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Raf kinase inhibitory protein knockout mice: expression in the brain and olfaction deficit

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Abstract

Raf Kinase Inhibitory Protein (RKIP-1) is involved in the regulation of the MAP kinase, NF- κ B, and GPCR signaling pathways. It is expressed in numerous tissues and cell types and orthologues have been documented throughout the animal and plant kingdoms. RKIP-1 has also been reported as an inhibitor of serine proteases, and a precursor of a neurostimulatory peptide. RKIP-1 has been implicated as a suppressor of metastases in several human cancers. We generated a knockout strain of mice to further assess RKIP-1's function in mammals. RKIP-1 is expressed in many tissues with the highest protein levels detectable in testes and brain. In the brain, expression was ubiquitous in limbic formations, and homozygous mice developed olfaction deficits in the first year of life. We postulate that RKIP-1 may be a modulator of behavioral responses.

Keywords

RKIP; PEBP; gene trap; knockout mouse; brain; behavior; olfaction

1. Introduction

Raf Kinase Inhibitory Protein (RKIP), also known as Phosphatidylethanolamine Binding Protein (PEBP) [3], was first documented as an important regulator of the MAP kinase (MAPK) signal transduction pathway, whose role is to rapidly convert extracellular signals into activation of transcription factors [6,20,32,38]. To date, RKIP-1 is the only known cellular inhibitor of Raf kinase.

Other signaling pathways are also influenced by RKIP-1. For example, the inhibition of RKIP-1 enhances NF- κ B-induced transcription while over-expression reduces it [40]. In addition,

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RKIP-1 impinges on GPCR signaling by controlling the activity of the G-protein coupled receptor kinase-2 (GRK2) [21,23].

The functions of RKIP-1 in a variety of organisms are diverse and include inhibition of carboxypeptidase Y in yeast [5], an immunoprotective function in nematodes [12], odorant binding and detection in *Drosophila* [12], and control of shoot growth and flowering in plants [33]. In mammals, signaling proteins are often linked to disease states [22], and recent work indicates that RKIP-1 is a metastasis suppressor [7,13,19,31]. Although localized primarily in the cytoplasm, it is also the primary source of the extracellular hippocampal cholinergic neurostimulatory peptide (HCNP) [30].

Here, we report the generation of a knockout strain of mice. These mice were derived from ES cells carrying a gene trap in intron 1 of RKIP-1. We found that mice homozygous for the mutated allele are viable and appear normal up to 10 months of age. However, they develop an olfaction deficit, a phenotype that correlates with the expression pattern of the gene in the brain.

2. Materials and methods

2.1. ES cells

The ES cell line AQ0005 carrying the pGT01xr vector was obtained from The Wellcome Trust Sanger Institute.

2.2. Generation of mice

All breeding and procedures were carried according to NIH Guide for the Use and Care of Laboratory Animals and institutional regulations at Brown University animal facility. The AQ0005 ES cells were grown until 90% confluency and trypsinized before injection. E3.5 blastocysts were derived from C57BL/6-*Tyr^{c-Brd}* female mice and injected with 12-20 ES cells. The injected blastocysts were implanted into the uteri of day 2.5 pseudo-pregnant females for generation of chimeras. Eight to ten injected embryos were implanted per uterine horn. The chimeras were mated with C57BL/6-*Tyr^{c-Brd}* females to obtain F1 progeny. The strain carrying the germ line transmitted allele (designated *RKIP1^{Gt (pGT01xrBetageo) 1Jkl}*, and referred to hereafter to as the minus allele) was maintained on a mixed C57BL/6 -129Ola background.

2.3. Genotyping

Genomic DNA was prepared from ES cells or tail biopsies by incubation in 200µl of lysis buffer (20mM NaCl, 50 mM Tris-HCl, pH 8.8, 10 mM EDTA, 1.5% sodium dodecyl sulfate and 1mg/ml of proteinase K). DNA was extracted with phenol/chloroform (1:1), centrifuged 4 minutes at 10,000RPM, transferred to a new tube, extracted three times with chloroform, and precipitated with 500 µl of ethanol. The DNA pellet was washed twice with 80% ethanol and dissolved in 10 mM Tris-HCl, pH 7.5. PCR was performed using primers b+f for detecting the gene trap allele, and primers b+g for detecting wild-type allele (Fig 1A). A 600 bp product was produced with primers b+f, while primers b+g produced a 900 bp PCR band. The PCR consisted of an initial incubation at 94°C for 2 min, then 35 cycles at 94°C for 15 sec, 60°C for 15 sec, and 72°C for 60 sec. The amplified DNA was resolved on 2% agarose gels.

2.4. Staining of tissues and embryos

Tissues and embryos were dissected out from deeply anesthetized (ketamine) adult animals, placed in cold 1 x PBS buffer and fixed for 1–2 hours in 2% paraformaldehyde (Sigma) depending on the size. Tissues were then washed three times in PBS for 20 min each time. Staining for β -geo was done in 2mM MgCl₂-0.01%, deoxyxholate-0.02% NP-40-100mM phosphate buffer (pH.8.0), 5 mM K₄Fe(CN)₆, 5mM K₃Fe(CN)₆, and 1 mg/ml of X-Gal. After

staining, the tissues and embryos were washed in PBS and stored in 4% PFA at 4°C. Brains were isolated, post-fixed for 2 hours in 5% formaldehyde at 4°C, and embedded in 3% low melting temperature agarose (Invitrogen) pre-cooled to 37°C. After 10 min at 4°C, vibratome sections were made and used immediately for X-Gal staining.

2.5. Western Blots

Primary cultures of tail fibroblasts were established [35] and protein extracts prepared using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% NP40) supplemented with protein inhibitors (1 mM PMSF, 1mM Na₃VO₄, 1mM NaF). For preparation of tissue extracts, ten milligrams of tissue was homogenized on ice in 450 µl of RIPA buffer supplemented with protein inhibitors. The lysate was then centrifuged for 40 minutes, 16,000 x g at 4°C, and the collected supernatant was centrifuged a second time for 15 minutes at 16,000 x g at 4°C. The concentration of protein was determined using the Biorad D/C protein assay kit. The extract was mixed with Laemli sample buffer (containing SDS and β-mercaptoethanol), fifty micrograms of total protein per lane were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to immobilon-P, and probed with rat anti-RKIP antibodies (Upstate Biotechnology). After probing with a secondary antibody (HRP-conjugated goat-anti rabbit IgG, Jackson ImmunoResearch, Inc.) the filters were incubated with a chemiluminescent substrate and detected by exposing the blot to KODAK Biomax film.

2.6. Real time qPCR

Total cellular RNA was extracted with the Trizol reagent (Invitrogen) and transcribed into cDNA using random hexamer primers and the TaqMan (r) kit from Applied Biosystems. qPCR was performed using the SYBR green system from Applied Biosystems according to provided instructions and analyzed in the Prism 7700 sequence detector (Applied Biosystems). All reactions were performed in duplicate and glyceraldehyde phosphate dehydrogenase (GAPDH mRNA) was used as an internal standard. Default cycling parameters and threshold values, provided by Applied Biosystems for the Prism 7700 instrument were used. Relative abundance of RKIP mRNA was calculated using the $\Delta\Delta CT$ method provided by Applied Biosystems using the abundance of GAPDH mRNA as an internal standard to correct for sample recovery and experimental variability between samples.

2.7. Behavioral tests

For the odor object recognition test, mice (50% C57BL/6, 50% 129Ola) between 2–4 months (n = 18 per group) or 5–8 months of age (n = 13 per group) were selected randomly. Food and water were available *ad libitum*. The age distribution between the groups under comparison was always similar. The odor object (cheddar cheese) recognition test was performed essentially as described [8]. Briefly, the experimenter was blind to the genotypes of the investigated mice. The piece of cheese was hidden under the bedding in one corner of the clean testing arena. The mouse was placed in the opposite corner of the arena and positioned diagonally from the hidden odor food object. The time that elapsed to uncover the food was recorded in each case. The cut off time point was set at 20 min. Mice were maintained on a 12 hour day/night light cycle and tests performed during the dark phase.

RKIP-1^{-/-} (n=26) and wild-type (n=28) mice were also tested on a sensory discrimination task that included visual, tactile, and olfactory problems. For these experiments F1 mice were backcrossed three times to C57BL/6 and then intercrossed to generate testing animals. Twenty-six mice were tested at 3.5–4 months of age and 28 mice were tested at 5–5.5 months of age. Tested mice included males (n=30) and females (n=24). In this task, mice were presented with two clay pots one of which contained a hidden food reward (candy sprinkle, Sweet Toppings Carousel Mix, Betty Crocker). Mice were required to learn which pot contained the food reward. Three problems (pairs of pots) were presented. Stimulus pots were distinguished by

digging medium inside (green or blue shredded tissue), texture on the outside (smooth or textured ribbon), or odor on the rim (Seabreeze or Citrus, Glade). The medium and texture problems were designed to test visual and tactile modalities, respectively, and did not include an olfactory component. Mice were first shaped to dig in a clay pot for the food reward. For the first four trials of each problem, the mouse was allowed to dig in the correct pot after making an incorrect choice. Thereafter, the trial was terminated if the incorrect pot was chosen. The measure of learning was the number of trials needed to reach a criterion of six sequential correct choices. For each problem, a probe trial was inserted into the test trials in which the reward was placed in the correct pot only after a choice was made. The probe was included to verify that choices were not guided by the odor of the reward. Each mouse was tested on all three discrimination problems. Testing was generally completed in one session. Food and water were available *ad libitum* until two days prior to testing, at which time food was restricted. At the completion of testing, food was again made available *ad libitum*. Mice were maintained on a 12 hour day/night light cycle and tests were performed during the light phase.

2.8. Statistical analyses

For the olfactory odor object recognition test, comparisons between two groups were performed with Student's *t* test. For the sensory discrimination test, the effects of group on number of trials to criterion was analyzed using repeated measures analysis of variance (rANOVA) with discrimination problem as the repeated factor. Planned comparisons were conducted for individual discrimination problems.

3. Results

3.1. Characterization of gene trapped ES cells

Although gene trap vectors integrate randomly within the genome of ES cells, the integration events that occur within transcribed units are selectable since the splicing acceptor site and the β -geo fusion function is provided by the vector. In the case of the clone AQ0005 used to generate RKIP1^{-/+} mice, the initial 5'RACE revealed that the disruption of the gene occurred 3' to the exon I of the RKIP-1 gene. We further confirmed the identity of the AQ0005 clone and farther defined the position of the integration site (Fig. 1A). A series of forward primers spanning a distance of 1.8kb downstream of exon I were synthesized (Fig. 1B). These primers were located within exon 1, intron 1, exon II and intron II. The sequence of the gene trap pGT01xr vector was used to design the reverse primer. The combinations of primers a+f and b+f produced bands consistent with the integration of the trap within intron I (Fig. 1C). The PCR products were not obtained using the remaining primer combinations (i.e., c+f, d+f, and e+f) suggesting a 3' position of the forward primer sequences relative to the integration site. The intron I of the mouse RKIP gene is 1164 bp, and this analysis narrows the integration site to position 580 ± 50 with respect to the start of intron.

3.2. RKIP-1^{-/-} mutants are viable

The AQ0005 ES cells were used to obtain heterozygous F1 animals. The mating of RKIP-1^{-/+} pairs produced RKIP-1^{+/+}, RKIP-1^{-/+}, and RKIP-1^{-/-} F2 progeny. Among 181 pups examined, the segregation of the disrupted allele was close to the expected Mendelian 1:2:1 distribution with no gender bias in all three individual categories (Table I). Thus, we conclude that the gene-trapped allele constitutes a recessive mutation, and animals homozygous for the allele are viable. RKIP-1 function appears to be largely dispensable during embryonic development.

3.3. Expression of the RKIP gene family in the mouse

Given the reported importance of RKIP-1 in the regulation of signaling cascades, the viability of the knockout mice could be a result of functional substitution by related genes. A number of RKIP orthologues have been noted in mammalian genomes by sequence analysis, but their expression has not been rigorously tested in any species. We searched the mouse genome and found five RKIP-like sequences on chromosomes 5, 6, 10, 12, and 19 (hereafter referred to as RKIP-1, RKIP-2, RKIP-3, RKIP-4 and RKIP-5, respectively). The locus on chromosome 5 is RKIP-1 and contains three introns. The remaining loci contain open reading frames (ORFs) but are intronless. The locus on chromosome 6, designated as RKIP-2, has been reported to be both transcribed and translated with a highly testis-specific expression [16]. The expression of the other loci has not been previously analyzed.

Using genome sequence information we designed primer pairs for real-time qPCR specific for each of the RKIP-like loci in the mouse genome. To test the specificity of the primers, we first amplified portions of the genomic loci that encompass the amplicon of each primer pair with outside primers (Fig. 2A, primers h-r), and sequenced the amplified fragments to ascertain that we in fact amplified the correct genomic locus in each case. The amplified fragments were then used as templates with each of the five qPCR primer pairs (Fig. 2A, primer pairs 1–5). Amplification of the genomic RKIP-like loci by qPCR indicates that the qPCR primers do amplify their intended target sequences.

In tail fibroblasts, relative to wild-type cells, the level of RKIP-1 mRNA expression was 51% and 3% in RKIP-1^{+/-} and RKIP-1^{-/-} cells, respectively (Fig. 2B). Since the qPCR primers are downstream of the insertion, the low level expression seen in RKIP-1^{-/-} cells may be due to either read-through or use of cryptic downstream promoters. In either case, translation from such an mRNA would not result in a functional protein. RKIP-2 mRNA was detected at a level of 1% relative to RKIP-1 in wild-type fibroblast cells. Primer pairs 3, 4 and 5 (corresponding to RKIP-3, RKIP-4, and RKIP-5) did not detect any signal in any of the fibroblast cells (Fig. 2B). Consistent with these observations, Western blot analysis revealed no RKIP-1 protein present in fibroblasts derived from RKIP-1^{-/-} mice, and RKIP-1^{+/-} fibroblasts displayed a 50% reduction in protein level relative to wild-type cells (Fig. 2C).

We next examined the expression of each of the RKIP-like mRNAs in extracts prepared from a variety of mouse tissues. In wild-type mice, RKIP-1 was expressed in a large number of tissues; the highest expression levels were detectable in testis, brain and liver (Fig. 2D). Expression was, however, clearly measurable in all tissues tested. Very low level of RKIP-1 expression was seen in RKIP-1^{-/-} animals (not shown). RKIP-2 was expressed in the testes in both wild-type and RKIP^{-/-} animals (not shown). Expression of RKIP-3 and RKIP-4, and RKIP-5 was not detected in any tissue in any of the animals examined. Based on the absence of detectable mRNA expression for any RKIP family member other than RKIP-1 in any of the major organs examined (except RKIP-2 in testes), it can be concluded that RKIP-3, RKIP-4 and RKIP-5 are silent pseudogenes and the viability of the RKIP-1 knockout mouse is not caused by compensation.

Immunoblot analysis of RKIP-1 expression in a variety of tissues (Fig. 2E) was in good agreement with the observed mRNA expression. Highest levels were seen in testes and brain, followed by liver and kidney. These observations are in good agreement with a prior analysis in rat tissues [12]. No detectable RKIP-1 protein was observed in any tissue from RKIP-1^{-/-} animals (not shown), with the exception of a low signal in testis (Fig. 2E, lane 2). Since our antibody is polyclonal and was raised against full-length recombinant RKIP-1, this result indicates that the antibody cross-reacts with RKIP-2, which is expressed in testes and shares a high degree of sequence identity with RKIP-1. The low level signal in the RKIP-1^{-/-} testis

is also in agreement with the low level of RKIP-2 mRNA expression we observed in the same tissue.

Thus, Real Time qPCR and immunoblotting analysis allow us to conclude that only RKIP-1 and RKIP-2 genes are expressed in the mouse. While RKIP-1 seems to be ubiquitous, RKIP-2 expression is limited to testes. RKIP-3, RKIP-4, and RKIP-5 represent pseudogenes that are not expressed. The gene trap insertion results in a true loss of function allele in the mutant mouse.

3.4. Tissue expression analysis using β -geo reporter activity

The pGT01xr vector used for the insertional mutagenesis carries the β -geo selectable marker. β -geo is a recombinant chimeric protein that contains two enzymatically active domains: β -galactosidase at its N-terminus, and neomycin phosphotransferase (specifying G418 resistance) at its C-terminus. After insertional mutagenesis this cassette is placed under control of the target gene promoter. Histochemical detection of β -galactosidase activity can thus be used to follow the expression of the targeted gene in the mouse.

Hand cut sections of various tissues were isolated from 10 week old RKIP-1^{+/-} and wild-type mice and used for comparative X-Gal staining to assess the distribution of RKIP-1 expression throughout the organisms. We tested skeletal muscle, heart, skin, spleen, liver, brain, testes, epididymis, seminal vesicles, duodenum, stomach, colon, lung, ovaries, uterus, brain, desidual swellings from pregnant mothers, yolk sacs from developing E12.5 embryos, fibroblasts derived from tail biopsies, and ES cells. Within the sensitivity of this assay, testes and brain produced a signal indicating differential *LacZ* expression in RKIP^{+/-} but not in wild-type tissues (not shown). Remaining tissues were found either negative or produced no obviously visible staining difference between RKIP^{+/-} and wild-type animals. This detection system is not as sensitive as immunohistochemistry or immunofluorescence. Accordingly, we detected strong staining only in the testis and brain, the two tissues with the highest RKIP-1 mRNA and protein expression.

3.5. Expression of β -geo reporter in the brain of adult mice

Over the course of these studies some RKIP-1^{-/-} animals exhibited depressive behavior manifested through withdrawal from group activities such as huddling and allogrooming. To explain such behavior, and since a high expression level of the RKIP-1 gene has been detected in the brain using qPCR, we analyzed the expression pattern of β -geo reporter by staining coronal brain sections of RKIP-1^{-/-} animals with X-Gal. We found the β -geo expression ubiquitous, and particularly strong in limbic formations. Staining in some areas was stronger than in others (Fig. 3A and B). The intensity ranged from undetectable, through weak to moderate and strong. The differences resulted from both the level of β -geo expression within the positive cells as well as from the number of positive cells within the given formation.

In telencephalon, fewer yet clearly detectable cells stained in the olfactory nuclei surrounding the anterior commissure. The anterior commissure itself expressed β -geo below the detectable level. In the middle part of the forebrain, a moderate to strong staining nuclei included: the core accumbens nucleus, lateral and medial septal nuclei, nucleus of the vertical limb of the diagonal band, and islands of Calleja. β -geo expression within the caudate putamen (striatum) was moderate. The oriens layer of the hippocampal formation was moderately positive while pyramidal cell layer and granular layer of dentate gyrus reacted rather strongly with X-Gal. This elevated reactivity extended further to all three CA fields of hippocampus.

Moderate to high levels of expression were detected in diencephalon. The almost total absence of labeling in the dorsal thalamus was striking. However, neurons surrounding the third

ventricle, including the medial habenular nucleus and the paraventricular thalamic nucleus were positive. The preoptic area was positive. The hypothalamus expressed the β -geo reporter most intensely. Virtually all nuclei of the hypothalamus displayed a high density of strongly reactive cells. This reactivity extended towards the parasubthalamic nucleus, zona incerta and magnocellular nucleus. Notably, a very high intensity staining was detectable in all nuclei of the amygdala.

The mammalian neocortexes consist of six layers of cells. Except for layer I, all exhibited a moderate level of β -geo expression. However, locally, a high concentration of β -geo-expressing cells was apparent, particularly within the piriform cortex and the retrosplenial granular and agranular cortices.

Widespread reporter expression continued throughout the mesencephalon. In the midbrain tectum, the superficial gray layer, optic nerve layer, and the intermediate gray layer of the superior colliculus all reacted strongly, along with the external cortex and central neuron of the inferior colliculus. The cuneiform nucleus, subbrachial nucleus, and deep mesencephalic nucleus, the Edinger-Westphal nucleus, along with cells of the periaqueductal gray, all expressed a high level of β -geo. The superior cerebellar peduncle, and subbrachial neurons displayed moderate staining intensities. Also, there was some evidence of staining within the substantia nigra.

Within the metencephalon, cells positive for β -geo were widely distributed. However, neurons of the locus coeruleus, Barrington's nucleus, and dorsal tegmental area exhibited the strongest staining. This intense area of reactivity extended to the alpha part of the central gray and the central gray of the pons. Other reporter-positive nuclei included the dorsomedial and ventrolateral principal sense nucleus 5, and reticular nuclei such as the paraventricular reticular nucleus alpha, intermediate reticular nucleus, caudal pontine reticular nucleus, and the gigantocellular reticular nucleus alpha. Interestingly, raphe pallidus and magnus nuclei were also positive along with the dorsal periolivary region, lateral superior olive, superior paraolivary nucleus and nucleus of the trapezoid body. In contrast, the level of β -geo expression in the cerebellum was relatively low, and mostly contained within cerebellar nuclei. A single layer of Purkinje cells was positive (Fig. 3B), but the overall reactivity of cerebellar structures was weak relative to the other formations of the brain.

Within the myelencephalon there was exceptionally strong staining detectable in all neurons of the solitary tract. The spinal trigeminal tract was negative, however, scattered cells of the dorsomedial and ventrolateral principal sense nucleus 5, parvocellular reticular nucleus, lateral reticular nucleus, ambiguus nucleus, and gigantocellular reticular nuclei were positive.

3.6. Behavioral deficits

The strong expression of the reporter in the limbic formations of the brain suggests that RKIP-1 may be involved in the control of emotions and complex behaviors, including reproductive behaviors. The β -geo expression was readily detectable in olfactory neurons. Thus, it was of interest to determine if RKIP-1^{-/-} mice suffer from a decline of the sense of smell. In an odor object recognition test, mice were randomly selected and subjected to the task of finding an odorous food object hidden under the bedding. Three to four month old homozygous and heterozygous animals performed equally well (Fig. 4A). Older RKIP-1^{+/-} mice were able to complete the task within ~3 minutes (Fig. 4B), while the RKIP-1^{-/-} mice were either not able to find the object, or recognized the object with a significant delay (average greater than 8 min). RKIP^{-/-} males performed somewhat better than RKIP-1^{-/-} females, but both sexes were affected (not shown). Thus, we found that RKIP-1^{-/-} mutants suffer from an olfaction deficit that develops in the first year of life.

The sensory discrimination test included three discrimination problems, one visual, one tactile, and one olfactory (Fig. 4C). There were no effects of test age or sex on the task, and the analysis of probe trials verified that neither group of mice was using the odor of the food reward to solve the discriminations. The RKIP-1^{-/-} mice exhibited an overall impairment in acquiring the discriminations [$F(1,52)=5.41$, $p<0.24$]. Although there was no statistical interaction between modality and genotype, planned comparisons indicated that the mutant and wild type mice were significantly different on the olfactory discrimination ($p<0.038$), but not on the visual or tactile discriminations ($p>0.18$). The results of the sensory discrimination task confirm that RKIP-1^{-/-} mice have olfactory deficits. Moreover, these deficits are measurable by four months of age. These results also suggest that the mutation is associated with a general learning deficit.

4. Discussion

We found that the β -geo reporter expression pattern in the RKIP-1^{-/-} mouse brain follows the majority of limbic formations, many of which control complex mammalian behaviors and reproduction. Most notably, we found that the reporter is expressed in nucleus accumbens involved in the reward feelings, pleasure and addiction, hippocampal formations that are required for the formation of long-term memories, hypothalamic nuclei that regulate heart rate, hunger, thirst, sexual arousal, and sleep-wake cycle, and the olfactory neurons. RKIP-1 appears to be expressed at high levels in the aggression and fear controlling amygdala, and in the preoptic area. These are the regions of the brain known to control neuroendocrine-related homeostasis. The medial preoptic area (MPA) plays a critical integrative role of converging the neuronal pathways that determine sexual behavior in several species. Changes in dopamine levels, aromatase levels, and signaling pathways mediated by N-methyl-D-aspartate receptors of MPA were linked to abnormal sexual behavior in rodents [9,10,17,18,25]. The hypothalamus, by controlling peripheral endocrine glands of the body through the pituitary circuit, regulates reproduction in mammals through hormonal control of mating, pregnancy, and lactation. Ubiquitous expression of RKIP-1 was also found in cortexes and olfactory nuclei, which help to regulate eating and reproduction. Consistent with these data, we found that RKIP-1^{-/-} mutants display a reproductive decline without any obvious fertility defects (manuscript in preparation).

The RKIP^{-/-} mice display an olfaction deficit that is apparent by four months of age but increases in severity by 5–8 months. This phenotype may be evolutionarily reminiscent of the RKIP-1 function previously seen in *Drosophila* [12] and plants [33]. In *Drosophila*, RKIP orthologues reside in antennae and olfactory hairs and play a role in odorant binding. In plants, an orthologue of RKIP-1 was found to be involved in shoot growth and flowering. In mammals, olfaction is critical in sexual recognition and in establishing food preferences. It is widely accepted that the amygdala is the brain center for gustation and food preference [2,4,28]. The smell information from nasal mucosa is sensed by chemosensitive peripheries of olfactory neurons and then projected to the olfactory bulbs. Second-order neurons project from the bulbs to numerous cerebral structures such as amygdala, the piriform and endorhinal cortexes [26]. These are the brain centers affected in several age-related neurodegenerative disorders, including Alzheimer disease (AD). Beside dementia, memory loss, impairment of judgment and language [11], and motor complications in severe cases [27,34], the decline in olfactory perception is one of the prominent features of AD [36]. Tests for loss of olfactory discrimination can actually distinguish between AD patients and depressed individuals in which the sense of smell seems to be less affected [36]. RKIP-1 has been implicated in AD [14,39], although it has not been linked to the loss of the sense of smell. Behavioral testing also revealed a general learning deficit associated with the loss of RKIP-1 function. The role of RKIP-1 in AD emerges through its diminished expression in affected neurons [14]. An inverse correlation between the severity of AD and the RKIP-1 level in human and mouse brains has been reported [14,24].

A strong expression of the β -geo reporter in the parabrachial nuclei, the nuclei of the solitary tract, area postrema and surrounding nuclei was striking. RKIP-1 was highly expressed in the autonomic systems such as Edinger-Westphal nucleus, dorsal motor nucleus of the vagus, and the amygdaloid hypothalamic nuclei. These are the areas of the brain involved in the integration of the autonomic nervous system. Visceral sensory information from the vagus passes through the solitary tract and then is relayed to the hypothalamus. The projected information includes blood pressure, gut distention, and signals from the glossopharyngeal and facial nerves. Although expression of RKIP-1 in the brain appears consistent with autonomic visceral functions, further experiments are needed to test such assumptions.

The sequence of eleven N-terminal amino acids of RKIP-1 was found to be identical to the hippocampal cholinergic neurostimulatory peptide that stimulates acetylcholine synthesis and secretion in the brain [30]. It has been suggested that RKIP-1 protein is a precursor of HCNP [29,30,37]. The reporter expression pattern in the mouse brain is consistent with potential endocrine relevance of the gene [1,15]. However, the mutation used herein does not disrupt exon I, preserving the N-terminal HCNP sequence for processing and function.

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Fig. 1.
Genomic structure of RKIP-1 locus and the identity of gene-trapped ES cells. **A** - Genomic structure of the RKIP gene and the gene trapped allele. **B** - Primers used to verify integration site of the gene trap at RKIP-1 locus. **C** - Gel electrophoresis of PCR products obtained using AQ0005 DNA as a template and different combinations of primers.

Fig. 2.

Real time qPCR and Western analysis of RKIP expression. **A** – Primer pairs used for amplification of genomic sequences (h-r), and primer pairs used for qPCR of RKIP cDNAs (1a-5b). **B** - Real time qPCR analysis of RKIP family mRNA expression in wild-type, RKIP-1^{+/+} and RKIP-1^{-/-} tail fibroblasts. cDNA was prepared from early passage fibroblasts. Primer pairs were specific for each locus: 1, RKIP-1 on chr. 5; 2, RKIP-2 on chr. 6; 3, pseudogene on chr. 10; 4, pseudogene on chr. 12; 5, pseudogene on chr. 19. Signals were normalized to GAPDH and are shown relative to RKIP expression in wild-type cells. **C** – Western blot of protein extracts obtained from wild-type, RKIP-1^{+/+} and RKIP-1^{-/-} tail fibroblasts. **D** - Real time qPCR expression of RKIP-1 mRNA in the tissues of wild-type mice. **E** – RKIP-1 protein detection by Western blotting in tissues prepared from wild-type and RKIP-1 knockout mice.

Fig. 3.

β-geo reporter expression in the brain isolated from five months old homozygous male mouse.

A - Coronal sections stained for lacZ. Abbreviations: PrL, prelimbic cortex; FrA, frontal association cortex; Mo, medial orbital cortex; Lo, lateral orbital cortex; Vo, ventral orbital cortex; DLO, dorsolateral orbital cortex; AOD, anterior olfactory nucleus, dorsal; GrO, granular cell layer of olfactory bulb; AOV, anterior olfactory nucleus, ventral; aci, intrabulbar anterior commissure, fmi, forceps minor corpus callosum; Pir, piriform cortex; aca, anterior commissure; AcbC, accumbens nucleus, core; Cg1, cingulate cortex, area 1; Cg2, cingulate cortex, area 2; S1FL, S1 cortex, forelimb region; S2, secondary somatosensory cortex; Cl, claustrum; Den, dorsal endopiriform nucleus; Cpu, caudate putamen (striatum); LSV, lateral

septal nucleus, ventral part; IcjM, islands of Calleja; VDB, nucleus of the vertical limb of the diagonal band; HT, hypothalamic area; Or, oriens layer of hippocampus; Amy, amygdala; RSA, retrosplenial agranular cortex; RSG, retrosplenial granular cortex; Au, auditory cortex; Py, pyramidal cell layer of hippocampus; pc, posterior commissure; PVP, paraventricular thalamic nucleus; VLGMC, magnocellular nucleus; Rc, rhinal cortices; PH, posterior hypothalamus; V2 secondary visual cortex; V1, primary visual cortex; AuD, dorsal auditory cortex; Au1, primary auditory cortex; GrDG, granular layer of dentate gyrus; Apir, amygdalopiriform transition area; SuG, superficial gray layer of superior colliculus; PAG, periaqueductal gray; EW, Edinger-Westphal nucleus; IF, interfascicular nucleus; SubB, subbrachial nucleus; IpL, interpeduncular nucleus, lateral; ECIC, external cortex of inferior colliculus; scp, superior cerebellar peduncle; mlf, medial longitudinal fasciculus; CnF, cuneiform nucleus; ILL, intermediate nucleus of lateral lemniscus; KF, Kolliker-Fuse nucleus; PC5, paraventricular motor nucleus; Cb, cerebellar nuclei; Med, medial cerebellar, nuclei; Pr, prepositus nucleus; sol, solitary tract; PCrt, parvicellular reticular nucleus; LPGi, paragigantocellular nucleus; 6Cb, 6th cerebellar lobule; 7Cb, 7th cerebellar lobule; 8Cb, 8th cerebellar lobule; 9Cb, 9th cerebellar lobule; Sp5I, spinal 5 nucleus; Ro, nucleus of Roller; Amb, ambiguus nucleus. Pta, parietal association cortex; cc, corpus callosum; AHIAL, amygdalohippocampal area; BLV, basolateral amygdaloid nucleus; BMP, basomedial amygdaloid nucleus; VEn, ventral endopiriform nucleus; LaVM, lateral amygdaloid nucleus; LSI, lateral septal nucleus; LSV, lateral septal nucleus, ventral part; MS, medial septal nucleus. **B** – higher power magnification of selected areas shown in (A). Abbreviations as in (A).

Fig. 4. Behavioral tests. **A** – Latency to find hidden food in an odor object recognition test using three to four months old homozygous ($-/-$) and heterozygous ($-/+$) animals ($n=36$, $p<0.5$). **B** – Odor object recognition test using 5–8 months old $RKIP-1^{-/-}$ and $RKIP-1^{-/+}$ animals ($n=26$, $p<0.024$). **C** – Trials to criterion for three sensory discrimination problems using 4–6 months old homozygous ($-/-$) and wild type ($+/+$) animals ($n=54$, $p<0.024$). Data are in means $\pm$ SEM.

Table 1

Distribution of genotypes and gender in offspring from matings of heterozygous parents.

Genotype	Total pups	Gender	Number of pups
+/+	50	Male	24
		Female	26
+/-	91	Male	46
		Female	45
-/-	40	Male	21
		Female	19