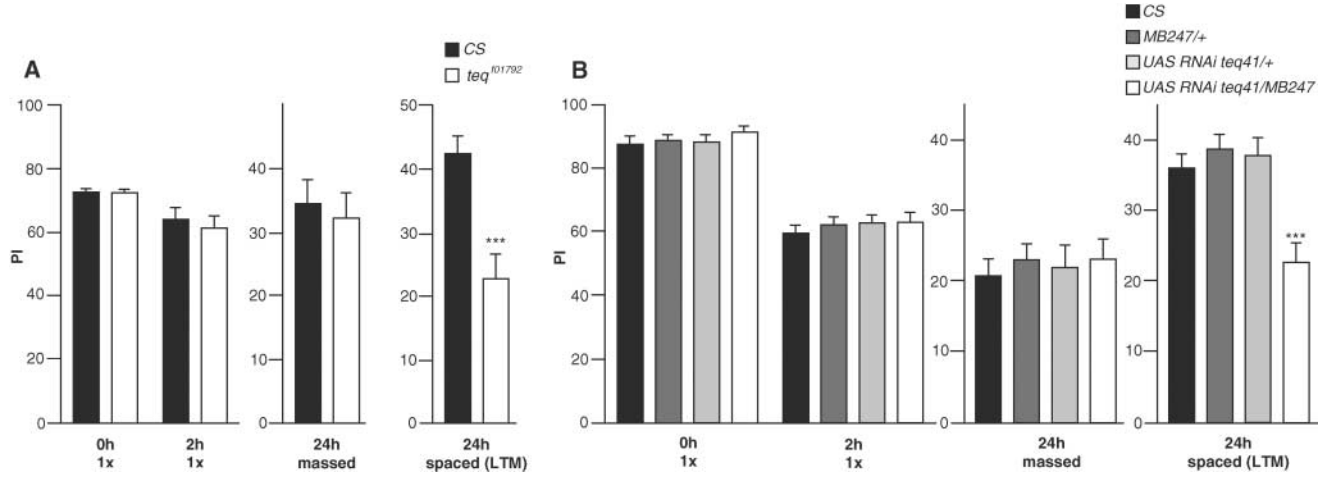

Fig. 1. *teq*^{f01792} mutants show an LTM-specific defect. (**A**) *teq*^{f01792} is an LTM-specific mutant. Performance indices (PIs) were measured at 0 hours (n = 10 groups) and 2 hours (n = 18 or 19 groups) after a single conditioning cycle, 24 hours after massed (n = 13 or 14 groups) or spaced training (n = 14 to 16 groups). Results are means ± SEM; ***$P < 0.001$ (t test). (**B**) Inhibition of *teq* expression in the MBs leads to a LTM-specific defect. The *MB247* Gal4 line shows expression in neurons that project to all MB lobes. PIs were measured at 0 hours (n = 10 groups), 2 hours (n = 35 groups) after a single conditioning cycle, and 24 hours after massed (n = 20 groups) or spaced training (n = 29 to 33 groups). Results are means ± SEM; ***$P < 0.001$ (t test).

to two distinct odors, one of which is accompanied by an electric shock (*4*). With repeated and spaced training bouts, LTM is formed that is dependent on new protein synthesis (*5*). In the absence of rest intervals between training sessions ("massed training"), a distinct form of memory is produced that does not require protein synthesis (*5*). A *piggyBac* insertion in the *teq* gene (referred to as teq^{f01792}) (*6*) was shown to decrease Teq expression (fig. S2). The mutation was first outcrossed over 10 generations to shift its genetic background to that of the reference strain *Canton-Special* (*CS*). Interestingly, teq^{f01792} displayed a decrease in 24-hour LTM after spaced training, whereas a normal 24-hour memory capacity was observed after massed training (Fig. 1A). After a single conditioning, teq^{f01792} learning and 2-hour memory were also normal, showing that teq^{f01792} is a LTM-specific mutant.

The mushroom bodies (MBs) are bilateral symmetrical structures of the *Drosophila* brain (*7*) (Fig. 2B) essential for olfactory learning and memory. MBs play a key role in LTM (*8*, *9*). We therefore examined the integrity of these structures in the *teq* mutant by immunohistochemistry using antibodies to fasciclin II (*10*) or to protein kinase A catalytic subunit (*11*) and paraffin sections (figs. S3 and S4). No structural defect was detected in MBs, which suggested that the teq^{f01792} mutation does not impair LTM via an abnormal development of MBs. However, more subtle developmental defects may not have been detected at this level of resolution.

To specifically silence the *teq* gene in the MBs, we took advantage of the GAL4-UAS system combined with RNA interference (RNAi) (*12*). We generated transgenic flies expressing a *teq* RNAi construct under the control of

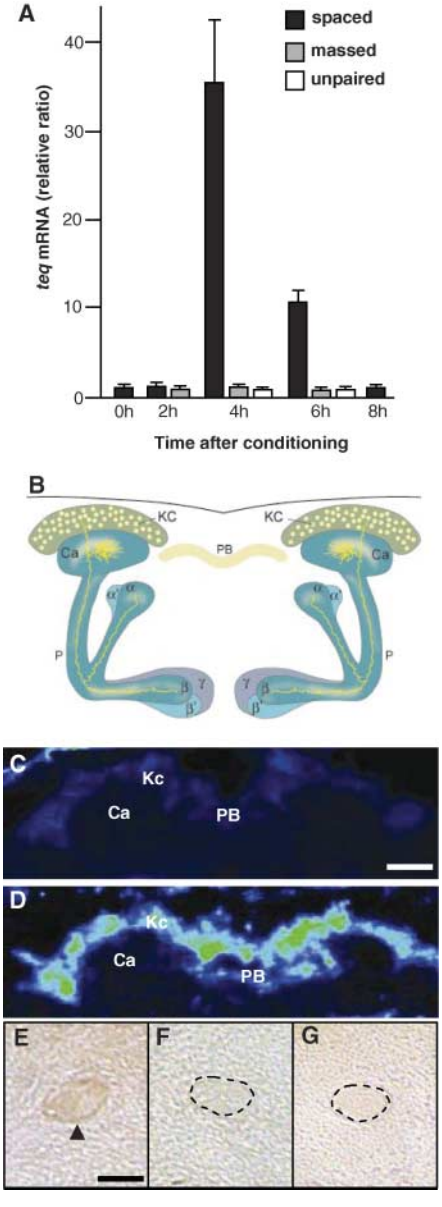

Fig. 2. *teq* mRNA expression is up-regulated after LTM conditioning. (**A**) The level of head *teq* mRNA is up-regulated after LTM conditioning. Heads of *CS* flies were collected at different times after spaced training, total RNA was extracted, and quantitative RT-PCR was performed. Quantitative RT-PCR experiments indicate that the level of *teq* RNA is up-regulated from 4 to 6 hours after spaced training (n = 4 to 9 groups). No significant changes were observed after massed (n = 5 to 7 groups) or unpaired training (n = 4 to 6 groups). The ratio represents [*teq* mRNA (trained)/*tub* mRNA (trained)]/[*teq* mRNA (naïve)/*tub* mRNA (naïve)]. (**B**) Schematic representation of the adult *Drosophila* MBs. Each of the MBs comprises about 2500 parallel-packed neurons that are organized into distinct computational networks. The MB cell bodies (Kenyon cells, KC) are located at the dorsal cortex, extending their dendrites into the calyx (Ca), which receives olfactory information from the antennal lobes. More distally, MB axons project to the anterior portion of the brain via a dense structure known as the peduncle (P), where they give rise to five major lobes (α, α′, β, β′, and γ) (*6*). PB, protocerebral bridge. (**C** and **D**) FISH of *CS* brain sections at the protocerebral bridge level. Each probed slide carried a mixture of brains from naïve (C) and trained flies (D) to ensure identical treatment. *teq* mRNA is expressed in Kenyon cells 4 hours after conditioning. Scale bar, 50 μm. (**E** to **G**) Teq immunostaining on brain sections at the peduncle level. Five hours after the end of the training, Teq is detected in the MB peduncle (arrowhead) of the conditioned flies (E), whereas no staining is detected in conditioned teq^{f01792} mutant (F) or naïve *CS* flies (G). Dotted lines outline the peduncle limits. Scale bar, 20 μm.

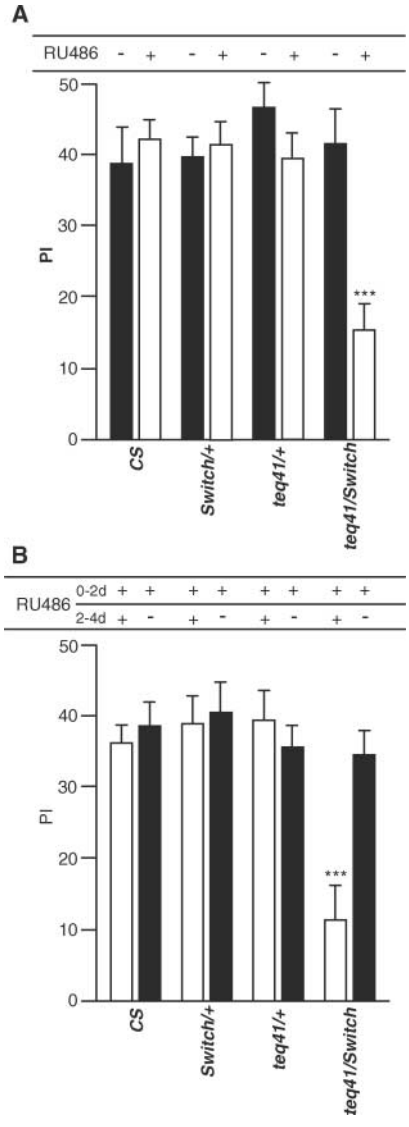

Fig. 3. *teq* is acutely required for LTM formation. (**A**) Expression of *teq* RNAi in adult MBs leads to an LTM defect. PIs were measured 24 hours after spaced training (n = 11 to 14 groups). Flies were fed during 2 days with food supplemented with 200 μM RU486. There was no effect of RU486 administration on LTM of control flies. Results are means ± SEM. ***P < 0.001 (t test) with the appropriate genetic control: *UAS RNAi teq 41/MB247-Switch* (+RU486) versus *UAS RNAi teq 41/MB247-Switch* (–RU486). (**B**) LTM impairment in conditional *teq* mutant is reversible. Flies were fed during 2 days with food supplemented with 200 μM RU486 to induce *teq* RNAi expression. After this period, flies were transferred onto regular food or food with RU486. Flies recovered a normal LTM capacity after 2 days without RU486, whereas continuous expression of the *teq* RNAi led to the typical LTM defect. PIs were measured 24 hours after a spaced training (n = 15 to 18 groups). Results are means ± SEM. ***P < 0.001 (t test) with the appropriate genetic control: *UAS-RNAi teq 41/MB247-Switch* (–RU486) versus *UAS-RNAi teq 41/MB247-Switch* (+RU486).

MB247, a specific GAL4 driver of larval and adult MBs (*4*, *13*). RNAi-mediated *teq* knockdown induced a decrease in 24-hour LTM, whereas 24-hour memory after massed training was unaffected, as were learning and 2-hour memory after single conditioning (Fig. 1B). This effect was found to be independent of the insertion site of the RNAi construct (fig. S5A). Thus, the inactivation of *teq* in the MBs resulted in a LTM-specific defect similar to that induced by the *piggyBac* constitutive mutation. Similar LTM defects were observed with two additional MB GAL4 drivers, Gal5122 (*14*) and 238Y (*15*) (fig. S5B). As expected, no alteration of MB morphology was observed in *Gal4/UAS-RNAi teq* mutants (figs. S3 and S4).

Because LTM formation requires de novo protein synthesis that depends partly on transcriptional regulation (*5*, *14*, *16*), we investigated whether *teq* expression was regulated after LTM conditioning in the wild-type fly. Head RNAs were extracted at various times after spaced training, and levels of *teq* mRNA were assayed by quantitative reverse transcription polymerase chain reaction (RT-PCR) (*4*). The *teq* mRNA level was increased 4 to 6 hours after the end of LTM training (Fig. 2A), whereas no change was observed 2 or 8 hours after training. No variation in *teq* expression was observed in flies subjected to massed training, nor in pseudo-conditioned flies that received the odor and electric shock stimuli in a temporally dissociated manner, a protocol that does not induce learning (*5*) (Fig. 2A).

To identify brain structures in which the *teq* gene was overexpressed, we performed fluorescence in situ hybridization (FISH) on brain sections after a spaced training (*4*). *teq* mRNA was strongly expressed in MB cells 4 hours after LTM training, whereas it was weakly expressed in naïve flies (Fig. 2, C and D). The time course of the gene expression observed in situ correlated with that observed after quantitative RT-PCR. In a second step, polyclonal antibodies were raised against Teq protein. Five hours after LTM training, the Teq protein was detected by antibodies to Teq in the MB peduncles (*4*) (Fig. 2E). No noticeable staining was found in the peduncle of naïve wild-type flies or in trained teq^{f01792} flies (Fig. 2, F and G). A signal was observed in the cortical region of the MB in naïve and trained CS flies (fig. S6). It is noteworthy that Teq expression was observed neither in the dendritic region of the MBs nor in MB lobes in naïve or trained CS flies (fig. S6).

The observation that *teq* expression is up-regulated in MBs after LTM conditioning strongly suggests that Teq is physiologically involved in brain plasticity. To further support this hypothesis, we used the inducible Gene-Switch system (*17*). In this system, the DNA binding domain of the GAL4 protein is fused to the progesterone receptor to generate the RU486-inducible chimeric activator. In the absence of RU486, the Gene-Switch is in the "off" state. In the presence of hormone, the chimeric protein undergoes a conformational change and it can bind to a UAS sequence and activate transcription of the RNAi construct. Specific expression of *teq* RNAi in adult MBs led to a strong LTM deficit (Fig. 3A and fig. S5C) with no obvious structural anomalies in the MBs (fig. S3).

To further study the dynamics of *teq* involvement in this process, we addressed the question of whether expressing Teq in a previously Teq-defective fly restored normal LTM capacity. Hence, *teq* RNAi was first induced for 2 days in adult MBs. *MB247-Switch/UAS-RNAi* flies were then transferred to food without RU486 to restore MB *teq* mRNA expression. These flies regained a normal 24-hour LTM capacity (Fig. 3B), thus demonstrating that the lack of Teq has reversible consequences for *Drosophila* brain function.

Several studies have emphasized a role of serine proteases in the nervous system (*18*, *19*). During neural development, serine proteases contribute to cell migration, axon outgrowth, and synapse elimination (*20*). In adult life, they play a role in neuropeptide processing, regulation of neuronal survival, and structural plasticity associated with learning and memory processes. Mentally retarded children with neurotrypsin mutations have normal milestones of psychomotor development over the first 18 months and become retarded starting at 2 years of age (*1*), suggesting a specific role of neurotrypsin in postnatal cognition processes (*20*). It is therefore important to determine whether their cognitive disorder is due to improper brain maturation at the ultrastructural level or to a physiological dysfunction. The results obtained with *Drosophila teq* support the view that the Teq pathway (and by analogy the neurotrypsin pathway in humans) is essential for information processing and functional plasticity because (i) *teq* mutants have a LTM-specific defect, (ii) *teq* mRNA is up-regulated during a short time window after spaced training, (iii) *teq* is specifically required in adult MBs, and (iv) impairment of LTM capacity after transient *teq* silencing is reversible. Further experiments will be required to determine the function of Teq within the adult MBs, in particular at the level of the peduncle.

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