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Sex is a major determinant of neuronal dysfunction in Neurofibromatosis Type 1

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Abstract

Objective—Children with Neurofibromatosis-1 (NF1) are at risk for developing numerous nervous system abnormalities, including cognitive problems and brain tumors (optic pathway glioma). Currently, there are few prognostic factors that predict clinical manifestations or outcomes in patients, even in families with an identical *NF1* gene mutation. In this study, we leveraged *Nf1* genetically-engineered mice (GEM) to define the potential role of sex as a clinically-relevant modifier of NF1-associated neuronal dysfunction.

Methods—De-identified clinical data were analyzed to determine the impact of sex on optic glioma-associated visual decline in children with NF1. In addition, *Nf1* GEM were employed as experimental platforms to investigate sexually dimorphic differences in learning/memory, visual acuity, retinal ganglion cell (RGC) death, and *Nf1* protein (neurofibromin)-regulated signaling pathway function (RAS activity, cyclic AMP and dopamine levels).

Results—Female patients with NF1-associated optic glioma were twice as likely to undergo brain MRI for visual symptoms and three times more likely to require treatment for visual decline than their male counterparts. As such, only female *Nf1* GEM exhibited a decrement in optic glioma-associated visual acuity, shorter RGC axons, and attenuated cAMP levels. In contrast, only male *Nf1* GEM showed spatial learning/memory deficits, increased RAS activity, and reduced dopamine levels.

Interpretation—Collectively, these observations establish sex as a major prognostic factor underlying neuronal dysfunction in NF1, and suggest that sex should be considered when interpreting future preclinical and clinical study results.

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Keywords

neurofibromin; cyclic AMP; dopamine; Ras; NF1; learning; vision; central nervous system

Introduction

Neurofibromatosis type 1 (NF1) is a clinically heterogeneous neurologic disorder characterized by germline *NF1* gene mutations[1], such that children with NF1 are prone to the development of brain tumors (optic gliomas)[2,3], cognitive problems[4,5], and attention deficits[6]. While affected individuals are at risk for all of these abnormalities, it is currently not possible to predict who will develop which neurologic problem and what clinical outcome will ensue. This challenge is further underscored by the observation that individuals from the same family (with the identical *NF1* gene mutation) can exhibit significantly different clinical features and disease severities.

Recent studies using *Nf1* genetically-engineered mice (GEM) have begun to reveal potential genomic loci that influence tumor susceptibility[7,8]. For example, astrocytoma resistance is conferred by a modifier gene (*Arlm1*) located on mouse chromosome 12, which operates in a sex-specific manner. The finding that sex interacts with this genomic modifier to influence gliomagenesis raises the intriguing possibility that other NF1 clinical abnormalities may also be sexually dimorphic. To define the role of sex as a modifier for neurologic dysfunction in NF1, we leveraged *Nf1* GEM that develop optic glioma and learning/memory abnormalities. In this report, we establish that sex is a major determining factor underlying learning/memory deficits and optic glioma-associated visual decline in *Nf1* mutant mice through sexually-dimorphic effects on *Nf1* protein (neurofibromin)-mediated neuronal cyclic AMP (cAMP), Ras and dopamine signaling.

Materials and Methods

Human subjects

De-identified data from individuals ≤18 years with an established diagnosis of NF1[9] managed in the St. Louis Children's Hospital Neurofibromatosis Clinical Program (1994-2013) were collected under an approved Human Studies Protocol at the Washington University School of Medicine.

Mice

Nf1+/-^{GFAP}CKO (*Nf1*$^{flox/mut}$;GFAP-Cre, *Nf1*-CKO) and littermate control (*Nf1*$^{flox/flox}$; CTL) mice were maintained on an inbred C57BL/6 background with *ad libitum* access to food and water. All experiments were performed on 3-4-month-old mice, unless otherwise stated, under an approved Animal Studies Committee protocol at the Washington University School of Medicine.

Immunostaining

Western blots were performed using pDARPP32 (1:500, Cell Signaling), DARPP32 (1:1000, Cell Signaling), pERK1/2 (1:1000, Cell Signaling), and ERK1/2 (1:1000, Cell

Signaling) primary antibodies. Ras activation (Millipore), cAMP (New East Biosciences), TUNEL (Roche Diagnostics), and dopamine (Rocky Mountain Diagnostics) measurements were determined following manufacturer's protocols. Each experiment was performed with samples from at least three independently-generated cohorts.

Behavioral testing

Morris Water Maze testing for spatial learning and memory was conducted as previously described[10]. Three days after completing cued (visible but variable platform location; 4 trials/day, 2 consecutive days) trials, spatial learning acquisition was evaluated during the place condition (submerged, hidden platform, constant location; 4 trials/day, 5 consecutive days). Escape path length and latency, and swimming speeds were calculated for all cued and place trials. Retention performance was evaluated during probe trials (platform removed) conducted 1-h after the last place trial on the third and fifth days. Time spent in the target quadrant and spatial bias (time in the target quadrant versus each of the other quadrants) were also analyzed. Visual acuity (n=20 mice per group) was assessed using the virtual optokinetic system (VOS) under photopic conditions (1.8 log cd/m^2)[10], as previously described. Contrast thresholds were measured at a frequency of 0.128 cycles/degree and a speed of 5.4 degrees/sec.

Results

Sex determines visual outcome in children and mice with NF1

15-20% of children with NF1 develop optic pathway gliomas; however, only one-third to one-half of these children will require treatment, typically as a consequence of visual decline[11,12]. While previous studies have indicated that glioma location and patient age are negative predictors of visual decline[13], we sought to determine whether patient sex influences clinical outcome. From the St. Louis Children's Hospital NF Clinical Program (1994-2013), 431 individuals younger than 19 years of age were identified who met diagnostic criteria for NF1 (205 males, 226 females) (Fig 1A). Brain magnetic resonance imaging (MRI) was performed on 255 individuals based on clinical indications (Fig 1B; 122 males, 133 females), revealing 96 children with optic gliomas (38%; 40 males, 56 females). While boys and girls with NF1 exhibited similar frequency of optic glioma, girls with NF1-associated optic gliomas were twice as likely to have undergone neuroimaging for visual symptoms (p=0.005, Fig 1B) and 3 times more likely than boys to require treatment due to visual decline (p=0.0007, Fig 1A). There were no significant differences in the age at optic glioma diagnosis or tumor location between boys and girls (Fig 1A, C). However, it is important to note that females with optic gliomas in all locations, except the post-chiasmal tracts, exhibited higher frequencies of visual decline requiring treatment (Fig 1D). The independent prognostic value of post-chiasmal involvement has been recently reported[13].

To determine whether sex influences the outcome of murine optic glioma, we leveraged an *Nf1* GEM strain that develops optic nerve/chiasmal gliomas (*Nf1*-CKO)[14,15]. While optic nerve volumes were indistinguishable between male and female *Nf1*-CKO mice (Fig 2A), only female *Nf1*-CKO mice exhibited reduced visual acuity on VOS testing (Fig 2B). Moreover, female *Nf1*-CKO mice had ~2-fold more retinal ganglion cell death (RGC

apoptosis; Fig 2C) relative to their male counterparts. This increase in RGC apoptosis is also observed *in vivo*, as evidenced by a time-dependent degeneration of optic nerve axons[16, 17], as well as reduced RGC neuronal lengths *in vitro*[18]. Consistent with these sex-specific observations, reduced axon lengths were only observed in female *Nf1*-CKO primary RGC neurons relative to controls *in vitro* (Fig 2D, E).

Spatial learning impairments in male Nf1-CKO mice

While 30-70% of children with NF1 exhibit specific learning deficits, only two reports have specifically examined sex. In these studies, a 2:1 male bias in the prevalence of these NF1-associated learning problems was observed[5, 14]. To explore the impact of sex on learning/memory in mice, *Nf1*-CKO mice were evaluated in the Morris Water Maze. Whereas sex did not influence performance during the cued and place trials (Supplementary Fig 1), only *Nf1*-CKO male mice showed no spatial preference and spent equal time in all quadrants (Fig 3A, B) during both the memory acquisition (probe trial 1) and retention (probe trial 2) trials. Similarly, male *Nf1*-CKO mice exhibited a 25% reduction in time spent and number of entries into the target quadrant relative to littermate controls (Fig 3C-F). In contrast, *Nf1*-CKO female mice performed comparably to controls.

Sex-dependent differences in neurofibromin function underlie the sexual dimorphic deficits in Nf1-CKO mice

Neurofibromin regulates several downstream signaling pathways within the nervous system[10,19-22] (Fig 4A). As such, previous studies have demonstrated that reduced RGC axon lengths and survival results from impaired neurofibromin generation of cyclic AMP (cAMP), and treatment with agents that restore cAMP levels ameliorate the neurite length and survival defects *in vitro* and attenuate the optic glioma-associated RGC apoptosis *in vivo*[18]. Additionally, learning/memory deficits in *Nf1*-CKO mice reflect impairments in neurofibromin regulation of both hippocampal Ras activity and dopamine levels, such that either Ras inhibition (e.g., Lovastatin[21] or dopamine elevation (e.g., methylphenidate[23]) corrects these learning/memory impairments in *Nf1* mutant mice (Fig 4A).

We sought to determine whether sex-dependent differences in neurofibromin regulation of cAMP, Ras and dopamine homeostasis account for the sexually-dimorphic abnormalities in learning and optic glioma-associated vision loss. First, we show that only female *Nf1*-CKO mice had lower retinal cAMP levels relative to controls (Fig 4B). Second, only *Nf1*-CKO male mice had reduced hippocampal dopamine levels and DARPP32 phosphorylation compared to controls (Fig 4C, D). Third, only male *Nf1*-CKO mice exhibited increased hippocampal Ras activation and ERK1/2 phosphorylation (activation) relative to controls (Fig 4E, F).

Discussion

While NF1 is a monogenetic disorder, the specific clinical manifestations and outcomes are not determined solely by the germline *NF1* gene mutation. However, the individual factors that contribute to patient outcome are multi-factorial and often difficult to establish in human clinical studies. For this reason, *Nf1* GEM strains provide experimentally-controlled

platforms to assess potential risk factors. Using this approach, modifier genes have been identified in rodents that influence susceptibility to astrocytoma and malignant peripheral nerve sheath tumor (MPNST) development[7,8]. Although these autosomal genomic modifiers reflect differences between inbred mouse strains, we now establish for the first time that sex differentially impacts on NF1-associated neurologic dysfunction.

In children with NF1-associated optic glioma, we demonstrate that girls are more likely to require treatment as a result of visual decline. The increase in visual loss secondary to optic glioma in girls was not attributable to differences in patient age or tumor location, and did not reflect an increased prevalence of these tumors in girls with NF1. Based on these intriguing clinical findings, we leveraged *Nf1* mutant mice to show that sex strongly influences the impact of an inactivating germline *Nf1* gene mutation on optic glioma-associated visual loss. In addition, we demonstrate that this sexual dimorphic effect exists at both the tissue (retina) and molecular (cAMP) level. As such, only female *Nf1*-CKO mice exhibit reduced visual acuity due to reduced RGC survival and cAMP generation. Coupled with previous experiments demonstrating that cAMP elevation (Rolipram treatment) nearly completely ameliorated the optic glioma-induced retinal apoptosis *in vivo*[18], these new observations underscore the need to consider sex when interpreting *Nf1* preclinical GEM results as well as evaluating completed and future NF1 optic glioma therapeutic clinical studies.

While previous reports have revealed sex-related differences in cognition and behavior in both mice[24] and in people with other neurologic conditions, including autism[25] and reading disability[26], there is currently a paucity of clinical data that specifically examined the impact of sex on cognitive function in children with NF1. In the two clinical studies evaluating sex as a potential risk factor, specific learning deficits were more prevalent in boys[5,14]. Using *Nf1*-CKO mice, we demonstrate that learning/memory deficits were only observed in males. This sex-specific abnormality results from selective impairments in both hippocampal dopamine and Ras signaling, and is consistent with studies that show that restoration of dopamine levels[23] or inhibition of Ras hyperactivation[21] reverse the learning/memory deficits in *Nf1* mutant mice. While sexually dimorphic changes in Ras regulation have not been previously reported, embryonic midbrain dopaminergic cell number is determined by sex[27,28]. Current studies are focused on examining the relationship between dopamine homeostasis and Ras signaling in hippocampal neurons.

Previous reports have revealed that neurofibromin regulates different downstream signaling effectors (e.g., cAMP, dopamine, RAS/mTOR) in a both cell type- and brain region-specific manner[19-22]; however, the molecular mechanisms responsible for these sex-dependent effects are currently unknown. One possibility is potential differential sex hormonal influences, which will require castration/ovariectomy and hormonal manipulations to evaluate. Another complementary approach would entail the use of a novel complement of genetically-engineered mouse strains (four core genotype model), which allows for a direct assessment of sex chromosome contributions independent of gonadal sex[29,30].

It is also plausible that sex interacts with a germline *Nf1* gene mutation through epigenetic mechanisms, such as differential methylation or imprinting[31,32] to produce global changes in

gene expression[33,34]. In preliminary gene expression profiling experiments using *Nf1*+/- and wild-type embryonic mouse brains stratified by sex, the female *Nf1*+/- brain transcriptome clustered separately from the three other conditions (wild-type male and female brains, male *Nf1*+/- brains); however, no specific causative genes were identified (data not shown). Additional studies are planned to explore the etiology for these sexually-dimorphic effects.

Collectively, these observations support a model in which the clinical heterogeneity seen in individuals with NF1 likely results from the interplay between genomic determinants (e.g., sex) and neurofibromin function in specific tissues. Further elucidation of the underlying mechanisms may yield new predictive markers of patient outcome or unique targets for future therapeutic drug design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A

	Male	Female	p-value
Individuals with NF1	205	226	0.77
MRI obtained (%)	122 (59.5)	133 (58.8)	0.92
Optic glioma (% on MRI)	40 (32.8)	56 (42.1)	0.15
Average age at OPG diagnosis (range)	5.76 (1-12)	5.75 (1-14)	0.99
Treatment (%)	7 (17.5)	29 (51.8)	0.0007

B

Indication for brain MRI	Male	Female	p-value
Development delay (%)	47 (39)	38 (29)	0.11
Headache (%)	27 (22)	32 (24)	0.77
Visual symptoms (%)	16 (13)	37 (28)	0.005
Facial/neck plexiform neurofibroma (%)	18 (15)	15 (11)	0.46
Seizure (%)	6 (5)	7 (5)	0.99
Precocious puberty (%)	6 (5)	4 (3)	0.53
Other* (%)	2 (2)	0 (0)	0.23

C

Location of OPG	Male	Female	p-value
Optic nerve (%)	18 (45)	24 (43)	0.83
Optic nerve + chiasm (%)	11 (28)	21 (38)	0.51
Optic nerve + chiasm + tracts (%)	6 (15)	7 (15)	0.76
Hypothalamus (%)	4 (10)	5 (9)	0.99

D

Treatment	Male	Female	p-value
Optic nerve (%)	1 of 18 (6)	10 of 24 (42)	0.01
Optic nerve + chiasm (%)	2 of 11 (18)	10 of 21 (48)	0.14
Optic nerve + chiasm + tracts (%)	4 of 6 (67)	6 of 7 (86)	0.56
Hypothalamus (%)	0 of 4 (0)	3 of 5 (60)	0.17

Figure 1. Optic glioma location and size is not impacted by sex

(A) Girls with NF1-associated optic glioma were 3 times more likely to be treated for visual decline than their male counterparts (p=0.0007). **(B)** Indications for brain MRI. *Other includes one individual each imaged for weakness and the presence of a facial port-wine stain. (**C**) Locations of optic pathway gliomas (OPGs) in male and female children with NF1 occur at similar frequencies. (**D**) Frequency of visual decline necessitating treatment in girls and boys stratified by tumor location.

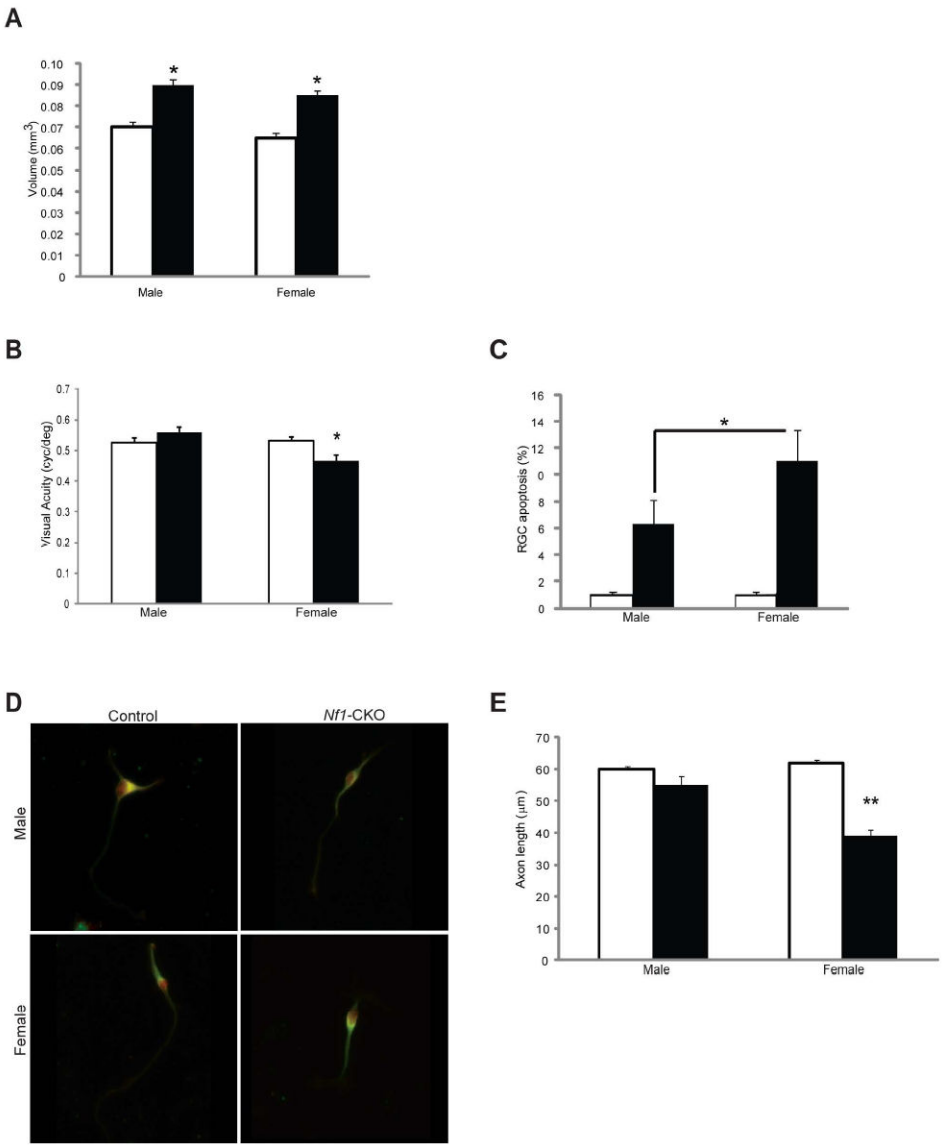

Figure 2. Optic glioma-associated vision disturbances are greater in girls with NF1 and in female *Nf1*-CKO mice

(A) Optic nerve volumes in *Nf1*-CKO mice (black bars) are larger than those observed in control littermate mice (white bars), regardless of sex. Asterisks denotes $p<0.05$. **(B)** Only female *Nf1*-CKO mice have impaired visual acuity (VOS; cycles/degree) relative to controls. **(C)** Greater retinal ganglion cell apoptosis (%TUNEL+ cells) was observed in female *Nf1*-CKO mice (11.5-fold over female controls) relative to *Nf1*-CKO males (6.2-fold over male controls). **(D)** Representative images of retinal ganglion cells in culture demonstrate that female, but not male, *Nf1*-CKO neurons have reduced axon lengths relative to controls. **(E)** Female, but not male, *Nf1*-CKO neurons exhibit shorter axon lengths (~50% reduction) relative to controls. Open (white) bars denote control (CTL) mice; closed (black) bars denote *Nf1*-CKO mice. *$p<0.05$, **$p<0.01$.

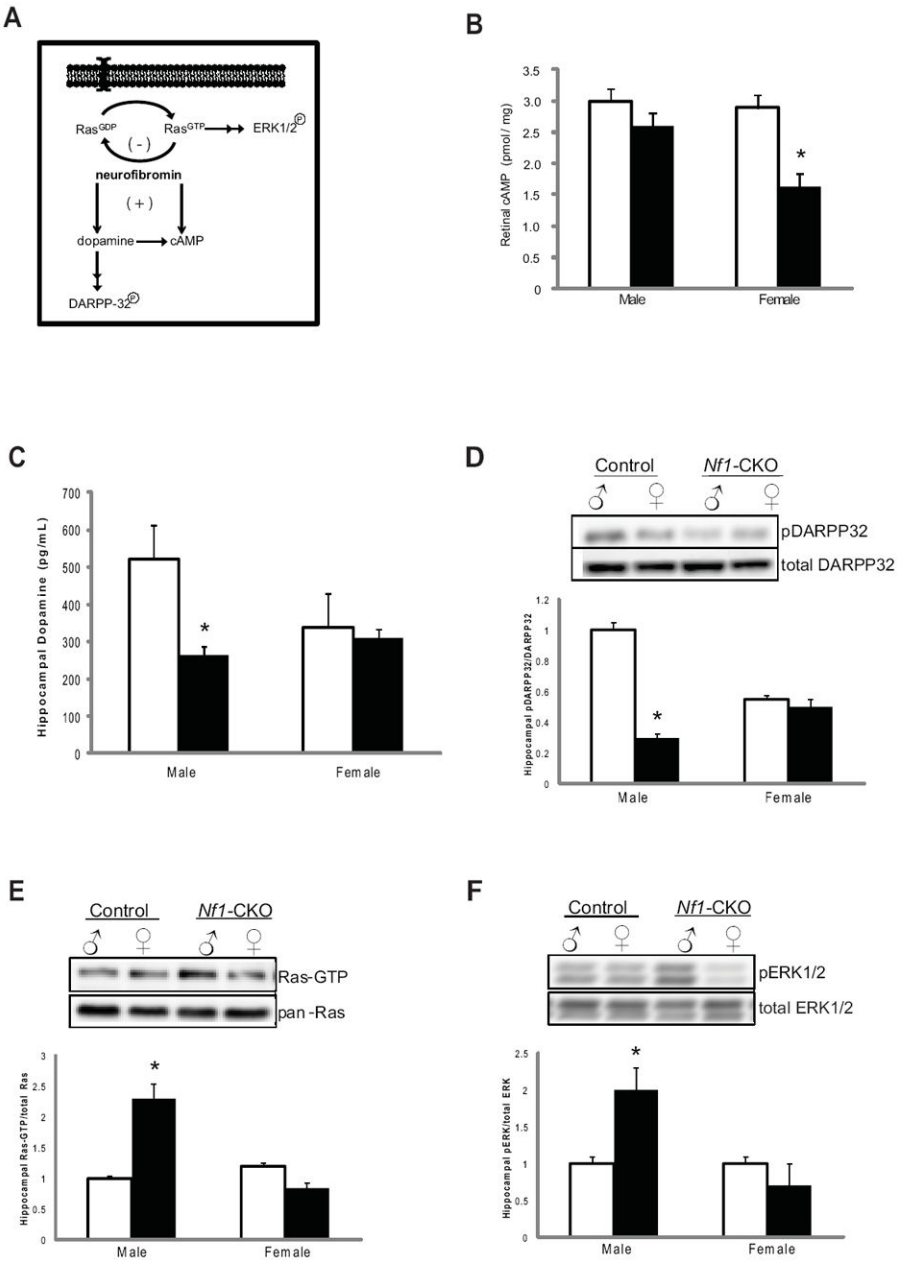

Figure 3. Spatial learning impairments in the Morris Water Maze occur only in *Nf1*-CKO male mice

(A-B) During the first **(A)** and second **(B)** probe trials, control mice and female *Nf1*-CKO mice spent significantly more time in the target quadrant than all the other quadrants. In contrast, male *Nf1*-CKO mice showed no spatial preference and spent equal time in all quadrants. **(C-D)** Time spent in the target quadrant during the first (**C**) and second (**D**) probe trials was reduced by 25% and 23%, respectively in male *Nf1*-CKO mice. (**E-F**) Male *Nf1*-CKO mice entered the target 1.5-fold less frequently than controls in both the first (**E**) and second (**F**) probe trials. *p<0.05, **p<0.01.

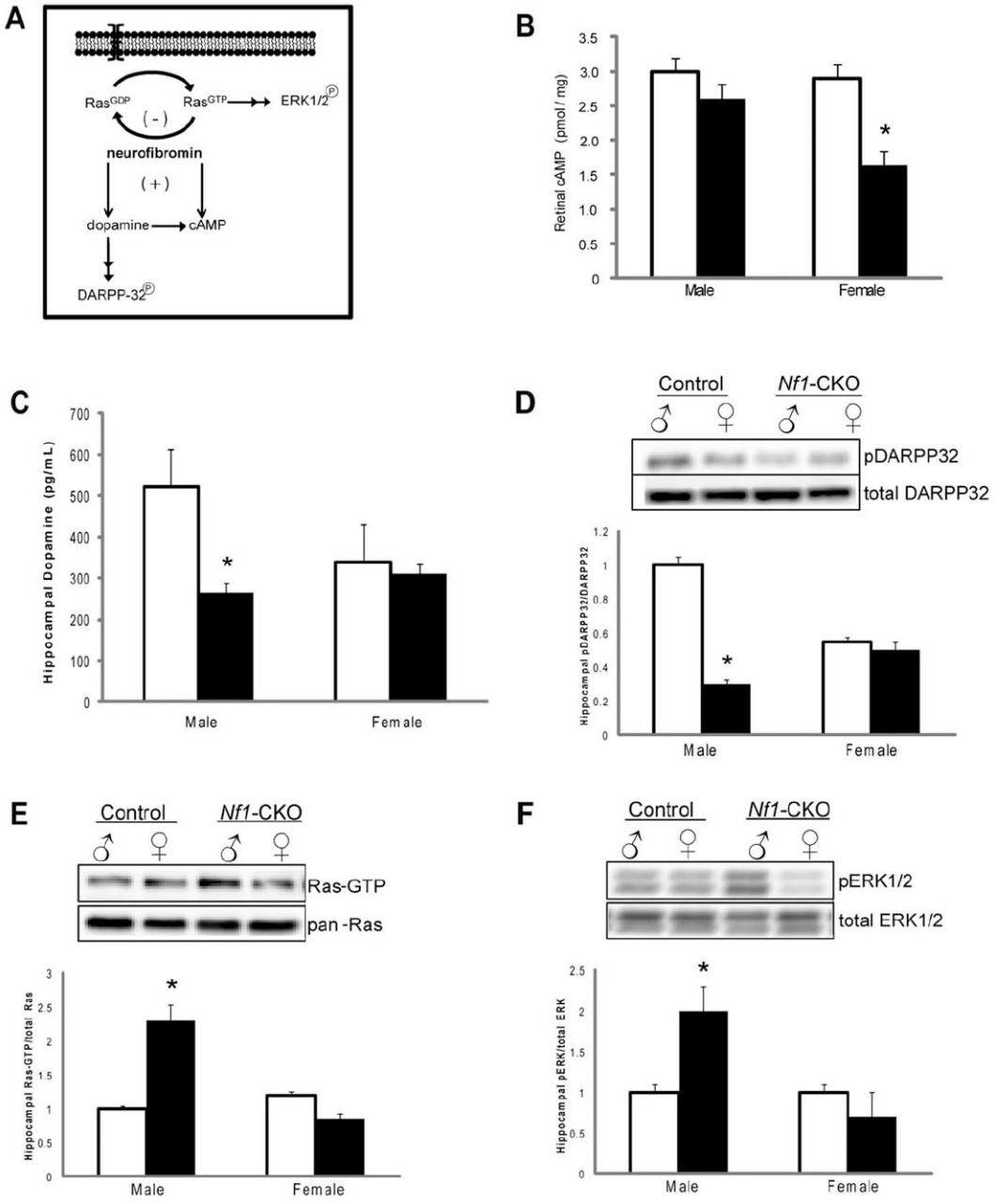

Figure 4. Sexually dimorphic regulation of neurofibromin signaling pathways

(A) Established molecular pathways regulated by the *NF1* gene product, neurofibromin. (B) Retinal cyclic adenosine monophosphate (cAMP) levels were reduced by 47% in female *Nf1*-CKO mice relative to female controls. No change in retinal cAMP levels was observed in male *Nf1*-CKO mice relative to male controls. (C) Hippocampal dopamine levels were reduced 2-fold in male *Nf1*-CKO mice compared to male controls. No differences were observed in female *Nf1*-CKO mice relative to female controls. (D) DARPP32 phosphorylation was reduced in the hippocampus of *Nf1*-CKO male, but not female, mice. (E, F) Densitometric quantification reveals increased active (guanosine triphosphate [GTP]-bound) Ras (Ras-GTP levels relative to total Ras; E) and ERK (phospho-ERK1/2 relative to

total ERK1/2; F) activation in male, but not female, *Nf1*-CKO hippocampi relative to controls. *p <0.05.