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ORIGINAL PAPER

KF-1 Ubiquitin Ligase: Anxiety Suppressor Model

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Abstract Anxiety disorders are the most popular psychiatric disease in any human societies irrespective of nation, culture, religion, economics or politics. Anxiety expression mediated by the amygdala may be suppressed by signals transmitted from the prefrontal cortex and hippocampus. KF-1 is an endoplasmic reticulum (ER)-based E3-ubiquitin (Ub) ligase with a RING-H2 finger motif at the C-terminus. The *kf-1* gene expression is up-regulated in the frontal cortex and hippocampus in rats after anti-depressant treatments. The *kf-1* null mice show no apparent abnormalities, but exhibit selectively pronounced anxiety-like behaviors or increased timidity-like responses. The *kf-1* orthologous genes had been generated after the Poriferan emergence, and are found widely in all animals except insects, arachnids and threadworms such as *Drosophila*, *Ixodes* and *Caenorhabditis*, respectively. This suggests that the *kf-1* gene may be relevant to some biological functions characteristic to animals. Based on these observations, the Anxiety Suppressor Model has been proposed, which assumes that KF-1 Ub ligase may suppress the amygdala-mediated anxiety by degrading some anxiety promoting protein(s), such as a neurotransmitter receptor, through the ER-associated degradation pathway in the frontal cortex and hippocampus. According to this model, the emotional sensitivity to environmental stresses may be regulated by the cellular protein level of KF-1 relative to that of the putative anxiety promoter. The *kf-1* null mice should be useful in elucidating the molecular mechanisms of the anxiety regulation and for screening novel anxiolytic compounds, which may block the putative anxiety promoter.

Keywords Anxiety · Timidity · Emotional control · Frontal cortex · Hippocampus · Amygdala

Introduction

Anxiety, or learned fear, may be an instinct acquired during the animal evolution to promote adaptive survival by evading unnecessary danger. Anxiety disorders may be ascribed to enhanced sensitivity against the environmental stresses, which bring about heterogeneous phenotypes such as obsessive–compulsive disorders, panic disorder, adaptive disorder, post-traumatic stress disorder, social withdrawal (hikikomori) disorder, various phobias, and so on. Furthermore, anxiety can be a basic symptom of other psychiatric diseases, including depression and schizophrenia. Patients with anxiety disorders interpret circumstantial incidences such as episodes, comments, expressions, relations to others and social and physical positions in a negative way. Such interpretations may well lead individuals to pronounced capture or delusions and cause emotional disruption. Under the evolutionary constraint, it is likely that

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complex regulation of emotional expression has developed in animals. This is most apparent in highly socialized mammals like humans [1]. The amygdala may primarily be responsible for the expression of anxiety or fear, and the prefrontal cortex and hippocampus may play an important role in fear extinction by suppressing the amygdala-mediated fear expression [2, 3]. Nevertheless, the molecular mechanisms involved in the regulation of anxiety expression are not clear and remain largely unknown. However, it has been demonstrated, using genetic mouse models, that the serotonin receptor and transporter and Disc1 protein are involved in the onset of depression and schizophrenia [4–6]. Although some genes have been reported to affect behavioral anxiety and despair [4, 7–14], anxiety- and despair-like behaviors are often confused or complicated and it may not even be clear whether or not anxiety and depressive disorders are distinguishable at the molecular levels in the animal models. As far as the authors are aware, until recently there were no reports of specific genes being responsible for anxiety- or timidity-like behaviors, and not being implicated in other abnormalities.

KF-1 Ubiquitin (Ub) Ligase

Ubiquitylation plays an essential role in regulating critical eukaryotic cellular processes. Proteasomal degradation through the endoplasmic reticulum-associated degradation (ERAD) pathway is no exception. These processes are involved in a variety of human somatic diseases such as cancer, viral infection, hypertension, diabetes, inflammation, muscle wastage, and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases [15, 16]. However, no ubiquitin ligases have been reported until recently as being involved selectively in mental disorders or emotional sensitivity to environmental stresses. KF-1 facilitates E2-dependent ubiquitylation and thus is an E3-ubiquitin Ub ligase [17]. KF-1 may be the first example of such Ub ligases, which may be responsible for suppressing the anxiety response without affecting other behavioral features [18, 19]. KF-1 was originally identified as a gene whose transcripts were found more frequently in the frontal cortex of human Alzheimer's disease (AD) patients compared to normal subjects [20]. The *kf-1* gene is located on the human chromosome 2p11.2, and expressed at the basal levels in many tissues but prominently in brain tissue, particularly in the cerebellum and hippocampus [20]. Interestingly, the nucleotide sequence of *kf-1* mRNA is highly conserved between mouse and human not only in the coding region (94% homologous) but also in the 3′-untranslated region (91.3%), the latter of which may form a stable twofold-sheet structure [20]. Consequently, the mRNA half-life may be strictly controlled to stabilize *kf-1* transcripts for the steady synthesis of KF-1 protein, suggesting that the basal cellular concentration of KF-1 protein may be important. The human KF-1 protein consists of 685 amino acid (aa) residues and contains a possible leader sequence and a RING-H2 finger motif at the N- and C-termini, respectively, with two closely located membrane-spanning domains in the middle of the molecule with 3 aa residues apart from each other (Fig. 1). *kf-1* gene expression is up-regulated in the frontal cortex and hippocampus of rats after anti-depressant treatments such as chronic administration of serotonin selective re-uptake inhibitor (SSRI) [21], electroconvulsive therapy [22], and repetitive transcranial magnetic stimulation [23]. These observations suggest that the elevated *kf-1* transcription is associated with some physiological responses to the anti-depressant pathway but not merely with chemical reactions to SSRIs. However, it is not clear which anti-depressant pathways this up-regulation is associated with, anxiolytic or anti-despair pathway, or both. In co-transfected cells, KF-1 molecules are co-localized with Presenilins, responsible for familial AD, located on the endoplasmic reticulum [19]. Therefore, KF-1 Ub ligase may be involved in ERAD pathway for degrading some membrane proteins such as Presenilins, neurotransmitter receptors, and so on.

Phylogenetic Studies on KF-1

The *kf-1* orthologous genes have been found in many animals, but not in any species of other eukaryotes such as plants and fungi, and prokaryotes (Fig. 2). Tablet animals such as *Trichoplax adhaerens* [24] possess the *kf-1* gene, but sponges such as *Amphimedon queenslandica* [25] and choanoflagellates such as *Monosiga brevicollis* do not [26], the latter of which belong to Protista and are believed to be unicellular origins of the Animalia (Fig. 2). This implies that the *kf-1* gene may have been generated between the poriferan and placozoan emergences (Fig. 2). Interestingly, the *kf-1* genes are found in blood flukes (*Schistosoma mansoni*), but not Insecta (*Drosophila melanogaster*), Arachnida (*Ixodes scapularis*) or Nematoda (*Caenorhabditis elegans*). Nevertheless, it is likely that waterfleas (*Daphnia pulex*) may possess a KF-1 protein, although its deduced structure corresponds to only a half of the protein proximal to the C-terminus probably due to the incomplete determination of genome sequences. Therefore, it can be concluded, if the present phylotaxonomy is correct as in Fig. 2, that the *kf-1* gene was lost independently three times in the lineages leading up to *D. melanogaster*, *I. scapularis*, and *C. elegans*, respectively (Fig. 2). In any case, it is clear that *Drosophila* cannot be an animal model for human diseases due to KF-1 deficiency, if any, unlike Parkinson's disease model in *D. melanogaster* lacking the gene for Parkin Ub ligase.

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MWLKLFFLLLYFLVLFVLAR FFEAIVWYETGIFATQLVDP VALSFKKLKTILECRGLGYS   60
GLPEKKDVRELVEKSGDLME GELYSALKEEEASESVSSTN FSGEMHFYELVEDTKDGIWL  120
VQVIANDRSPLVGKIHWEKM VKKVSRFGIRTGTFNCSSDP RYCRRRGWVRSTLIMSVPQT  180
STSKGKVMLKEYSGRKIEVE HIFKWITAHAASRIKTIYNA EHLKEEWNKSDQYWLKIYLF  240
ANLDQPPAFFSALSIKFTGR VEFIFVNVENWDNKSYMTDI GIYNMPSYILRTPEGIYRYG  300
NHTGEFISLQAMDSFLRSLQ PEVNDLFVLSLVLVNLMAWM DLFITQGATIKRFVVLISTL  360
GTYNSLLIISWLPVLGFLQL PYLDSFYEYSLKLLRYSNTT TLASWVRADWMFYSSHPALF  420
LSTYLGHGLLIDYFEKKRRR NNNNDEVNANNLEWLSSLWD WYTSYLFHPIASFQNFPVES  480
DWDEDPDLFLERLAFPDLWL HPLIPTDYIKNLPMWRFKCL GVQSEEEMSEGSQDTENDSE  540
SENTDTLSSEKEVFEDKQSV LHNSPGTASHCDAEACSCAN KYCQTSPCERKGRSYGSYNT  600
NEDMEPDWLTWPADMLHCTE CVVCLENFENGCLLMGLPCG HVFHQNCIVMWLAGGRHCCP  660
VCRWPSYKKKQPYAQHQPLS NDVPS 685
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Fig. 1 Critical domains and SNP positions in human KF-1 Ub ligase. *Solid*, *dashed*, and *double underlines* indicate a possible leader sequences, membrane-spanning regions and RING-H2 finger motif domain, respectively. *Bold letters* indicated consensus amino acid residues conserved in animals. Note that *kf-1* genes are not found in *D. melanogaster*, *I. scapularis* and *C. elegans*. *Four boxed letters* indicate amino acid residues, in which critical SNPs are found in their codons according to the NIH databases, at T180 (frame shift), S251 (S to P substitution), P502 (P to H substitution), and E626 (termination) as rs35921467, rs17857046, rs17853383, and rs11695337, respectively

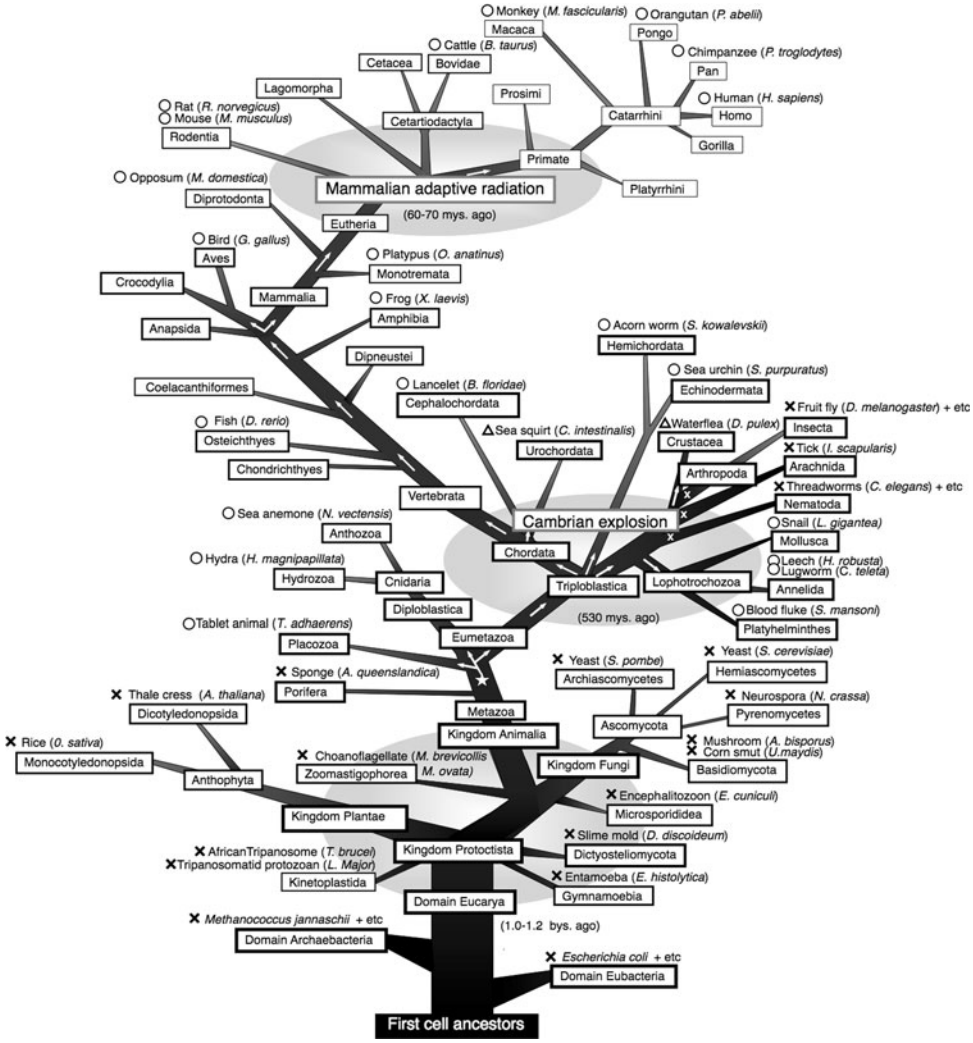

Fig. 2 Presence, absence, generation, and transmission of *kf-1* gene on the evolutionary tree. The tree is as presented previously with modifications and corrections [19, 29]. *An open star*, *open arrows*, and *three open crosses* in the tree branches indicate the generation, transmission, and termination of the *kf-1* gene, respectively. The genomic information of waterflea (*Daphnia pulex*) was obtained at <http://genome.jgi-psf.org/Dappu1/Dappu1.home.html>. *Circles*, *two triangles* or *crosses* in front of the names for organisms indicate the presence, probable presence or absence, respectively, of *kf-1* as confirmed in the GenBank database on 24 September 2010 and SUPERFAMILY database release 1.73 <http://supfam.cs.bris.ac.uk/SUPERFAMILY_1.73/index.html> [30, 31] 'etc' for 'Insecta', 'Nematoda', 'Domain Archaebacteria', and 'Domain Eubacteria' means that the absence of the *kf-1* gene was confirmed in more than 11, 6, 11, and 50 species, respectively

kf-1 Null Mice

To elucidate the biological functions of KF-1, the *kf-1* null mice should be a useful tool for future studies. In fact, *kf-1* knockout mice have been generated by the gene-targeting procedure using Cre–loxP recombination system [18]. The resulting *kf-1* null mice showed no apparent abnormalities in anatomy, pathology, physiology, neuroethology or in

Table 1 Behavioral test battery conducted between *kf-1*$^{-/-}$ and *kf-1*$^{+/+}$ littermate mice

Tests	Measurements	Phenotypes
General health examination	Whisker, coat, reflexes	=
Physical test	Body weight	=
	Body temperature	=
	Wire hanging time	↑
	Grip strength	=
	Auditory capability	=
Light/dark transition test	Anxiety-like behaviors	↑
Open field test	Exploratory locomotion	=
Elevated plus maze test	Anxiety-like behaviors	↑
Hot plate test	Pain sensitivity	=
Social interaction test	Social interaction	=
Rotarod test	Motor coordination	=
Prepulse inhibition (PPI) test	Startle amplitude in startle-stimulus-only trials	↓
	Prepulse inhibition	↑
Porsol forced swim test	Behavioral despair (immobility time)	=
T-maze test	Spatial working memory	=
Fear conditioning test	Immediate freezing during conditioning phase with aversive footshock	↑
	Contextual testing conducted after conditioning	=
	Cued test with altered context	=
Tail suspension test	Behavioral despair (immobility time)	=

The details of these experiments have been described in Tsujimura et al. [18], and partly in the section, *kf-1* null mice. Arrows, '↑' and '↓', indicate significant increase and decrease, respectively, in *kf-1*$^{-/-}$ mice compared to *kf-1*$^{+/+}$ littermate mice. '=' indicates no significant differences between *kf-1*$^{-/-}$ and *kf-1*$^{+/+}$ mice

reproductive capability, and were then used for a behavioral test battery. The detailed procedures and apparatuses used have been described [18]. As summarized in Table 1, *kf-1* null mice exhibit selectively enhanced anxiety-like behaviors or increased timidity-like responses. The mouse anxiety-like behaviors are determined on the bases of their nocturnal and geotaxis natures by using the light/dark transition and elevated plus maze tests. In the light/dark transition test, the apparatus is used, which consists of a cage ($21 \times 42 \times 25$ cm^3) divided into two chambers, namely light and dark boxes. Mice are individually placed in the dark box at the beginning, and allowed to move freely between the two boxes with door open. In the elevated plus maze test, plus-shaped four arms of the same size (25×5 cm^2) are placed at a height of 55 cm above the floor. The arms and central square (5×5 cm^2) are made of opaque and white plastic plates, and two of the four arms at opposite sides are enclosed with 15 cm high transparent walls (closed arms). Mice are placed individually in the crossing center to face one of the closed arms. In both the experiments, mouse movements are automatically traced and recorded. Compared to the *kf-1*$^{+/+}$ littermates, the distance travelled and stay time in the light box are significantly reduced in the light/dark transition test (Fig. 3a, b), and similarly in the elevated plus maze test (Fig. 3c, d), significantly the total distance travelled and the number of entries into the crossing center is significantly reduced. The reduced distance travelled in these tests should not be ascribed to physical alteration in locomotive activities of mice, but rather to mental or emotional alterations in the mice, because no significant differences in locomotive activity are observed in the open field test between *kf-1*$^{-/-}$ and *kf-1*$^{+/+}$ littermate mice ($P = 0.2879$, $F_{1,38} = 1.162$) [18]. One of the striking features in these results is that the P values are strikingly small ($P = 0.0004$–0.0005) compared to those usually obtained in such tests, implying that these results must be reproducible.

Consistently, the *kf-1* null mice exhibit significantly increased timidity-like responses as observed in prolonged wire hanging time and enhanced immediate freezing to aversive foot shock compared to *kf-1*$^{+/+}$ littermates (Table 1). These observations should also reflect mental but not physical alterations in *kf-1*$^{-/-}$ mice, since no significant differences existed in grip strength and body weight between *kf-1*$^{-/-}$ and *kf-1*$^{+/+}$ mice ($P = 0.4060$, $F_{1,38} = 0.706$ and $P = 0.6254$, $F_{1,38} = 0.242$, respectively).

Another example of timidity-like responses is observed in the startle-stimulus-only trials of the startle response/prepulse inhibition (PPI) test, in which *kf-1*$^{-/-}$ mice show significantly lower startle amplitudes than the *kf-1*$^{+/+}$ littermates do (Fig. 3e). This reduced startle amplitude may be interpreted similarly to the enhanced freezing as observed after aversive foot shock. The PPI test is used widely to measure deficits in information-processing abilities in schizophrenic patients [27], and can be employed in both human and animal experiments [28]. PPI of the startle

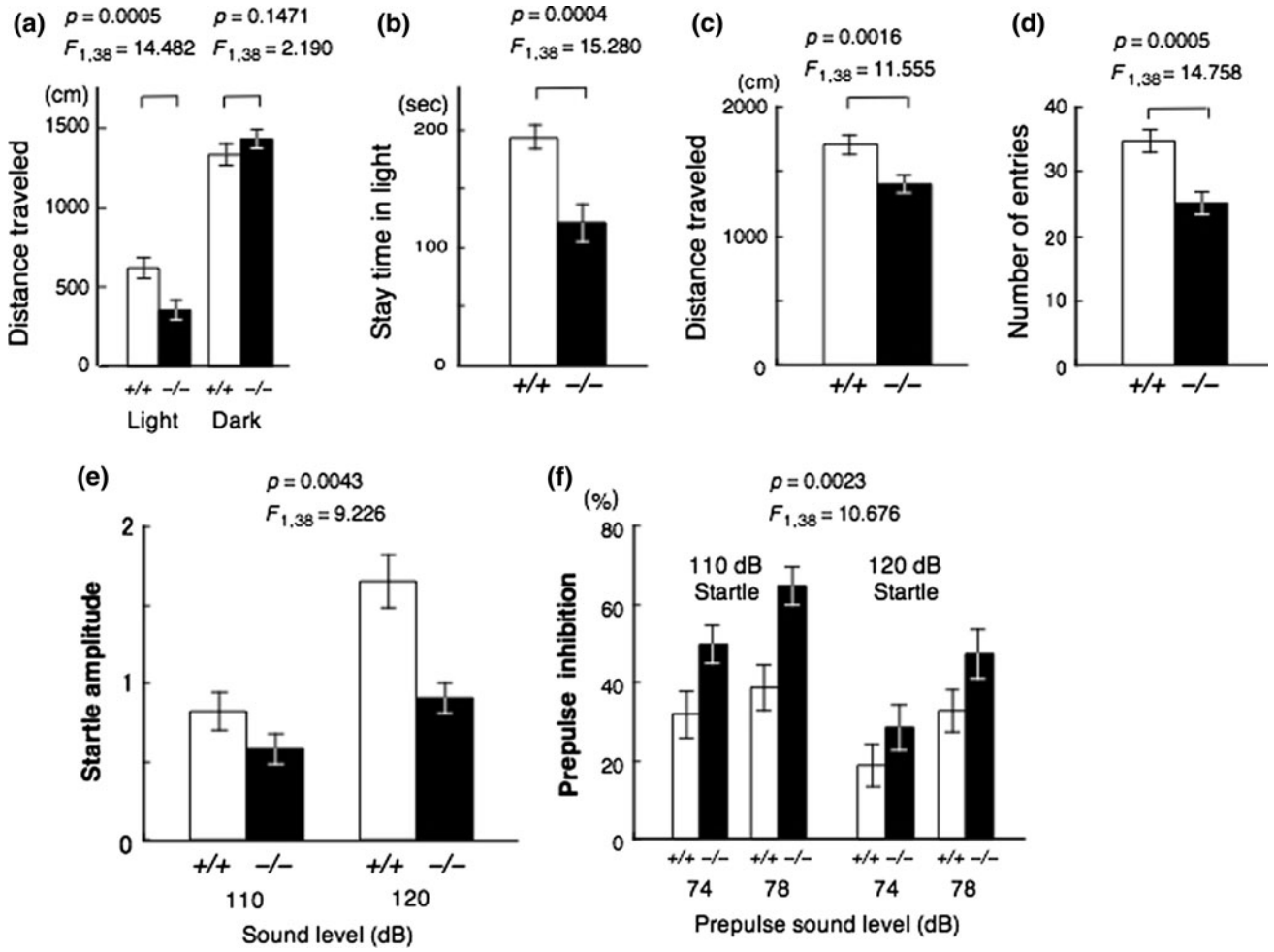

Fig. 3 Increased anxiety-like behaviors or timidity-like responses in *kf-1*$^{-/-}$ mice in comparison to their *kf-1*$^{+/+}$ littermates. Significantly reduced locomotive activities in the light but not dark (**a**) and decreased stay time in the light (**b**) in the light/dark transition test. Significantly reduced total distance travelled (**c**) and decreased number of entries into the crossing center (**d**) on heights in the elevated plus maze test. Significantly reduced startle amplitude in startle-stimulus-only trials (**e**) and increased PPI values (**f**) in the startle response/prepulse inhibition test. These data are taken from Tsujimura et al. [18]

response is defined as the degree (%) to which the startle response to a sudden noise (110 and 120 dB) is reduced when the noise is preceded shortly by a mild and low-intensity sound (74 and 78 dB). The PPI values were significantly greater in *kf-1*$^{-/-}$ than in *kf-1*$^{+/+}$ (Fig. 3f; overall ANOVA including all four combinations, $P = 0.0023$, $F_{1,38} = 10.676$). This could be due to the reduced baseline startle amplitudes in the *kf-1*$^{-/-}$ mice (Fig. 3e, suggesting that reduced startle amplitudes may be similar to the reduced immediate freezing responses in the aversive foot shock. Nevertheless, if PPI is independent of the baseline startle, one cannot rule out the possibility that increased KF-1 activity may reduce PPI and thus could be a risk factor of schizophrenia.

No other significantly altered behaviors have been observed in the *kf-1*$^{-/-}$ mice compared to *kf-1*$^{+/+}$ mice, namely exploratory locomotion, pain sensitivity, social interaction, motor coordination, despair-like behaviors, spatial working memory, cognitive functions. In conclusion, the KF-1 Ub ligase may function specifically as an anxiety suppressor by degrading, through the ERAD pathway, its substrate protein(s) responsible for promoting emotional sensitivity to environmental stresses. The putative anxiety promoter could be a neurotransmitter-related protein, but is unlikely to be a serotonin receptor or transporter because a lack of either protein increases anxiety-like behaviors [4, 10, 19].

Anxiety Suppressor Model

The observations as described in the preceding section suggest that the reduction of KF-1 activity could be a risk factor selective to anxiety disorder, but may not be relevant

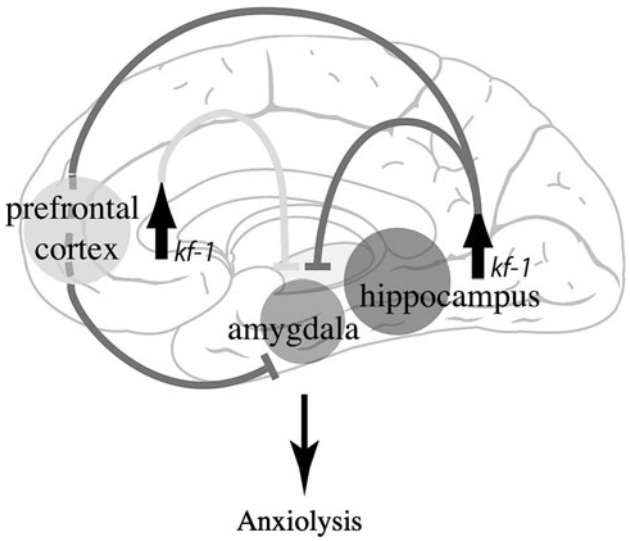

Fig. 4 A circuitry presentation of the anxiety suppressor model by the up-regulation of kf-1 expression. The model has been proposed previously [19]. The anxiolytic pathway after *kf-1* up-regulation is incorporated into the model

to other mental disorders such as despair and cognitive deficit. In this context, it may be worth noting that some critical SNPs are found in the human *kf-1* coding region according to the NCBI database. These include a frame shift, two aa substitutions and a termination codon (Fig. 1). On the basis of these observations, the Anxiety Suppressor Model has been proposed, assuming that KF-1 Ub ligase may suppress amygdala-mediated anxiety when over-produced in the frontal cortex and hippocampus by transmitting some signals to the amygdala (Fig. 4).

The *kf-1* null mice may be a useful tool to study the molecular mechanisms controlling the emotional sensitivity to environmental stresses. Moreover, these mice may also be used to identify putative anxiety promoters and for screening novel anxiolytic compounds against them.

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