Sex Is a Major Determinant of Neuronal Dysfunction in Neurofibromatosis Type 1

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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: Deidentified 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 adenosine monophosphate [cAMP], and dopamine levels).
Results: Female patients with NF1-associated optic glioma were twice as likely to undergo brain magnetic resonance imaging for visual symptoms and 3× 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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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] Although 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

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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 adenosine monophosphate (cAMP), Ras, and dopamine signaling.

Materials and Methods

Human Subjects

Deidentified data from individuals ≤18 years old 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- to 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 Technology, Danvers, MA), DARPP32 (1:1,000; Cell Signaling Technology), pERK1/2 (1:1,000; Cell Signaling Technology), and ERK1/2 (1:1,000; Cell Signaling Technology) primary antibodies. Ras activation (Millipore, Billerica, MA), cAMP (NewEast Biosciences, King of Prussia, PA), terminal deoxynucleotide transferase–mediated deoxyuridine triphosphate nick-end labeling (Roche Diagnostics, Mannheim, Germany), and dopamine (Rocky Mountain Diagnostics, Colorado Springs, CO) measurements were determined following manufacturers' protocols. Each experiment was performed with samples from at least 3 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 hour 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 vs 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 candela/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/s.

Results

Sex Determines Visual Outcome in Children and Mice with NF1

Fifteen to 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] Previous studies have indicated that glioma location and patient age are negative predictors of visual decline[13]; however, we sought to determine whether patient sex influences clinical outcome. From the St Louis Children's Hospital Neurofibromatosis 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 1). Brain magnetic resonance imaging was performed on 255 individuals based on clinical indications (122 males, 133 females), revealing 96 children with optic gliomas (38%; 40 males, 56 females). Although 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 ($p = 0.005$) and 3× more likely than boys to require treatment due to visual decline ($p = 0.0007$). There were no significant differences in the age at optic glioma diagnosis or tumor location between boys and girls. However, it is important to note that females with optic gliomas in all locations, except the postchiasmal tracts, exhibited higher frequencies of visual decline requiring treatment. The independent prognostic value of postchiasmal involvement has been recently reported.[13]

To determine whether sex influences the outcome of murine optic glioma, we leveraged *Nf1* GEM that develop optic nerve/chiasmal gliomas (*Nf1*-CKO).[14,15] Although optic nerve volumes were indistinguishable between male and female *Nf1*-CKO mice, only female *Nf1*-CKO mice exhibited reduced visual acuity on VOS testing (Fig 2). Moreover, female *Nf1*-CKO mice had ~2-fold more retinal ganglion cell death (RGC apoptosis) relative to their male counterparts. This increase in RGC apoptosis was 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.

Spatial Learning Impairments in Male Nf1-CKO Mice

Although 30 to 70% of children with NF1 exhibit specific learning deficits, only 2 reports have specifically examined sex. In these studies, a 2:1 male bias in the

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 are not impacted by sex. (A) Girls with neurofibromatosis type 1 (NF1)-associated optic glioma were 3× more likely to be treated for visual decline than their male counterparts ($p = 0.0007$). (B) Indications for brain magnetic resonance imaging (MRI). *Other includes 1 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.

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), 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 (see 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 (Fig 4A).[10,19–22] As

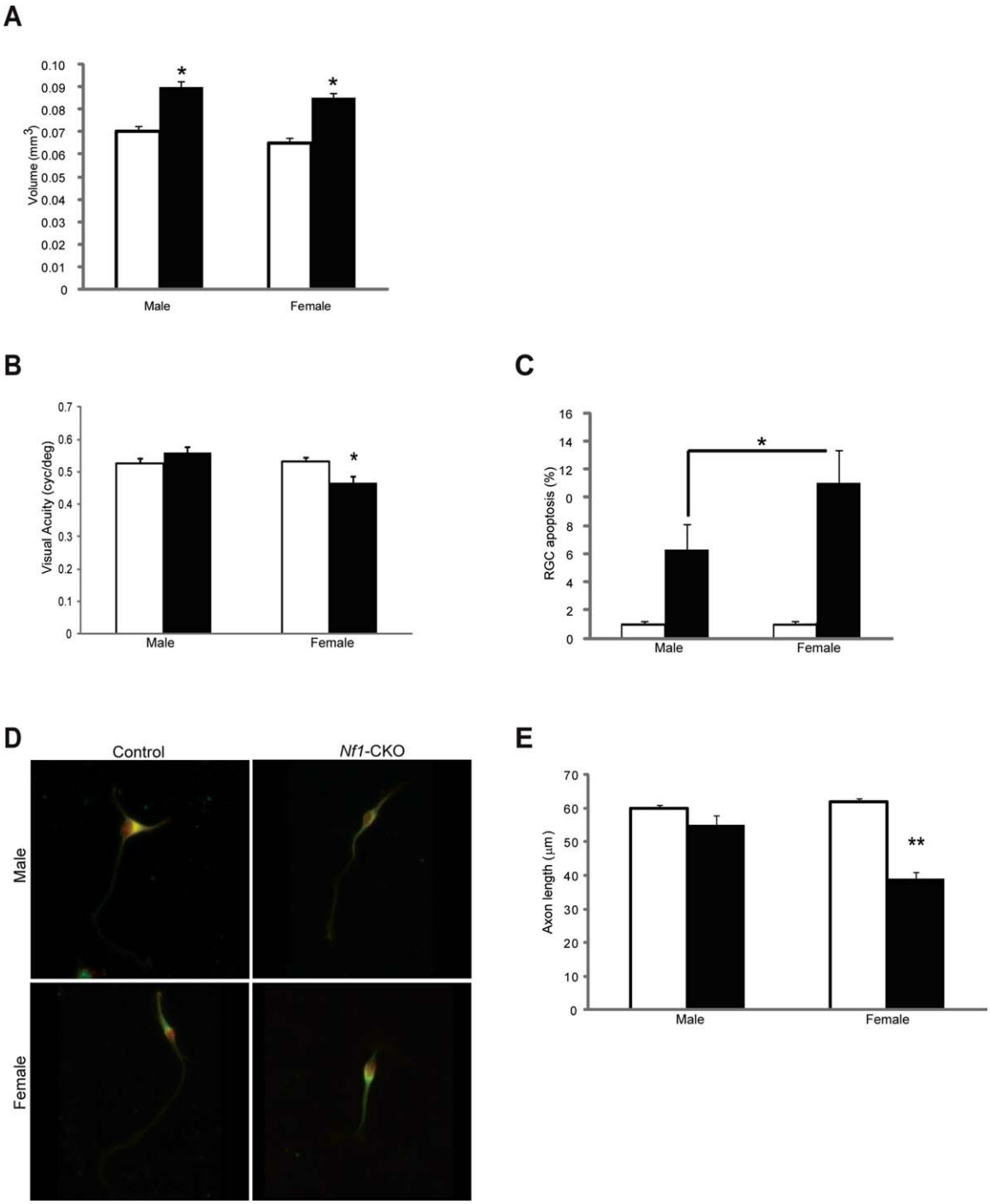

FIGURE 2: Optic glioma–associated vision disturbances are greater in female *Nf1*-CKO mice. (A) Optic nerve volumes in *Nf1*-CKO mice are larger than those observed in control littermate mice, regardless of sex. (B) Only female *Nf1*-CKO mice have impaired visual acuity (cycles/degree) relative to controls. (C) Greater retinal ganglion cell (RGC) apoptosis (percentage terminal deoxynucleotide transferase–mediated deoxyuridine triphosphate nick-end labeling–positive 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 RGCs 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. White bars denote control mice; black bars denote *Nf1*-CKO mice. *$p < 0.05$, **$p < 0.01$. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

such, previous studies have demonstrated that reduced RGC axon lengths and survival results from impaired neurofibromin generation of 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

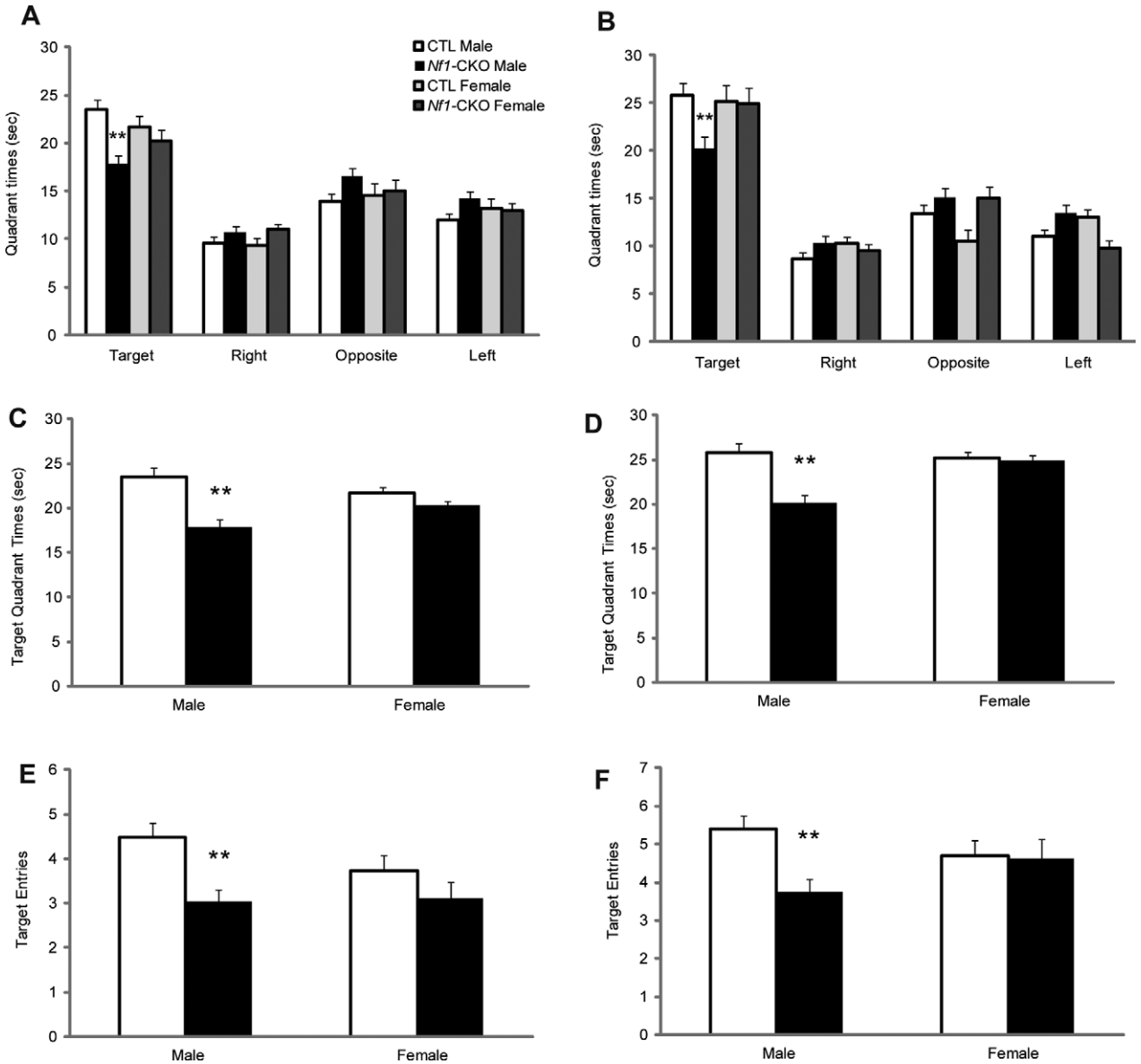

FIGURE 3: Spatial learning impairments in the Morris Water Maze occur only in *Nf1*-CKO male mice. (A, B) During the first (A) and second (B) probe trials, control (CTL) mice and female *Nf1*-CKO mice spent significantly more time in the target quadrant than all the other quadrants. In contrast, male *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. **$p < 0.01$.

hippocampal Ras activity and dopamine levels, such that either Ras inhibition (eg, lovastatin)[21] or dopamine elevation (eg, methylphenidate[23]) corrects these learning/memory impairments in *Nf1* mutant mice (see 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 (see Fig 4). Second, only *Nf1*-CKO male mice had reduced hippocampal dopamine levels and DARPP32 phosphorylation compared to controls. Third, only male *Nf1*-CKO mice exhibited increased hippocampal Ras activation and ERK1/2 phosphorylation (activation) relative to controls.

Discussion

Although 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 multifactorial and often difficult to establish in human clinical studies. For this reason, *Nf1* GEM strains provide

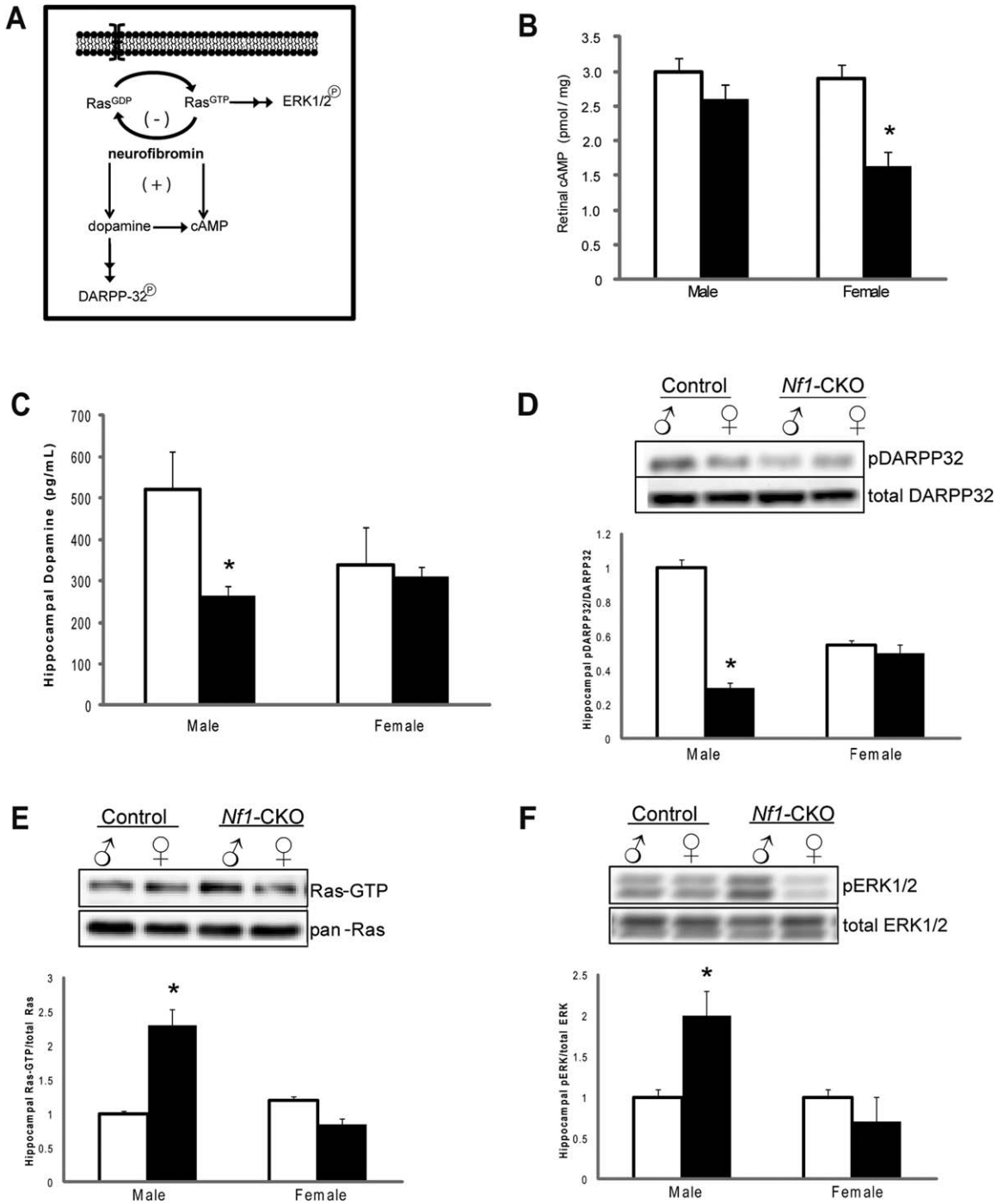

FIGURE 4: Sexually dimorphic regulation of neurofibromin signaling pathways. (A) Established molecular pathways regulated by the *NF1* gene product, neurofibromin. (B) Retinal cyclic adenosine monophosphate (cAMP) levels were reduced by 47% in female *Nf1*-CKO mice relative to female controls. No change 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. *$p < 0.05$.

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 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 demonstrated 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.

Although 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 2 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. Although 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/mammalian target of rapamycin) in a manner that is specific to both cell type and brain region[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 (4 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 3 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 (eg, 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.

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Authorship

K.A.D.-A., J.A.B., and S.M.G. performed the experiments and analysis. K.A.D.-A. and D.H.G. wrote and edited the manuscript, with comments from D.F.W. and J.B.R.

Potential Conflicts of Interest

D.F.W.: grants/grants pending, NIH. D.H.G.: grants/grants pending, NIH, JSMF, NBTS, Department of Defense; patents, NF1 gene patent, NF1 mTOR patent; royalties, NF1 gene license.

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