

AWARD NUMBER: W81XWH-14-1-0076

TITLE: Zebrafish as a Model to Study NF1-Associated Learning Deficits

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REPORT DATE: July 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE July 2016		2. REPORT TYPE Final		3. DATES COVERED 15 Apr 2014 - 14 Apr 2016	
4. TITLE AND SUBTITLE Zebrafish as a Model to Study NF1-Associated Learning Deficits				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-14-1-0076	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Michael Granato, Ph.D. E-Mail: granatom@mail.med.upenn.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Pennsylvania Cell & Developmental Biology 1110 BRB II/III, 421 Curie Blvd. Philadelphia, PA 19104-6058				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Neurofibromatosis type 1 (NF1) is a neurogenetic disorder best known to cause predisposition to central and peripheral nervous system tumors. At the same time, NF1 causes significant cognitive impairments, and 50-70% of children with NF1 exhibit cognitive dysfunction, most prominently scholastic under-performance characterized by attention deficit and learning disabilities. The NF1 protein govern distinct aspects of cognitive behavior: the NF1-GRD attenuates Ras-pathway and GABA signaling to regulate memory, while the NF1 C-terminal region activates adenylate cyclase - mediated cAMP homeostasis to govern learning. Despite clear evidence of cAMP-mediated learning in <i>Drosophila Nf1</i> models, it is unclear whether NF1-dependent cAMP signaling is critical for vertebrate learning and/or memory. The aims of this proposal are to take advantage of the zebrafish system to 1) determine whether cAMP signaling contributes to NF1-dependent learning and memory in vertebrates and 2) identify compounds that attenuate the learning deficiency in NF1 through screening libraries of bioactive small molecules.					
15. SUBJECT TERMS Neurofibromatosis; zebrafish; NF1; cAMP; Ras; learning; memory					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	21	19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION

Neurofibromatosis type 1 (NF1) is a neurogenetic disorder best known to cause predisposition to central and peripheral nervous system tumors. At the same time, NF1 causes significant cognitive impairments, and 50-70% of children with NF1 exhibit cognitive dysfunction, most prominently scholastic under-performance characterized by attention deficit and learning disabilities. The NF1 protein governs distinct aspects of cognitive behavior: the NF1-GRD attenuates Ras-pathway and GABA signaling to regulate memory, while the NF1 C-terminal region activates adenylate cyclase-mediated cAMP homeostasis to govern learning. Despite clear evidence of cAMP-mediated learning in *Drosophila Nf1* models, it is unclear whether NF1-dependent cAMP signaling is critical for vertebrate learning and/or memory. The aims of this proposal are to take advantage of the zebrafish system to 1) determine whether cAMP signaling contributes to NF1-dependent learning and memory in vertebrates and 2) identify compounds that attenuate the learning deficiency in NF1 through screening libraries of bioactive small molecules.

2. KEYWORDS:

Neurofibromatosis; zebrafish; NF1; cAMP; Ras; learning; memory;

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Determine whether cAMP signaling is critical for NF1 dependent learning and memory in vertebrates.

Specific Aim 2: Identify chemical modifiers of *Nf1* dependent learning.

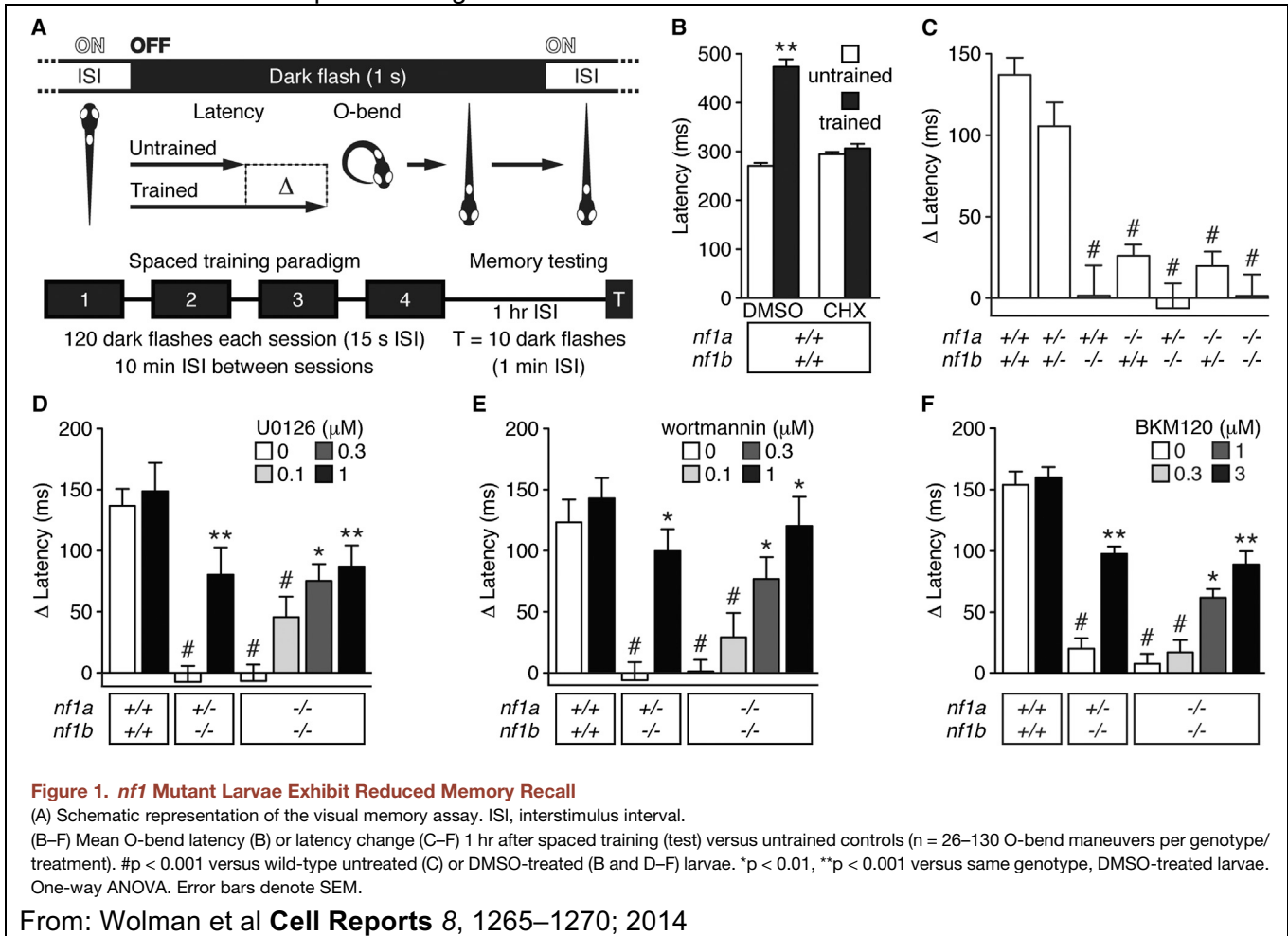
What was accomplished under these goals?

Specific Aim 1: This Aim has been completed, and the results have been published (**Cell Reports** 8, 1265–1270; 2014). **Below** I outline in accordance with the approved Statement of work what was accomplished.

Specific Aim 1, Task 1 was to determine concentrations of forskolin, rolipram, and 8-Br-cAMP which would not affect baseline O-bend performance. For this we tested each of these drugs at concentrations from 1nM – 100 μ M in 1% DMSO. Specifically, we found that 20 minutes to 4 hour exposures for each of these drugs at 1, 3 and 10 μ M in 1% DMSO did not reduce baseline performance (frequency of O-bend response initiation and O-bend behavior performance kinematics). We therefore used these three concentrations in tasks 2 and 3, see below. During the course of the project (although not part of the original statement), we tested additional modulators of learning and memory pathways for overall toxicity and O-bend performance. Specifically, we tested the inhibitors of the Ras effectors MAPK (UO126) and PI3K (wortmannin, and BKM120). Again, we found that 20 minutes to 4 hour exposures for each of these drugs at 1, 3 and 10 μ M in 1% DMSO did not reduce baseline performance (frequency of O-bend response initiation and O-bend behavior performance kinematics). We therefore used these three concentrations in tasks 2 and 3, see below.

Specific Aim 1, Task 3 was to determine whether increasing cAMP signaling rescues NF1 **memory** deficit. Larval zebrafish show a remarkable capacity for behavioral plasticity including habituation to visual stimuli. We have previously shown that after a period of light adaptation, exposing the larvae to a sudden absence of light, termed a dark flash, elicited a highly stereotyped yet habituable reorientation maneuver known as an O-bend (Burgess and Granato, 2007). Importantly, habituation reflects a highly conserved form of attention-based learning and memory that is similar to the type of cognition impairment found in NF1 children. We first confirmed that delivering repetitive dark flashes through a spaced training paradigm elicited protein-synthesis-dependent memory formation (**Figures 1A and 1B** below). We next utilized a zebrafish model of NF1 that harbors null alleles in the *NF1* orthologs *nf1a*

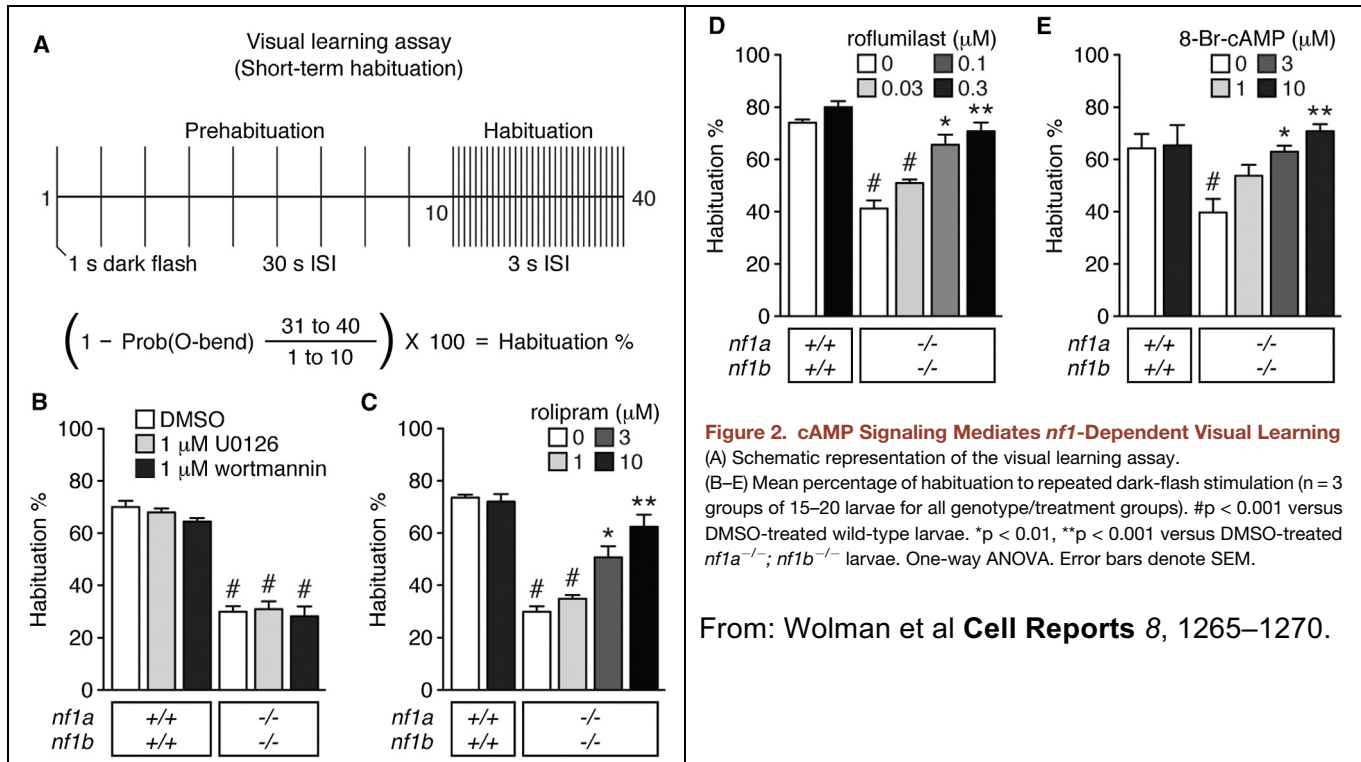
and *nf1b* to evaluate memory recall. Larvae null for *nf1a* or *nf1b* showed impaired memory (Figure 1C, see below). This memory deficit is consistent with cognitive impairment observed in NF1 patients and in other animal models of NF1, and supports the use of *nf1* mutant zebrafish to probe the mechanisms of NF1-dependent cognition.



Memory impairment in *Drosophila* and mouse NF1 models is due at least in part to elevated Ras signaling. We therefore asked whether acute pharmacological inhibition of the Ras effectors MAPK and PI3K could improve memory recall in *nf1* mutants. Small molecules readily cross the developing blood-brain barrier of larval zebrafish until at least 8 days of age. We treated wild-type, *nf1a*^{+/-}; *nf1b*^{-/-}, and *nf1a*^{-/-}; *nf1b*^{-/-} larvae with inhibitors of MAPK (U0126) or PI3K (wortmannin, BKM120) for 30 min before and throughout training and testing for memory recall. Each compound improved memory recall in *nf1* mutant larvae in a dose-dependent manner (Figures 1D–1F, see above). Treatment with 1 mM wortmannin restored memory to wild-type levels, and 1 mM U0126 or 3 mM BKM120 yielded significant memory improvement. Although each of these Ras pathway antagonists exhibits known off-target effects, their different selectivity profiles suggest that nonspecific effects are unlikely to underlie the observed increase in memory recall. Therefore, these results support a conserved function for the neurofibromin GRD domain in regulation of memory formation through the Ras/MAPK/PI3K signaling pathway.

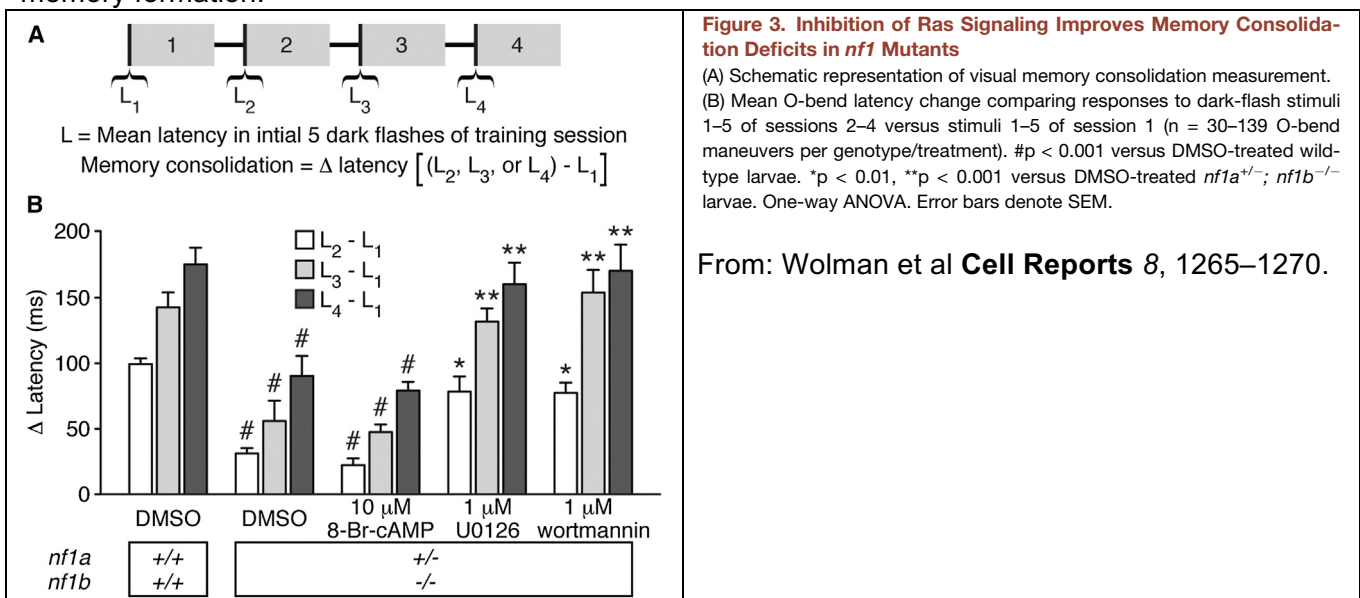
Specific Aim 1, Task 2 was to determine whether increasing cAMP signaling rescues NF1 learning deficit. Learning (the acquisition of information) is critical for establishing memory. We evaluated learning by exposing larvae to dark-flash stimuli delivered at 3 second interstimulus intervals (ISIs) and measuring short-term habituation, as indicated by a reduction in the probability of initiating an O-

bend response (Figure 2A, see below). *nf1a*^{-/-}; *nf1b*^{-/-} larvae showed markedly reduced short-term visual (Figure 2B, see below) and acoustic habituation compared with wild-type controls. Larvae with at least one wild-type allele of either *nf1a* or *nf1b* did not show a learning deficit, despite dramatic memory deficits (Figure 1C, see above). It is possible that our nonassociative habituation assay lacks the necessary sensitivity to detect relatively subtle learning deficiencies in larvae with these genotypes. Attenuating Ras signaling by acute pharmacological inhibition of MAPK (U0126) or PI3K (wortmannin) failed to improve the learning deficit of *nf1a*^{-/-}; *nf1b*^{-/-} larvae (Figures 2B, see below), suggesting that a distinct pathway mediates NF1-dependent learning.

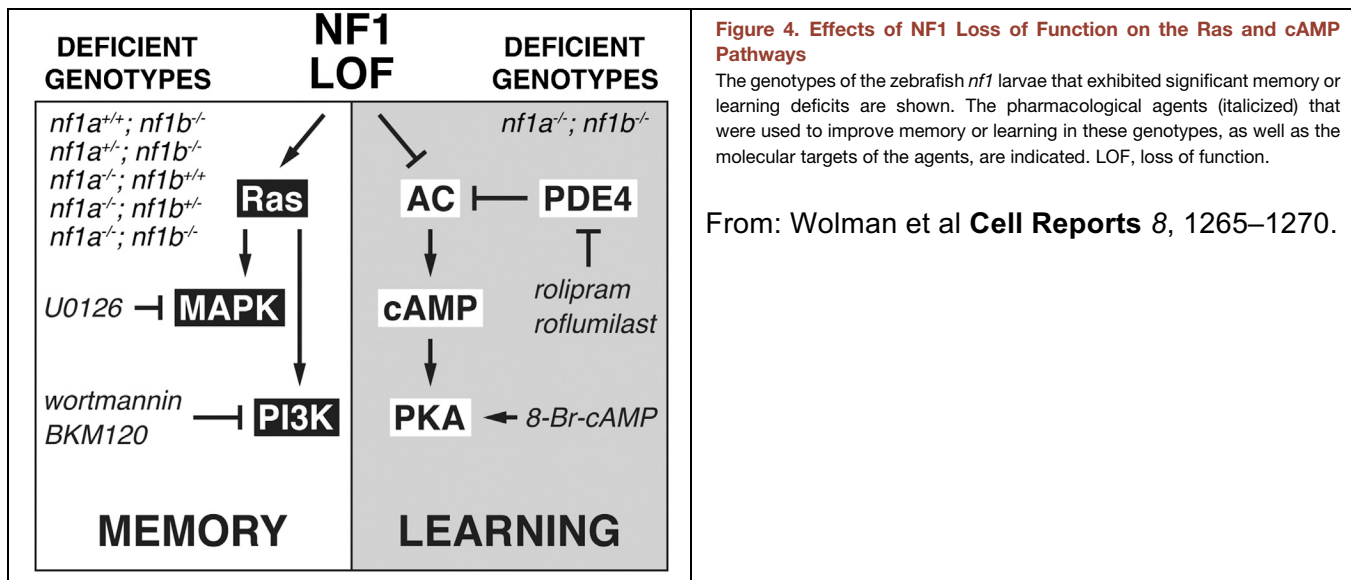


To determine whether reduced cAMP signaling contributed to the learning deficits in *nf1a*^{-/-}; *nf1b*^{-/-} mutants, we tested whether enhancing cAMP signaling by acute pharmacological inhibition of phosphodiesterase 4 (PDE4) or stimulation of PKA could improve learning. Inhibition of PDE4 by rolipram or roflumilast, or PKA stimulation by 8-Br-cAMP improved learning behavior in *nf1a*^{-/-}; *nf1b*^{-/-} mutants in response to both repetitive visual (Figures 2C–2E). Treatment with at least 10 mM rolipram, 0.1 mM roflumilast, or 3 mM 8-Br-cAMP improved habituation to wild-type levels. These results provide evidence that cAMP signaling regulates NF1-dependent learning in a vertebrate system. We next asked whether cAMP signaling regulates NF1-dependent memory in addition to learning. We tested *nf1a*^{-/-}; *nf1b*^{-/-} larvae, which show reduced learning and a failure to recall memory, and *nf1a*^{+/-}; *nf1b*^{-/-} larvae, which learn normally but fail to form memory, and compared them with wild-type controls. Treatment with 10 mM 8-Br-cAMP, a sufficient dose to restore learning in *nf1a*^{-/-}; *nf1b*^{-/-} larvae (Figures 2E, see above), failed to improve memory recall in either *nf1a*^{+/-}; *nf1b*^{-/-} or *nf1a*^{-/-}; *nf1b*^{-/-} larvae. These results suggest that **cAMP signaling regulates NF1-dependent learning but not memory**. Moreover, these results indicate that the memory defects in *nf1a*^{-/-}; *nf1b*^{-/-} mutants are not simply attributable to their learning deficit. These data strongly imply that molecularly distinct pathways that control learning and memory are affected in NF1.

Learned behavior requires consolidation to form stable memory. Despite consensus that defective neurofibromin function can result in learning and memory impairments, whether impaired consolidation contributes to memory deficits remains unclear. *nf1a*^{+/-}; *nf1b*^{-/-} larvae learn normally but show reduced memory recall (Figure 1C, above). Therefore, we asked whether reduced memory was due to a **consolidation** deficit. We determined memory consolidation by calculating the difference between the mean O-bend latency in response to the first five dark-flash stimuli of training session 1 and subsequent training sessions (Figure 3A, see below). Long ISIs between training sessions promote memory consolidation, and therefore spaced training paradigms elicit more stable memory than do massed training paradigms. After each session, *nf1a*^{+/-}; *nf1b*^{-/-} larvae showed reduced consolidation compared with wild-type larvae (Figure 3B, see below), suggesting that the memory-recall deficit observed in *nf1a*^{+/-}; *nf1b*^{-/-} larvae (Figure 1C, above) may be due to a defect in memory consolidation. To determine the contribution of cAMP and Ras signaling to NF1-dependent memory consolidation, we attempted to improve consolidation in *nf1a*^{+/-}; *nf1b*^{-/-} larvae by pharmacologically enhancing cAMP or attenuating Ras. Enhancing cAMP in *nf1a*^{+/-}; *nf1b*^{-/-} larvae by treatment with 10 mM 8-Br-cAMP did not increase consolidation (Figure 3B, see below). Pharmacological inhibition of MAPK (1 mM U0126) or PI3K (1 mM wortmannin) improved memory consolidation in *nf1a*^{+/-}; *nf1b*^{-/-} larvae to levels indistinguishable from those observed in DMSO-treated wild-type larvae (Figure 3B, see below). These results reveal that deficits in memory consolidation contribute to the etiology of memory dysfunction in NF1 and support a specific role for Ras signaling in mediating NF1-dependent memory formation.



In summary, the data we generated in Specific Aim 1 provide compelling evidence that in vertebrates NF1 affects at least two distinct signaling pathways that independently modulate learning and memory (Figure 4, see below). Furthermore we demonstrate that deficits produced by genetic loss of NF1 function are reversible. These findings support the investigation of cAMP signaling enhancers as a companion therapy to Ras inhibition in the treatment of cognitive dysfunction in NF1.



Specific Aim 2: Identify chemical modifiers of learning in *Nf1* mutant zebrafish.

Task 1 was to screen 4080 small molecules for increased or decreased learning in *Nf1* mutant zebrafish larvae. This task required to generation and testing of approximately 130,000 larvae (32 larvae per compound). As we were to begin task 1 of Specific Aim 2 we noticed a very strong loss in fertility of our $Nf1a^{-/-}, Nf1b^{+/-} \times Nf1a^{+/-}, Nf1b^{-/-}$ adults, i.e. the breeders of the larvae to be tested ($Nf1a^{-/-}; Nf1b^{-/-}$ double homozygous animals survive until ~10 day post fertilization, but are not viable as adults). Since only a quarter of the larvae are expected to be of the desire genotype $Nf1a^{-/-}, Nf1b^{-/-}$, the loss of fertility almost instantly halted progress on this task. The cause of this reduced fertility was unclear, but we suspected that it might be due to keeping the $Nf1a^{-/-}$ and $Nf1b^{-/-}$ gene as homozygotes. After 3 month of trying to generate embryos from the $Nf1a^{-/-}, Nf1b^{+/-} \times Nf1a^{+/-}, Nf1b^{-/-}$ adults, we decided to ‘rejuvenate’ the stocks by outcrossing $Nf1a^{-/-}, Nf1b^{+/-}$ or $Nf1a^{+/-}, Nf1b^{-/-}$ adults to wild type adults. It took us ~6 month to finally have enough $Nf1a^{-/-}, Nf1b^{+/-} \times Nf1a^{+/-}, Nf1b^{-/-}$ adults to start the screen. In the retaining time of the funding period we were able to test a total of 424 compounds, or 10.4% of the proposed compounds. This did not yield any compounds that increased or decreased learning in *Nf1* mutant zebrafish larvae. Although we had hoped to identify at least one or two compounds by testing 10% of all compounds, the results did confirm the specificity of our assay. Below is a list of all tested compounds.

Task 2 was evaluate potential dose-response effect of compounds affecting learning. As outlined above, after testing only a small fraction of all compounds we did not identify any compound to further evaluate potential dose-response effects.

Task 3 was to evaluate specificity of compounds affecting learning. As outlined above, after testing only a small fraction of all compounds we did not identify any compound to further evaluate specificity.

List of all tested compounds:

S1003	Linifanib (ABT-869)	S1101	Vatalanib (PTK787) 2HCl
S1017	Cediranib (AZD2171)	S1109	BI 2536
S1026	Imatinib Mesylate (STI571)	S1119	Cabozantinib (XL184, BMS-907351)
S1039	Rapamycin (Sirolimus)	S1147	Barasertib (AZD1152-HQPA)
S1055	Enzastaurin (LY317615)	S1010	Nintedanib (BIBF 1120)
S1075	SB216763	S1022	Ridaforolimus (Deforolimus, MK-8669)
S1092	KU-55933 (ATM Kinase Inhibitor)	S1035	Pazopanib HCl (GW786034 HCl)
S1105	LY294002	S1046	Vandetanib (ZD6474)
S1114	JNJ-38877605	S1068	Crizotinib (PF-02341066)
S1134	AT9283	S1084	Brivanib (BMS-540215)
S1005	Axitinib	S1102	U0126-EtOH
S1018	Dovitinib (TKI-258, CHIR-258)	S1111	Foretinib (GSK1363089)
S1028	Lapatinib (GW-572016) Ditosylate	S1120	Everolimus (RAD001)
S1040	Sorafenib Tosylate	S1152	PLX-4720
S1056	AC480 (BMS-599626)	S1011	Afatinib (BIBW2992)
S1076	SB203580	S1023	Erlotinib HCl (OSI-744)
S1093	GSK1904529A	S1036	PD0325901
S1106	OSU-03012 (AR-12)	S1048	VX-680 (Tozasertib, MK-0457)
S1116	Palbociclib (PD-0332991) HCl	S1070	PHA-665752
S1138	Brivanib Alaninate (BMS-582664)	S1088	NVP-ADW742
S1006	Saracatinib (AZD0530)	S1103	ZM 447439
S1019	Canertinib (CI-1033)	S1112	SGX-523
S1032	Motesanib Diphosphate (AMG-706)	S1124	BMS-754807
S1042	Sunitinib Malate	S1153	Roscovitine (Seliciclib,CYC202)
S1064	Masitinib (AB1010)	S1014	Bosutinib (SKI-606)
S1077	SB202190 (FHPI)	S1025	Gefitinib (ZD1839)
S1094	PF-04217903	S1038	PI-103
S1107	Danuserib (PHA-739358)	S1049	Y-27632 2HCl
S1117	Triciribine	S1072	ZSTK474
S1143	AG-490 (Tyrphostin B42)	S1091	OSI-906 (Linsitinib)
S1008	Selumetinib (AZD6244)	S1104	GDC-0879
S1020	PD184352 (CI-1040)	S1113	GSK690693
S1033	Nilotinib (AMN-107)	S1133	Alisertib (MLN8237)
S1043	Tandutinib (MLN518)	S1154	SNS-314 Mesylate
S1065	GDC-0941	S1164	Lenvatinib (E7080)
S1078	MK-2206 2HCl	S1179	WZ8040
S1100	MLN8054	S1234	AG-1024
S1108	TAE684 (NVP-TAE684)	S1314	Zoledronic Acid
S1118	XL147	S1392	Pelitinib (EKB-569)
S1145	SNS-032 (BMS-387032)	S1474	GSK429286A
S1009	BEZ235 (NVP-BEZ235, Dactolisib)	S1519	CCT129202
S1021	Dasatinib	S1533	R406 (free base)
S1034	NVP-AEW541	S1570	KU-60019
S1044	Temsirolimus (CCI-779, NSC 683864)	S2013	PF-573228
S1066	SL-327	S1167	CP-724714
S1080	SU11274	S1181	ENMD-2076
		S1244	Amuvatinib (MP-470)
		S1342	Genistein

S1451	Aurora A Inhibitor I	S1274	BX-795
S1475	Pimasertib (AS-703026)	S1363	Ki8751
S1523	SAR245409 (XL765)	S1462	AZD6482
S1536	CP-673451	S1490	Ponatinib (AP24534)
S1572	BS-181 HCl	S1531	BIX 02189
S2014	BMS-265246	S1561	BMS-777607
S1169	TGX-221	S1590	TWS119
S1194	CUDC-101	S2163	PF-4708671
S1249	JNJ-7706621	S1178	Regorafenib (BAY 73-4506)
S1352	TG100-115	S1226	KU-0063794
S1454	PHA-680632	S1275	BX-912
S1485	HMN-214	S1378	Ruxolitinib (INCB018424)
S1524	AT7519	S1470	TSU-68 (SU6668, Orantinib)
S1555	AZD8055	S1494	LY2228820
S1573	Fasudil (HA-1077) HCl	S1532	AZD7762
S2134	AZD8330	S1568	PD318088
S1170	WZ3146	S1802	Acadesine
S1205	PIK-75	S2179	LY2784544
S1264	PD173074	S2183	BGJ398 (NVP-BGJ398)
S1360	GSK1059615	S2205	OSI-420
S1458	VX-745	S2226	CAL-101 (Idelalisib, GS-1101)
S1486	AEE788 (NVP-AEE788)	S2310	Honokiol
S1526	Quizartinib (AC220)	S2622	PP121
S1556	PHT-427	S2638	NU7441 (KU-57788)
S1574	BIRB 796 (Doramapimod)	S2680	Ibrutinib (PCI-32765)
S2150	Neratinib (HKI-272)	S2696	GDC-0980 (RG7422)
S1171	CYC116	S2726	PH-797804
S1207	Tivozanib (AV-951)	S2742	PHA-767491
S1266	WYE-354	S2185	AST-1306
S1361	MGCD-265	S2207	PIK-293
S1459	Thiazovivin	S2227	PIK-294
S1487	PHA-793887	S2386	Indirubin
S1529	Hesperadin	S2624	OSI-027
S1557	KRN 633	S2658	GSK2126458 (GSK458)
S1577	Tie2 kinase inhibitor	S2681	AS-604850
S2158	KW-2449	S2697	A-769662
S1173	WZ4002	S2727	Dacomitinib (PF299804, PF299)
S1219	YM201636	S2743	PF-04691502
S1267	Vemurafenib (PLX4032, RG7204)	S2192	AZD8931 (Sapitinib)
S1362	Rigosertib (ON-01910)	S2214	AZ 960
S1460	SP600125	S2231	Telatinib
S1489	PIK-93	S2391	Quercetin
S1530	BIX 02188	S2625	Fostamatinib (R788)
S1558	AT7867	S2661	WYE-125132 (WYE-132)
S1582	H 89 2HCl	S2682	CAY10505
S2161	RAF265 (CHIR-265)	S2699	CH5132799
S1177	PD98059	S2728	AG-1478 (Tyrphostin AG-1478)
S1220	OSI-930	S2744	CCT137690

S2193	GSK461364	S2740	GSK1070916
S2216	Mubritinib (TAK 165)	S2751	Miliciclib (PHA-848125)
S2235	Volasertib (BI 6727)	S2752	HER2-Inhibitor-1
S2406	Chrysophanic Acid	S2769	Dovitinib (TKI-258) Dilactic Acid
S2626	LY2603618	S2796	WP1066
S2670	A-674563	S2817	Torin 2
S2683	CHIR-124	S2859	Golvatinib (E7050)
S2700	KX2-391	S2895	Tyrphostin 9
S2729	SB415286	S2922	Icotinib
S2745	CHIR-98014	S6005	VX-702
S2194	R406	S7039	PD168393
S2218	PP242	S7114	NU6027
S2238	Palomid 529 (P529)	S2755	Varlitinib
S2475	Imatinib (STI571)	S2770	MK-5108 (VX-689)
S2628	PF-05212384 (PKI-587)	S2801	AZD4547
S2671	AS-252424	S2820	TAE226 (NVP-TAE226)
S2686	NVP-BSK805 2HCl	S2864	IMD 0354
S2703	GSK1838705A	S2896	ZM 323881 HCl
S2730	Crenolanib (CP-868596)	S2924	CHIR-99021 (CT99021) HCl
S2746	AZ 628	S7000	AP26113
S2198	SGL-1776 free base	S7050	AZ20
S2219	CYT387	S7127	TIC10
S2243	Degrasyn (WP1130)	S2758	Wortmannin
S2542	Phenformin HCl	S2774	MK-2461
S2634	DCC-2036 (Rebastinib)	S2806	CEP-33779
S2672	PF-00562271	S2823	Tideglusib
S2688	R547	S2867	WHI-P154
S2718	TAK-901	S2897	ZM 306416
S2735	MK-8776 (SCH 900776)	S2928	TAK-715
S2747	AMG-458	S7007	MEK162 (ARRY-162, ARRY-438162)
S2201	BMS-794833	S7060	PP1
S2220	SB590885	S7136	CGK 733
S2247	BKM120 (NVP-BKM120, Buparlisib)	S2759	CUDC-907
S2617	TAK-733	S2783	AZD2014
S2635	CCT128930	S2807	Dabrafenib (GSK2118436)
S2673	Trametinib (GSK1120212)	S2824	TPCA-1
S2689	WAY-600	S2870	TG100713
S2719	AMG-900	S2899	GNF-2
S2736	TG101348 (SAR302503)	S3012	Pazopanib
S2749	BGT226 (NVP-BGT226)	S7008	PP2
S2202	NVP-BHG712	S7065	MK-8745
S2221	Apatinib	S7145	AZD1080
S2266	Asiatic Acid	S2761	NVP-BVU972
S2621	AZD5438	S2784	TAK-285
S2636	A66	S2808	GDC-0068
S2679	Flavopiridol HCl	S2827	Torin 1
S2692	TG101209	S2872	GW5074
S2720	ZM 336372	S2902	S-Ruxolitinib (INCB018424)

S3026	Piceatannol	S8058	P276-00
S7018	CZC24832	S1079	PD153035 HCl
S7083	LDK378	S7338	AZ191
S7153	10058-F4	S1041	STF-62247
S2762	Alectinib (CH5424802)	S1130	YM155 (Sepantronium Bromide)
S2788	INCB28060	S1165	ABT-751 (E7010)
S2811	INK 128 (MLN0128)	S7195	RKI-1447
S2842	SAR131675	S7293	ZCL278
S2882	IKK-16 (IKK Inhibitor VII)	S8004	ZM 39923 HCl
S2904	PF-477736	S8031	NSC 23766
S4907	SC-514	S8078	Bardoxolone Methyl
S7028	IPI-145 (INK1197)	S1579	PD 0332991 (Palbociclib) Isethionate
S7093	IPA-3	S7342	UNC2250
S7158	LY2835219	S1052	Elesclomol (STA-4783)
S2767	3-Methyladenine	S1140	Andarine
S2789	Tofacitinib (CP-690550, Tasocitinib)	S1166	Cisplatin
S2814	BYL719	S7198	BIO
S2843	BI-D1870	S7317	WZ4003
S2890	PF-562271	S8005	SMI-4a
S2911	Go 6983	S8032	PRT062607 (P505-15, BIIB057) HCl
S5001	Tofacitinib (CP-690550) Citrate	S1537	DMXAA (Vadimezan)
S7035	XL388	S2245	CP-466722
S7102	VE-822	S7440	LEE011
S7167	SSR128129E	S1057	Obatoclox Mesylate (GX15-070)
S2768	Dinaciclib (SCH727965)	S1141	17-AAG (Tanespimycin)
S2791	Sotrastaurin	S1172	JNJ-26854165 (Serdemetan)
S2816	Tyrphostin AG 879	S7207	Ro 31-8220 Mesylate
S2845	Semaxanib (SU5416)	S7319	EHop-016
S2893	NU7026	S8007	VE-821
S2913	BAY 11-7082	S8036	Butein
S5002	Fingolimod (FTY720) HCl	S2891	GW441756
S7036	XL019	S2725	PF-03814735
S7106	AZD3463	S7509	ML167
S7173	AVL-292	S1061	Nutlin-3
S7176	SKI II	S1142	17-DMAG (Alvespimycin) HCl
S7284	CO-1686 (AVL-301)	S1174	MK-2866 (GTx-024)
S8002	GSK2636771	S7214	Skepinone-L
S8023	TCS 359	S7320	TG003
S8057	Pacritinib (SB1518)	S8009	AG-18
S8042	GW2580	S8040	GDC-0349
S7327	ID-8	S7258	SKLB1002
S1029	Lenalidomide (CC-5013)	S7108	LGX818
S1121	TW-37	S1001	ABT-263 (Navitoclax)
S1159	Ganetespib (STA-9090)	S1067	SB431542
S7194	GZD824	S1148	Docetaxel
S7291	TAK-632	S1175	BIIB021
S8003	PQ 401	S7253	AZD2858
S8024	Tyrphostin AG 1296	S7397	Sorafenib

S8015	CEP-32496	S1231	Topotecan HCl
S8044	BMS-345541	S1289	Carmofur
S7357	PF-562271 HCl	S1364	Epothilone B (EPO906, Patupilone)
S7193	1-Azakenpaullone	S1522	Tosedostat (CHR2797)
S1002	ABT-737	S1655	Ezetimibe
S1069	AUY922 (NVP-AUY922)	S1721	Azathioprine
S1150	Paclitaxel	S1909	Fluvastatin Sodium
S1180	XAV-939	S1189	Aprepitant
S7257	CNX-774	S1208	Doxorubicin (Adriamycin)
S7435	AR-A014418	S1218	Clofarabine
S8019	AZD5363	S1235	Letrozole
S8050	ETP-46464	S1297	Epothilone A
S7523	GS-9973	S1379	Isotretinoin
S7270	SRPIN340	S1525	MK-1775
S1013	Bortezomib (PS-341)	S1672	Aminoglutethimide
S1097	BTZ043 Racemate	S1735	Mesna
S1156	Capecitabine	S2003	Maraviroc
S1186	BIBR 1532	S1190	Bicalutamide
S1188	Anastrozole	S1209	Fluorouracil (5-Fluoracil, 5-FU)
S1201	Dimesna	S1221	Dacarbazine
S1215	Carboplatin	S1237	Temozolomide

What opportunities for training and professional development has the project provided?

The postdoctoral fellow Dr. Kurt Marsden completed an IDP which served as a basis to openly discuss his achievements, plans to further develop their skills, and to prepare him for applying for academic positions. In addition, Dr. Marsden was my lab assistant for a zebrafish course in Woods Hole in which I teach about the use of zebrafish for neuroscience research. This course is a hands-on course for 22 students (faculty, postdocs and PhD students). In addition to gaining experience in teaching, Dr. Marsden interacted with all other course faculty (~12), all leaders in the field.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. IMPACT

Our publication has been cited 10 time since its publication in September 2014.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS

Significant changes in use or care of human subjects.

Nothing to Report.

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents.

Nothing to Report.

6. PRODUCTS.

Publications

Wolman, M.A, de Groh, E. D, McBride, S. M., Jongens, T. A., **Granato, M*** and Epstein, J.A*. Modulation of cAMP and Ras signaling pathways improves distinct behavioral deficits in a zebrafish model of Neurofibromatosis type 1. *Cell reports*, 8(5):1265-70. ***Co-senior authors.**

Other Products

Press Release UPENN:

http://www.uphs.upenn.edu/news/News_Releases/2014/09/epstein/

Zebrafish Model of a Learning and Memory Disorder Shows Better Way to Target Treatment
PHILADELPHIA — Using a zebrafish model of a human genetic disease called neurofibromatosis (NF1), a team from the [Perelman School of Medicine at the University of Pennsylvania](#) has found that the learning and memory components of the disorder are distinct features that will likely need different treatment approaches. They published their [results this month in Cell Reports](#).

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Michael Granato, Ph.D.
Project Role:	PI
Researcher Identifier:	n/a
Nearest person month worked:	1 Cal. Month
Contribution to Project:	Dr. Granato provided the intellectual framework and supervised this project.
Name:	Kurt C. Marsden, Ph.D.
Project Role:	Postdoctoral Researcher
Researcher Identifier:	n/a
Nearest person month worked:	5 Cal. Months
Contribution to Project:	Dr. Marsden performed some of the learning assays for Aim 1.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

N/A

9. APPENDICES:

Wolman, M.A, de Groh, E. D, McBride, S. M., Jongens, T. A., **Granato, M*** and Epstein, J.A*. Modulation of cAMP and Ras signaling pathways improves distinct behavioral deficits in a zebrafish model of Neurofibromatosis type 1. *Cell reports*, 8(5):1265-70. ***Co-senior authors.**

Modulation of cAMP and Ras Signaling Pathways Improves Distinct Behavioral Deficits in a Zebrafish Model of Neurofibromatosis Type 1

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<http://dx.doi.org/10.1016/j.celrep.2014.07.054>

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SUMMARY

Neurofibromatosis type 1 (NF1) is a common autosomal-dominant disorder associated with attention deficits and learning disabilities. The primary known function of neurofibromin, encoded by the *NF1* gene, is to downregulate Ras activity. We show that *nf1*-deficient zebrafish exhibit learning and memory deficits and that acute pharmacological inhibition of downstream targets of Ras (MAPK and PI3K) restores memory consolidation and recall but not learning. Conversely, acute pharmacological enhancement of cAMP signaling restores learning but not memory. Our data provide compelling evidence that neurofibromin regulates learning and memory by distinct molecular pathways in vertebrates and that deficits produced by genetic loss of function are reversible. These findings support the investigation of cAMP signaling enhancers as a companion therapy to Ras inhibition in the treatment of cognitive dysfunction in NF1.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is associated with a broad range of clinical characteristics, including a predisposition to develop benign and malignant tumors, pigmentation defects, and cognitive deficits (Cichowski and Jacks, 2001). As many as 50%–70% of children with NF1 exhibit attention deficits and learning disabilities that contribute to scholastic underachievement and impaired social development (Hyman et al., 2005, 2006; Levine et al., 2006). Genetic and pharmacological experiments performed in mice and *Drosophila* support a role for the Ras-GTPase activating domain (GRD), which functions to downregulate Ras activity in protein-synthesis-dependent memory (Costa et al., 2002; Cui et al., 2008; Guilding et al., 2007; Ho et al., 2007; Li et al., 2005; Silva et al., 1997). However, cognitive dysfunction

in NF1 has been linked to mutations throughout the *NF1* gene that do not cluster in the region encoding the GRD, leading to the proposal that neurofibromin serves additional cellular functions (Fahsold et al., 2000). Studies performed in *Drosophila* suggest that neurofibromin can also stimulate adenylyl cyclase (AC), cAMP production, and PKA to promote learning and memory (Guo et al., 2000; Hannan et al., 2006; The et al., 1997; Tong et al., 2002). *Nf1*-deficient *Drosophila* brains show reduced cAMP levels, and expression of a C-terminal neurofibromin fragment lacking the GRD is sufficient to rescue learning (Ho et al., 2007; Tong et al., 2002). Similarly, brains of *Nf1*^{+/-} mice exhibit reduced cAMP levels (Brown et al., 2010, 2012; Hegedus et al., 2007) and cAMP regulation of dopaminergic function in the hippocampus is disrupted (Diggs-Andrews et al., 2013). The mechanism by which neurofibromin regulates AC remains controversial, and both Ras-dependent and Ras-independent pathways have been suggested (Guo et al., 1997; Hannan et al., 2006; Tong et al., 2002). Studies in *Drosophila* models of NF1 further argue that the resulting elevation in Ras activity, mediated through the upstream activation of neuronal dAkt, is responsible for observed decreases in cAMP signaling (Gouzi et al., 2011; Walker et al., 2006, 2013). Neurofibromin is also known to modulate both neural and glial development from neuroglial progenitors, and both Ras and cAMP have been implicated (Hegedus et al., 2007). Recent studies suggest that pharmacological activation of the cAMP pathway may enhance cognition in murine models (Jayachandran et al., 2014; Peng et al., 2014; Richter et al., 2013). However, it remains unclear whether NF1-dependent cAMP signaling is critical for learning or memory in vertebrates. Furthermore, the contributions of developmental and structural abnormalities to learning and memory deficits in NF1 have not yet been clearly defined (Armstrong et al., 2012; Karlsgodt et al., 2012; Shilyansky et al., 2010).

RESULTS AND DISCUSSION

We utilized a zebrafish model of NF1 that harbors null alleles in the *NF1* orthologs *nf1a* and *nf1b* (Shin et al., 2012) to evaluate molecular signaling pathways that control NF1-dependent

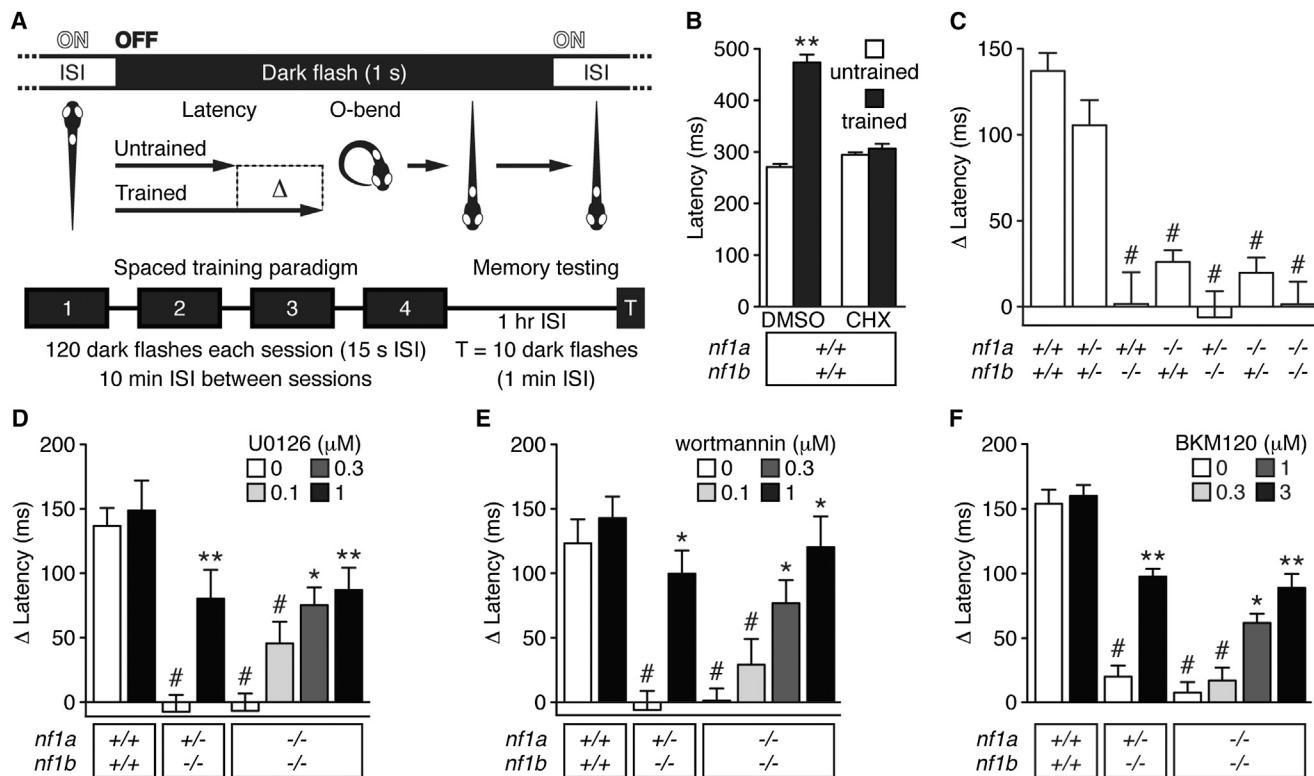


Figure 1. *nf1* Mutant Larvae Exhibit Reduced Memory Recall

(A) Schematic representation of the visual memory assay. ISI, interstimulus interval.

(B–F) Mean O-bend latency (B) or latency change (C–F) 1 hr after spaced training (test) versus untrained controls (n = 26–130 O-bend maneuvers per genotype/ treatment). #p < 0.001 versus wild-type untreated (C) or DMSO-treated (B and D–F) larvae. *p < 0.01, **p < 0.001 versus same genotype, DMSO-treated larvae. One-way ANOVA. Error bars denote SEM.

See also Figures S2 and S3.

learning and memory in vertebrates. Larval zebrafish show a remarkable capacity for behavioral plasticity in response to visual and acoustic stimuli, including habituation (Roberts et al., 2013; Wolman et al., 2011), as evidenced by a progressive decline in responsiveness to repeated, inconsequential stimuli (Thompson and Spencer, 1966). The duration of habituated behavior provides a metric for nonassociative learning (short-term habituation) and memory formation and recall (long-term, protein-synthesis-dependent habituation). Importantly, habituation reflects a highly conserved form of attention-based learning and memory that is similar to the type of cognition impairment found in NF1 children (Hyman et al., 2005; Isenberg et al., 2013; Levine et al., 2006). We tested 5-day-old larvae for protein-synthesis-dependent visual habituation to evaluate memory formation and recall. After a period of light adaptation, exposing the larvae to a sudden absence of light, termed a dark flash, elicited a highly stereotyped yet habituable reorientation maneuver known as an O-bend (Movie S1; Burgess and Granato, 2007a). Delivering repetitive dark flashes through a spaced training paradigm elicited protein-synthesis-dependent memory formation (Figures 1A and 1B). One hour after training, wild-type larvae showed a near doubling in the latency time period before initiating an O-bend compared with responses prior to training (Figure 1B). Treatment with the protein synthesis

inhibitor cycloheximide (CHX, 10 μM) abolished this increase (Figure 1B), consistent with a requirement for protein synthesis (Beck and Rankin, 1995; Davis and Squire, 1984). Larvae null for *nf1a* or *nf1b* showed impaired memory (Figure 1C). This memory deficit is consistent with cognitive impairment observed in NF1 patients and in other animal models of NF1, and supports the use of *nf1* mutant zebrafish to probe the mechanisms of NF1-dependent cognition.

Memory impairment in *Drosophila* and mouse NF1 models is due at least in part to elevated Ras signaling (Costa et al., 2002; Cui et al., 2008; Hannan et al., 2006; Li et al., 2005). Since *nf1* mutant larvae also show increased Ras activity (Shin et al., 2012), we asked whether acute pharmacological inhibition of the Ras effectors MAPK and PI3K could improve memory recall in *nf1* mutants. Small molecules readily cross the developing blood-brain barrier of larval zebrafish until at least 8 days of age (Fleming et al., 2013), facilitating pharmacogenetic approaches for identifying signaling pathways that underlie biological processes and screening of potential therapeutics for neuropsychiatric disorders such as NF1. We treated wild-type, *nf1a*^{+/-}; *nf1b*^{-/-}, and *nf1a*^{-/-}; *nf1b*^{-/-} larvae with inhibitors of MAPK (U0126) or PI3K (wortmannin, BKM120) for 30 min before and throughout training and testing for memory recall. Each compound improved memory recall in *nf1* mutant larvae in a

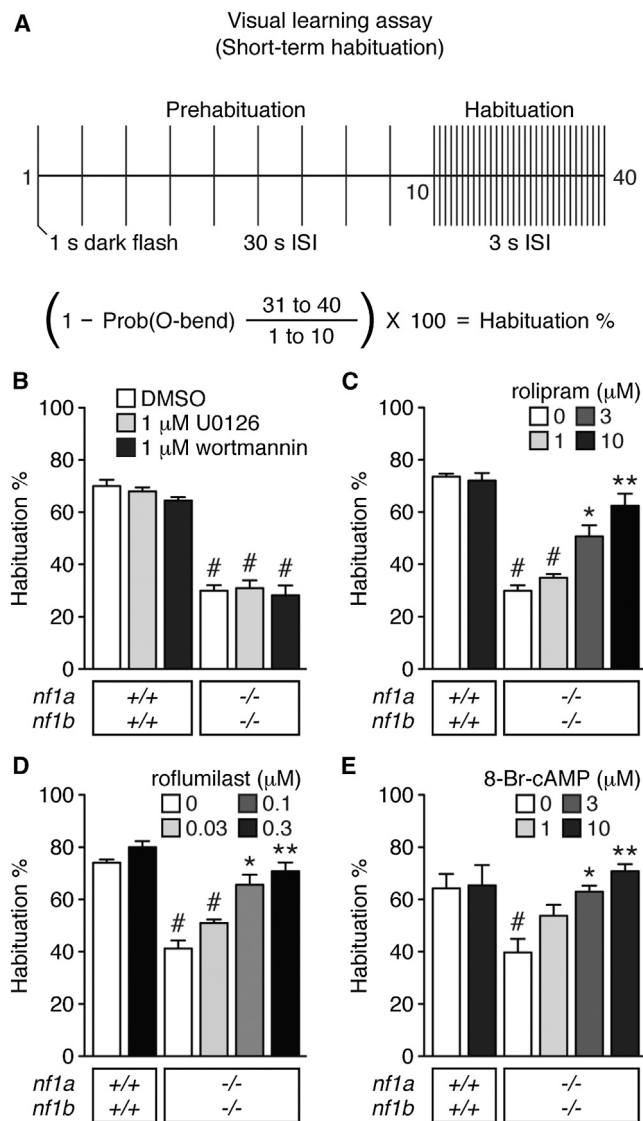


Figure 2. cAMP Signaling Mediates *nf1*-Dependent Visual Learning

(A) Schematic representation of the visual learning assay. (B–E) Mean percentage of habituation to repeated dark-flash stimulation ($n = 3$ groups of 15–20 larvae for all genotype/treatment groups). # $p < 0.001$ versus DMSO-treated wild-type larvae. * $p < 0.01$, ** $p < 0.001$ versus DMSO-treated *nf1a*^{-/-}; *nf1b*^{-/-} larvae. One-way ANOVA. Error bars denote SEM. See also Figures S1 and S3.

dose-dependent manner (Figures 1D–1F). Treatment with 1 μM wortmannin restored memory to wild-type levels, and 1 μM U0126 or 3 μM BKM120 yielded significant memory improvement. Although each of these Ras pathway antagonists exhibits known off-target effects, their different selectivity profiles (Bain et al., 2007; Liao and Laufs, 2005; Maira et al., 2012) suggest that nonspecific effects are unlikely to underlie the observed increase in memory recall. Therefore, these results support a conserved function for the neurofibromin GRD domain in regulation of memory formation through the Ras/MAPK/PI3K signaling pathway.

Learning (the acquisition of information) is critical for establishing memory. We evaluated learning by exposing larvae to dark-flash stimuli delivered at 3 s interstimulus intervals (ISIs) and measuring short-term habituation, as indicated by a reduction in the probability of initiating an O-bend response (Figure 2A). *nf1a*^{-/-}; *nf1b*^{-/-} larvae showed markedly reduced short-term visual (Figure 2B) and acoustic (Figures S1A and S1B; Shin et al., 2012) habituation compared with wild-type controls. Notably, *nf1a*^{-/-}; *nf1b*^{-/-} larvae showed some capacity for learning, which likely accounts for their potential to form memories in the presence of Ras pathway inhibitors (Figures 1D–1F). Larvae with at least one wild-type allele of either *nf1a* or *nf1b* did not show a learning deficit, despite dramatic memory deficits (Figure 1C; M.A.W. and E.D.d.G., unpublished data; Shin et al., 2012). It is possible that our nonassociative habituation assay lacks the necessary sensitivity to detect relatively subtle learning deficiencies in larvae with these genotypes. Attenuating Ras signaling by acute pharmacological inhibition of MAPK (U0126) or PI3K (wortmannin) failed to improve the learning deficit of *nf1a*^{-/-}; *nf1b*^{-/-} larvae (Figures 2B and S1B), suggesting that a distinct pathway mediates NF1-dependent learning.

Whole larval lysates revealed reduced cAMP levels in *nf1a*^{-/-}; *nf1b*^{-/-} mutants compared with wild-type controls (*nf1a*^{-/-}; *nf1b*^{-/-}: 33 fmol ± SEM 2.3 versus wild-type: 79 fmol ± SEM 7.8, $p < 0.001$). To determine whether reduced cAMP signaling contributed to the learning deficits in *nf1a*^{-/-}; *nf1b*^{-/-} mutants, we tested whether enhancing cAMP signaling by acute pharmacological inhibition of phosphodiesterase 4 (PDE4) or stimulation of PKA could improve learning. Inhibition of PDE4 by rolipram or roflumilast, or PKA stimulation by 8-Br-cAMP improved learning behavior in *nf1a*^{-/-}; *nf1b*^{-/-} mutants in response to both repetitive visual (Figures 2C–2E) and acoustic (Figures S1C and S1D) stimuli. Treatment with at least 10 μM rolipram, 0.1 μM roflumilast, or 3 μM 8-Br-cAMP improved habituation to wild-type levels. These results provide evidence that cAMP signaling regulates NF1-dependent learning in a vertebrate system.

We next asked whether cAMP signaling regulates NF1-dependent memory in addition to learning. We tested *nf1a*^{-/-}; *nf1b*^{-/-} larvae, which show reduced learning and a failure to recall memory, and *nf1a*^{+/-}; *nf1b*^{-/-} larvae, which learn normally but fail to form memory, and compared them with wild-type controls. Treatment with 10 μM 8-Br-cAMP, a sufficient dose to restore learning in *nf1a*^{-/-}; *nf1b*^{-/-} larvae (Figures 2E and S1D), failed to improve memory recall in either *nf1a*^{+/-}; *nf1b*^{-/-} or *nf1a*^{-/-}; *nf1b*^{-/-} larvae (Figure S2). These results suggest that cAMP signaling regulates NF1-dependent learning but not memory. Moreover, these results indicate that the memory defects in *nf1a*^{-/-}; *nf1b*^{-/-} mutants are not simply attributable to their learning deficit. These data strongly imply that molecularly distinct pathways that control learning and memory are affected in NF1.

Learned behavior requires consolidation to form stable memory. Despite consensus that defective neurofibromin function can result in learning and memory impairments, whether impaired consolidation contributes to memory deficits remains unclear. *nf1a*^{+/-}; *nf1b*^{-/-} larvae learn normally (M.A.W. and E.D.d.G., unpublished data; Shin et al., 2012) but show reduced memory recall (Figure 1C). Therefore, we asked whether reduced

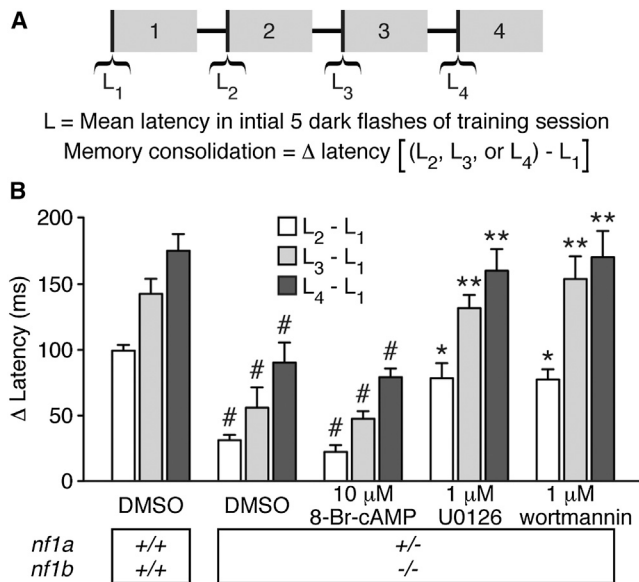


Figure 3. Inhibition of Ras Signaling Improves Memory Consolidation Deficits in *nf1* Mutants

(A) Schematic representation of visual memory consolidation measurement. (B) Mean O-bend latency change comparing responses to dark-flash stimuli 1–5 of sessions 2–4 versus stimuli 1–5 of session 1 (n = 30–139 O-bend maneuvers per genotype/treatment). #p < 0.001 versus DMSO-treated wild-type larvae. *p < 0.01, **p < 0.001 versus DMSO-treated *nf1a*^{+/-}; *nf1b*^{-/-} larvae. One-way ANOVA. Error bars denote SEM. See also Figure S3.

memory was due to a consolidation deficit. We determined memory consolidation by calculating the difference between the mean O-bend latency in response to the first five dark-flash stimuli of training session 1 and subsequent training sessions (Figure 3A). Long ISIs between training sessions promote memory consolidation, and therefore spaced training paradigms elicit more stable memory than do massed training paradigms (Beck and Rankin, 1997; Ebbinghaus, 1885). After each session, *nf1a*^{+/-}; *nf1b*^{-/-} larvae showed reduced consolidation compared with wild-type larvae (Figure 3B), suggesting that the memory-recall deficit observed in *nf1a*^{+/-}; *nf1b*^{-/-} larvae (Figure 1C) may be due to a defect in memory consolidation.

To determine the contribution of cAMP and Ras signaling to NF1-dependent memory consolidation, we attempted to improve consolidation in *nf1a*^{+/-}; *nf1b*^{-/-} larvae by pharmacologically enhancing cAMP or attenuating Ras. Enhancing cAMP in *nf1a*^{+/-}; *nf1b*^{-/-} larvae by treatment with 10 μ M 8-Br-cAMP did not increase consolidation (Figure 3B). Pharmacological inhibition of MAPK (1 μ M U0126) or PI3K (1 μ M wortmannin) improved memory consolidation in *nf1a*^{+/-}; *nf1b*^{-/-} larvae to levels indistinguishable from those observed in DMSO-treated wild-type larvae (Figure 3B). These results reveal that deficits in memory consolidation contribute to the etiology of memory dysfunction in NF1 and support a specific role for Ras signaling in mediating NF1-dependent memory formation.

Larvae deficient for *nf1* exhibit learning and memory deficits with characteristics reminiscent of those seen in human NF1

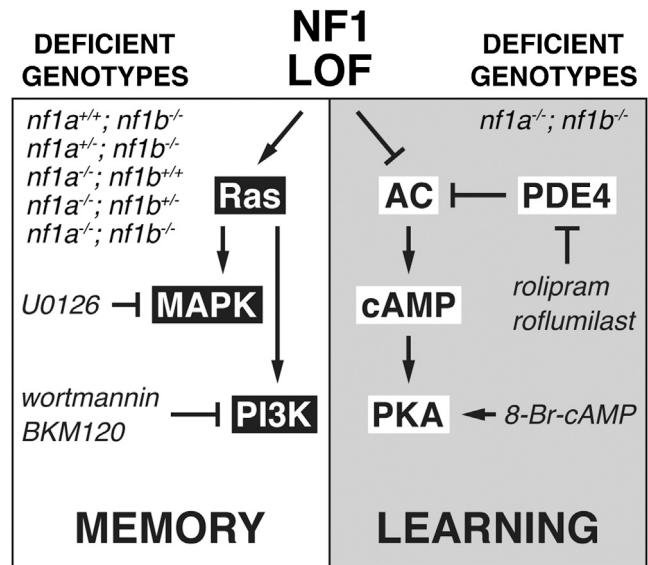


Figure 4. Effects of NF1 Loss of Function on the Ras and cAMP Pathways

The genotypes of the zebrafish *nf1* larvae that exhibited significant memory or learning deficits are shown. The pharmacological agents (italicized) that were used to improve memory or learning in these genotypes, as well as the molecular targets of the agents, are indicated. LOF, loss of function.

patients. We obtained strong evidence in a vertebrate system that NF1 affects at least two distinct signaling pathways that independently modulate learning and memory (Figure 4). A detailed understanding of the structure-function relationship among NF1 mutations, Ras and cAMP signaling, and phenotypes will allow for tailored and personalized therapies for cognitive defects in affected patients. It will also be interesting to determine whether the dynamic regulation of Ras or cAMP signaling in distinct areas of the brain correlates with unique behavioral outcomes. The fact that we observed robust improvements in learning and memory in our experiments even though we used only short-term treatments is encouraging for potential clinical application, and suggests that cognitive defects in this model are not developmental or irreversible. It will be exciting to determine whether these models can be validated in higher vertebrates and whether combination therapy with Ras and cAMP pathway effectors can improve the condition of some NF1 patients.

EXPERIMENTAL PROCEDURES

Generation and Maintenance of Zebrafish

The zebrafish (*Danio rerio*) larvae used in this study were generated from crosses of adults carrying the *nf1a*⁴⁵ and *nf1b*¹⁰ mutant alleles (Shin et al., 2012). Embryos were raised at 28°C in a 14 hr/10 hr light/dark cycle as previously described (Burgess and Granato, 2007a) and all behavioral experiments were conducted with 5 days postfertilization (dpf) larvae. For visual behavioral experiments, larvae were PCR genotyped by clipping a small region of the caudal fin at 3 dpf and genotyping as described previously (Shin et al., 2012). Larvae tested for acoustic habituation were tested individually in a 4 × 4 grid and genotyped after testing. All animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Behavioral Assays and Analysis

Dark-flash-induced O-bend responses were elicited, recorded, and measured as previously described (Burgess and Granato, 2007a; Wolman et al., 2011). Larvae were trained and tested at a density of 15 larvae per 9 ml E3 in 6 cm Petri dishes and kept in the dishes during training or testing. To elicit memory formation, larvae were exposed to a training paradigm comprised of four 30 min training sessions, each consisting of exposure to a 1 s dark flash delivered every 15 s. Training sessions were separated by 10 min ISIs. After the fourth session and a 1 hr ISI, larvae were exposed to ten dark flashes with 1 min ISIs to evaluate memory recall. To calculate memory recall, the average latency to initiate an O-bend in untrained larvae was subtracted from the latency to initiate an O-bend in trained larvae. Memory consolidation was calculated by subtracting the average latency to initiate an O-bend in response to dark-flash stimuli 1–5 of training session 1 from the latency to initiate an O-bend in response to dark flashes 1–5 of sessions 2–4.

To measure visual short-term habituation, a series of 40 1 s dark flashes were delivered. Stimuli 1–10 were delivered with 30 s ISIs and stimuli 11–40 were delivered with 3 s ISIs. The percentage of habituation was calculated by dividing the mean O-bend responsiveness to stimuli 31–40 by the mean O-bend responsiveness to stimuli 1–10, subtracting this value from 1, and multiplying by 100. An acoustic short-term habituation assay was performed as previously described (Wolman et al., 2011).

Pharmacology

All compounds were added to the larval media 30 min before and throughout the training and testing paradigm. Cycloheximide (C4859; Sigma-Aldrich), U0126 (9903, Cell Signaling Technology), wortmannin (9951; Cell Signaling Technology), BKM120 (S2247; Selleck Chemicals), rolipram (R6520; Sigma-Aldrich), roflumilast (S2131, Selleck Chemicals), and 8-Br-cAMP (B007; BIOLOG Life Science Institute) were dissolved in 100% DMSO and administered in a final concentration of 1% DMSO. Doses of each compound were prescreened for potential effects on baseline O-bend responsiveness to visual stimuli and short-latency C-bend responsiveness to acoustic stimuli. The defined, stereotyped kinematic parameters of both larval maneuvers were also examined (Burgess and Granato, 2007a, 2007b). Selected doses did not change baseline behavior responsiveness or kinematic performance after 30 min or 4 hr of incubation. Immunohistochemistry with anti-phospho-ERK (4377; Cell Signaling Technology) and anti-phospho-(Ser/Thr) PKA substrate (9621; Cell Signaling Technology) was performed on paraffin-embedded larval tissue after fixation in 4% paraformaldehyde, dehydration, and sectioning at 8 μ M thickness in order to demonstrate the pathway specificity of the pharmacologic inhibitors (Figure S3).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one movie and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.07.054>.

AUTHOR CONTRIBUTIONS

E.D.d.G. and M.A.W. designed and performed experiments together and wrote the manuscript. M.G. and J.A.E. designed experiments, supervised the work, and edited the manuscript. S.M.M. and T.A.J. contributed reagents and advice on the experimental design and approach.

ACKNOWLEDGMENTS

We thank Tom Look, Kurt Engleka, Kurt Marsden, and Min Min Lu for helpful discussions and technical support. This work was supported by grants from the NIH (2T32 HL007843 to E.D.d.G., R01 HL062974 to J.A.E., R01 MH092257 and HD 37975A11 to M.G., and GM086902 to T.A.J.) and the Department of Defense (NF110108 to J.A.E. and AR1101189 to T.A.J.). S.M. was supported by grants from the FRAXA Research Foundation and the University of Pennsylvania (R25 MH060490, Clinical Research Scholars Program in Psychiatry).

Received: January 24, 2014

Revised: July 20, 2014

Accepted: July 29, 2014

Published: August 28, 2014

REFERENCES

- Armstrong, B.C., Le Bouillier, J.C., and Petit, T.L. (2012). Ultrastructural synaptic changes associated with neurofibromatosis type 1: a quantitative analysis of hippocampal region CA1 in a Nf1(+/-) mouse model. *Synapse* 66, 246–255.
- Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C.J., McLauchlan, H., Klevvernic, I., Arthur, J.S., Alessi, D.R., and Cohen, P. (2007). The selectivity of protein kinase inhibitors: a further update. *Biochem. J.* 408, 297–315.
- Beck, C.D., and Rankin, C.H. (1995). Heat shock disrupts long-term memory consolidation in *Caenorhabditis elegans*. *Learn. Mem.* 2, 161–177.
- Beck, C.D.O., and Rankin, C.H. (1997). Long-term habituation is produced by distributed training at long ISIs and not by massed training or short ISIs in *Caenorhabditis elegans*. *Anim. Learn. Behav.* 25, 446–457.
- Brown, J.A., Gianino, S.M., and Gutmann, D.H. (2010). Defective cAMP generation underlies the sensitivity of CNS neurons to neurofibromatosis-1 heterozygosity. *J. Neurosci.* 30, 5579–5589.
- Brown, J.A., Diggs-Andrews, K.A., Gianino, S.M., and Gutmann, D.H. (2012). Neurofibromatosis-1 heterozygosity impairs CNS neuronal morphology in a cAMP/PKA/ROCK-dependent manner. *Mol. Cell. Neurosci.* 49, 13–22.
- Burgess, H.A., and Granato, M. (2007a). Modulation of locomotor activity in larval zebrafish during light adaptation. *J. Exp. Biol.* 210, 2526–2539.
- Burgess, H.A., and Granato, M. (2007b). Sensorimotor gating in larval zebrafish. *J. Neurosci.* 27, 4984–4994.
- Cichowski, K., and Jacks, T. (2001). NF1 tumor suppressor gene function: narrowing the GAP. *Cell* 104, 593–604.
- Costa, R.M., Federov, N.B., Kogan, J.H., Murphy, G.G., Stern, J., Ohno, M., Kucherlapati, R., Jacks, T., and Silva, A.J. (2002). Mechanism for the learning deficits in a mouse model of neurofibromatosis type 1. *Nature* 415, 526–530.
- Cui, Y., Costa, R.M., Murphy, G.G., Elgersma, Y., Zhu, Y., Gutmann, D.H., Parada, L.F., Mody, I., and Silva, A.J. (2008). Neurofibromin regulation of ERK signaling modulates GABA release and learning. *Cell* 135, 549–560.
- Davis, H.P., and Squire, L.R. (1984). Protein synthesis and memory: a review. *Psychol. Bull.* 96, 518–559.
- Diggs-Andrews, K.A., Tokuda, K., Izumi, Y., Zorumski, C.F., Wozniak, D.F., and Gutmann, D.H. (2013). Dopamine deficiency underlies learning deficits in neurofibromatosis-1 mice. *Ann. Neurol.* 73, 309–315.
- Ebbinghaus, H. (1885). *Memory: A Contribution to Experimental Psychology* (New York: Teachers College, Columbia University).
- Fahsold, R., Hoffmeyer, S., Mischung, C., Gille, C., Ehlers, C., Küçükceylan, N., Abdel-Nour, M., Gewies, A., Peters, H., Kaufmann, D., et al. (2000). Minor lesion mutational spectrum of the entire NF1 gene does not explain its high mutability but points to a functional domain upstream of the GAP-related domain. *Am. J. Hum. Genet.* 66, 790–818.
- Fleming, A., Diekmann, H., and Goldsmith, P. (2013). Functional characterisation of the maturation of the blood-brain barrier in larval zebrafish. *PLoS ONE* 8, e77548.
- Gouzi, J.Y., Moressis, A., Walker, J.A., Apostolopoulou, A.A., Palmer, R.H., Bernards, A., and Skoulakis, E.M. (2011). The receptor tyrosine kinase Alk controls neurofibromin functions in *Drosophila* growth and learning. *PLoS Genet.* 7, e1002281.
- Guiding, C., McNair, K., Stone, T.W., and Morris, B.J. (2007). Restored plasticity in a mouse model of neurofibromatosis type 1 via inhibition of hyperactive ERK and CREB. *Eur. J. Neurosci.* 25, 99–105.
- Guo, H.F., The, I., Hannan, F., Bernards, A., and Zhong, Y. (1997). Requirement of *Drosophila* NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides. *Science* 276, 795–798.

- Guo, H.F., Tong, J., Hannan, F., Luo, L., and Zhong, Y. (2000). A neurofibromatosis-1-regulated pathway is required for learning in *Drosophila*. *Nature* **403**, 895–898.
- Hannan, F., Ho, I., Tong, J.J., Zhu, Y., Nurnberg, P., and Zhong, Y. (2006). Effect of neurofibromatosis type 1 mutations on a novel pathway for adenylyl cyclase activation requiring neurofibromin and Ras. *Hum. Mol. Genet.* **15**, 1087–1098.
- Hegedus, B., Dasgupta, B., Shin, J.E., Emmett, R.J., Hart-Mahon, E.K., Elghazi, L., Bernal-Mizrachi, E., and Gutmann, D.H. (2007). Neurofibromatosis-1 regulates neuronal and glial cell differentiation from neuroglial progenitors in vivo by both cAMP- and Ras-dependent mechanisms. *Cell Stem Cell* **1**, 443–457.
- Ho, I.S., Hannan, F., Guo, H.F., Hakker, I., and Zhong, Y. (2007). Distinct functional domains of neurofibromatosis type 1 regulate immediate versus long-term memory formation. *J. Neurosci.* **27**, 6852–6857.
- Hyman, S.L., Shores, A., and North, K.N. (2005). The nature and frequency of cognitive deficits in children with neurofibromatosis type 1. *Neurology* **65**, 1037–1044.
- Hyman, S.L., Arthur Shores, E., and North, K.N. (2006). Learning disabilities in children with neurofibromatosis type 1: subtypes, cognitive profile, and attention-deficit-hyperactivity disorder. *Dev. Med. Child Neurol.* **48**, 973–977.
- Isenberg, J.C., Templer, A., Gao, F., Titus, J.B., and Gutmann, D.H. (2013). Attention skills in children with neurofibromatosis type 1. *J. Child Neurol.* **28**, 45–49.
- Jayachandran, R., Liu, X., Bosedasgupta, S., Müller, P., Zhang, C.L., Moshous, D., Studer, V., Schneider, J., Genoud, C., Fossoud, C., et al. (2014). Coronin 1 regulates cognition and behavior through modulation of cAMP/protein kinase A signaling. *PLoS Biol.* **12**, e1001820.
- Karlsgodt, K.H., Rosser, T., Lutkenhoff, E.S., Cannon, T.D., Silva, A., and Bearden, C.E. (2012). Alterations in white matter microstructure in neurofibromatosis-1. *PLoS ONE* **7**, e47854.
- Levine, T.M., Materek, A., Abel, J., O'Donnell, M., and Cutting, L.E. (2006). Cognitive profile of neurofibromatosis type 1. *Semin. Pediatr. Neurol.* **13**, 8–20.
- Li, W., Cui, Y., Kushner, S.A., Brown, R.A., Jentsch, J.D., Frankland, P.W., Cannon, T.D., and Silva, A.J. (2005). The HMG-CoA reductase inhibitor lovastatin reverses the learning and attention deficits in a mouse model of neurofibromatosis type 1. *Curr. Biol.* **15**, 1961–1967.
- Liao, J.K., and Laufs, U. (2005). Pleiotropic effects of statins. *Annu. Rev. Pharmacol. Toxicol.* **45**, 89–118.
- Maira, S.M., Pecchi, S., Huang, A., Burger, M., Knapp, M., Sterker, D., Schnell, C., Guthy, D., Nagel, T., Wiesmann, M., et al. (2012). Identification and characterization of NVP-BKM120, an orally available pan-class I PI3-kinase inhibitor. *Mol. Cancer Ther.* **11**, 317–328.
- Peng, S., Yang, X., Liu, G.J., Zhang, X.Q., Wang, G.L., and Sun, H.Y. (2014). The phosphodiesterase-4 inhibitor Ro 20-1724 reverses learning and memory impairments, and down-regulation of CREB in the hippocampus and cortex induced by ketamine anesthesia in immature rats. *J. Neurosurg. Sci.*
- Richter, W., Menniti, F.S., Zhang, H.T., and Conti, M. (2013). PDE4 as a target for cognition enhancement. *Expert Opin. Ther. Targets* **17**, 1011–1027.
- Roberts, A.C., Bill, B.R., and Glanzman, D.L. (2013). Learning and memory in zebrafish larvae. *Front Neural Circuits* **7**, 126.
- Shilyansky, C., Karlsgodt, K.H., Cummings, D.M., Sidiropoulou, K., Hardt, M., James, A.S., Ehninger, D., Bearden, C.E., Poirazi, P., Jentsch, J.D., et al. (2010). Neurofibromin regulates corticostriatal inhibitory networks during working memory performance. *Proc. Natl. Acad. Sci. USA* **107**, 13141–13146.
- Shin, J., Padmanabhan, A., de Groh, E.D., Lee, J.-S., Haidar, S., Dahlberg, S., Guo, F., He, S., Wolman, M.A., Granato, M., et al. (2012). Zebrafish neurofibromatosis type 1 genes have redundant functions in tumorigenesis and embryonic development. *Dis. Model. Mech.* **5**, 881–894.
- Silva, A.J., Frankland, P.W., Marowitz, Z., Friedman, E., Laszlo, G.S., Cioffi, D., Jacks, T., and Bourchuladze, R. (1997). A mouse model for the learning and memory deficits associated with neurofibromatosis type 1. *Nat. Genet.* **15**, 281–284.
- The, I., Hannigan, G.E., Cowley, G.S., Reginald, S., Zhong, Y., Gusella, J.F., Hariharan, I.K., and Bernards, A. (1997). Rescue of a *Drosophila* NF1 mutant phenotype by protein kinase A. *Science* **276**, 791–794.
- Thompson, R.F., and Spencer, W.A. (1966). Habituation: a model phenomenon for the study of neuronal substrates of behavior. *Psychol. Rev.* **73**, 16–43.
- Tong, J., Hannan, F., Zhu, Y., Bernards, A., and Zhong, Y. (2002). Neurofibromin regulates G protein-stimulated adenylyl cyclase activity. *Nat. Neurosci.* **5**, 95–96.
- Walker, J.A., Tchoudakova, A.V., McKenney, P.T., Brill, S., Wu, D., Cowley, G.S., Hariharan, I.K., and Bernards, A. (2006). Reduced growth of *Drosophila* neurofibromatosis 1 mutants reflects a non-cell-autonomous requirement for GTPase-Activating Protein activity in larval neurons. *Genes Dev.* **20**, 3311–3323.
- Walker, J.A., Gouzi, J.Y., Long, J.B., Huang, S., Maher, R.C., Xia, H., Khalil, K., Ray, A., Van Vactor, D., Bernards, R., and Bernards, A. (2013). Genetic and functional studies implicate synaptic overgrowth and ring gland cAMP/PKA signaling defects in the *Drosophila melanogaster* neurofibromatosis-1 growth deficiency. *PLoS Genet.* **9**, e1003958.
- Wolman, M.A., Jain, R.A., Liss, L., and Granato, M. (2011). Chemical modulation of memory formation in larval zebrafish. *Proc. Natl. Acad. Sci. USA* **108**, 15468–15473.