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Piconewton-Scale Analysis of Ras-BRaf Signal Transduction with Single-Molecule Force Spectroscopy

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Supporting Information

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Conflict of Interest

The authors declare no conflict of interest.

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Abstract

Intermolecular interactions dominate the behavior of signal transduction in various physiological and pathological cell processes, yet assessing these interactions remains a challenging task. Here, this study reports a single-molecule force spectroscopic method that enables functional delineation of two interaction sites (≈ 35 pN and ≈ 90 pN) between signaling effectors Ras and BRAF in the canonical mitogen-activated protein kinase (MAPK) pathway. This analysis reveals mutations on BRAF at Q257 and A246, two sites frequently linked to cardio-faciocutaneous syndrome, result in ≈ 10 – 30 pN alterations in Ras–BRAF intermolecular binding force. The magnitude of changes in Ras–BRAF binding force correlates with the size of alterations in protein affinity and in α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-sensitive glutamate receptor (-R)-mediated synaptic transmission in neurons expressing replacement BRAF mutants, and predicts the extent of learning impairments in animals expressing replacement BRAF mutants. These results establish single-molecule force spectroscopy as an effective platform for evaluating the piconewton-level interaction of signaling molecules and predicting the behavior outcome of signal transduction.

Intermolecular interactions hold the key to understanding of the behavior of signal transduction mediating various physiological and pathological cell processes, yet precisely assessing the interactions of signaling effectors remains a daunting task.^[1,2] Biochemical assays are the frequently used to evaluate the interactions since it directly measures the binding affinity of signaling effectors, although the approach often requires bulk samples and has limited resolution.^[3–7] Recently, the evolution- and/or structure-based computational modeling has emerged as an alternative approach to estimate protein–protein interactions, but the calculation may not always achieve the desired accuracy, and can be sometimes challenging.^[2,8,9] Single-molecule force spectroscopy, such as optical and magnetic tweezers, have been used as a powerful toolbox to investigate the forces and motions associated with cytoskeletal motors, ligand–receptor pairs, DNAs, RNAs, and viruses.^[10–14] However, whether this piconewton resolution approach may be adapted for dissecting inter-molecular interactions and interrogating signal transduction behavior is still unclear.

Rapid advances in genetic and genomics-sequencing techniques have identified nearly complete lists of genes and gene products involved in the major signaling networks in living organisms and associated large numbers of mutations on the genes with many human diseases.^[15–20] However, these gene products and mutations alone reveal relatively little about regulation of signaling networks and pathogenesis of human diseases. For example, an extensive amount of early work has led to the definition of several key signaling components in the canonical MAPK pathway that mediates growth, proliferation, differentiation, oncogenesis, cognition, and mental illness.^[2,17,18] In neuronal cells, this MAPK pathway

employs the Ca^{2+} /calmodulin-dependent protein kinase II–SynGap–Ras–BRaf–mitogen-activated protein kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) signaling cascade to control AMPA-R-mediated synaptic transmission.^[21–30] Moreover, genetic studies have linked large numbers of mutations on multiple MAPK signaling effector genes to mental disorders.^[31–35] In particular, 40 gain- or loss-of-function mutations on BRaf are associated with cardio-faciocutaneous syndrome (CFC), a sporadic developmental disorder characterized by distinctive craniofacial features, frequent heart defects, and prevalent cognitive deficit.^[36] It is also known that upon Ras activation, BRaf translocates to the plasma membrane and forms high- and low-affinity interactions with active Ras–guanosine-5'-triphosphate (GTP) via its Ras-binding domain (RBD) and cysteine-rich domain (CRD), which renders BRaf catalytically active to stimulate MEK–ERK signaling.^[3,37,38] Nevertheless, it remains elusive how Ras–BRaf interaction controls signal transduction and whether CFC-linked BRaf mutations cause learning deficits via altering Ras–BRaf interaction.

We have previously established an optical tweezers' system that permits direct measurement of the interaction force between single small GTPase Ras and protein kinase BRaf proteins,^[39] two key effector molecules in the canonical MAPK signaling pathway. In this study, we made both gain- and loss-of-function of on BRaf(Q257) and BRaf(A246) mutations, and carried out a series of experiments to determine whether optical tweezers' technique may be utilized to effectively interrogate the Ras–BRaf signal transduction. We verified that this single-molecule force spectroscopic method allowed delineation of two Ras–BRaf interaction sites, which form ≈ 35 pN and ≈ 90 pN intermolecular bonds, respectively. Systematical spectroscopic analysis revealed mutations on BRaf at Q257 and A246 resulted in ≈ 10 – 30 pN alterations in Ras–BRaf intermolecular binding force. Notably, the magnitude of changes in Ras–BRaf binding force correlated with the degree of alterations in protein affinity measured in vitro, matched to the amount of increases or decreases in AMPA-R-mediated responses in neurons expressing replacement BRaf mutants ex vivo, and predicted the extent of impairments in learning in behaving animals expressing replacement BRaf mutants. Together, our biochemistry, electrophysiology, and behavior experiments provide the first demonstration that single-molecule force spectroscopy is an effective method for interrogating intermolecular interactions and signal transduction of signaling effectors involved in physiological and pathological cell processes.

To quantitatively assess the molecular interaction between Ras and BRaf, we adapted a single-molecule force spectroscopic approach to measure the rupture force required to break the adhesion between single Ras and BRaf molecules that are linked to polystyrene beads (Figure 1a; see the “Experimental Section”). The adhesion between Ras- and BRaf-linked beads occurred in $<30\%$ of cases, as expected from the low density of proteins linked to the beads (see the “Experimental Section”). The rupture took place predominantly ($>90\%$) as a single step (Figure 1b), suggesting unbinding of single Ras and BRaf pairs. We first analyzed the single-step rupture forces required to separate wild-type (WT) Ras and BRaf pairs. The rupture force required to unbind Ras(WT) and BRaf(WT) pairs had a broad distribution, with three prominent peaks at 36, 88, and 128 pN, respectively (Figure 1b; Table S1, Supporting Information), representing three different force strengths for rupturing Ras–BRaf intermolecular bonds. Interestingly, the largest rupture force (i.e., 128 pN)

equaled approximately the summation of the two smaller rupture forces (i.e., 36 and 88 pN) (Figure 1b; Table S1, Supporting Information), which was suggestive of the existence of at least two Ras–BRAF intermolecular bonds and formation of one or both bonds between Ras and BRAF pairs.

We then examined the effect of two most frequent CFC-linked BRAF mutations (i.e., A246P and Q257R located in BRAF CRD) on Ras–BRAF intermolecular bonds by measuring the rupture force required to separate Ras(WT) and BRAF(A246P) or BRAF(Q257R) pairs. The rupture force to unbind Ras(WT) and BRAF(Q257R) pairs had a positively shifted distribution compared to Ras(WT) and BRAF(WT) pairs (Figure 1b). The rupture force distribution consisted of three prominent peaks at 64, 93, and 157 pN (Figure 1b; Table S1, Supporting Information), suggesting the enhanced Ras–BRAF (Q257R) intermolecular bonds. To determine whether the differences in charge and/or size of side chain of Q257R play a key role in increasing Ras–BRAF molecular bonds, we studied the rupture force required to separate Ras(WT) and BRAF(Q257E) or BRAF(Q257A) pairs. The rupture force to unbind Ras(WT) and BRAF(Q257E) had a negatively shifted distribution with four peaks of 28, 66, 99, and 126 pN, and that to unbind Ras(WT) and BRAF(Q257A) pairs had a negatively shifted distribution with four peaks at 35, 60, 96, and 126 pN (Figure 1b; Table S1, Supporting Information). These results suggest that both the size and charge changes associated with arginine mutation may contribute to the enhanced Ras–BRAF (Q257R) molecular bonds. Similarly, the rupture force required to unbind Ras(WT) and BRAF(A246P) pairs had a positively shifted distribution with three prominent discrete peaks at 48, 110, and 150 pN (Figure 1b; Table S1, Supporting information), suggesting the enhanced Ras–BRAF (A246P) intermolecular bonds. Analysis of additional mutations revealed that compared to that of Ras(WT) and BRAF(WT), the rupture force to unbind Ras(WT) and BRAF(A246F) pairs had a similar distribution with three peaks at 36, 93, and 122 pN (Figure 1b; Table S1, Supporting Information). In addition, the rupture force to unbind Ras(WT) and BRAF(A246G) pairs had a negatively shifted distribution with four peaks at 26, 68, 98, and 131 pN, and that to unbind Ras(WT) and BRAF(A246S) pairs had a negatively shifted distribution with four peaks at 33, 74, 101, and 146 pN (Figure 1b; Table S1, Supporting information). These results suggest that the size increase associated with proline mutation may contribute to the enhanced Ras–BRAF (A246P) molecular bonds. As with wild-type BRAF, the third rupture force required to separate Ras and mutant BRAF pairs equaled roughly to the summation of the first two (small) rupture forces (Table S1, Supporting Information), supporting the view that Ras and BRAF form intermolecular bonds via two interaction sites.

To examine whether BRAF mutations affect the protein–protein interaction between Ras and BRAF, we measured the binding affinity of Ras and wild-type and mutant BRAF using the biochemical pull-down assay (Figure 2a). Western blot analysis showed that BRAF(Q257R) and BRAF(A246P) bound more Ras(WT) than BRAF(WT) (Figure 2b,c), indicating that CFC-linked BRAF mutations had increased protein-binding affinity with Ras(WT). Moreover, BRAF(Q257E), BRAF(Q257A), BRAF(A246G), and BRAF(A246S) had reduced protein-binding affinity with Ras(WT), whereas BRAF(A246F) had the same affinity with Ras(WT) as BRAF(WT) (Figure 2b,c). Interestingly, the protein-binding affinities of Ras and BRAF correlated with the sum of two small basic rupture forces required to unbind Ras and BRAF

pairs (Figure 2d), suggesting that the strength of Ras–BRAF intermolecular bonds reflected the protein-binding affinity.

To determine whether altered Ras–BRAF intermolecular bonds may result in the parallel changes in Ras–BRAF signaling, which controls AMPA-sensitive glutamate receptors (–Rs)-mediated synaptic transmission,^[24,30] we examined AMPA responses in CA1 pyramidal neurons expressing wild-type and mutant BRAF in hippocampal cultured slices. Using our recently established lentivirus-based rapid gene replacement method,^[30] we made simultaneous short hairpin RNA-mediated knockdown of endogenous rat BRAF and quantitatively equivalent expression of wild-type or mutated human BRAF in CA1 neurons (Figure 3a; Figure S2, Supporting Information). After 84 h of expression, we made simultaneous patch-clamp recordings from shRNA-PalGFP-IRES-GFPBRAF(WT) expressing CA1 neurons, identified by the fluorescence from green fluorescent protein (GFP), and nearby control nonexpressing CA1 neurons to measure AMPA-R- and N-methyl-D-aspartate (NMDA)-R-mediated excitatory postsynaptic currents evoked by stimulation of afferent fibers. (cf. ref. [30]). Neurons expressing the replacing shRNA-PalGFP-IRES-GFP-BRAF(WT) had the same AMPA and NMDA responses compared to control nonexpressing neurons (Figure 3b,c), confirming the functionally equivalent replacement of endogenous BRAF with recombinant BRAF. Neurons expressing shRNA-PalGFP-IRES-GFP-BRAF(Q257R) had enhanced AMPA responses, whereas neurons expressing shRNA-PalGFP-IRES-GFPBRAF(Q257E) or -BRAF(Q257A) had reduced AMPA responses (Figure 3b,c). Similarly, neurons expressing shRNA-PalGFP-IRES-GFP-BRAF(A246P) had enhanced AMPA responses and neurons expressing shRNA-PalGFP-IRES-GFP-BRAF(A246F) had the same AMPA responses, whereas neurons expressing shRNA-PalGFP-IRES-GFPBRAF(A246G) or -BRAF(A246S) had reduced AMPA responses compared to control nonexpressing neurons (Figure 3b,c). NMDA responses in all expressing neurons were unchanged, suggesting postsynaptic effects. Notably, the size of AMPA responses, but not NMDA responses, in wild-type and mutant BRAF expressing CA1 neurons matched closely to the sum of two small basic rupture forces required to unbind Ras and BRAF pairs (Figure 3d), suggesting that the strength of Ras–BRAF intermolecular bonds predicted the efficacy of synaptic Ras–BRAF signaling.

To investigate whether altered Ras–BRAF intermolecular bonds may induce corresponding changes in behavior, we made massive viral expression of wild-type and mutant replacement shRNA-PalGFP-IRES-GFP-BRAF constructs in the hippocampal CA1 regions bilaterally in intact rats and performed the elevated Y-maze learning task, a hippocampus-dependent learning paradigm (Figure 4a; cf. ref. [30]). Rats expressing shRNA-PalGFP-IRES-GFP-BRAF(Q257R) or -BRAF(A246P) had impaired Y-maze learning compared to control nonexpressing rats, whereas rats expressing shRNAPalGFP-IRES-GFP-BRAF(WT) had no difference in Y-maze learning compared to control nonexpressing rats (Figure 4b). A full analysis of all BRAF mutant constructs revealed that learning impairments in BRAF expressing animals correlated well with the altered rupture forces required to unbind Ras and BRAF pairs (Figure 4c).

To confirm the behavioral effects of BRAF mutations, we performed bilateral, massive lentiviral expression of shRNAPalGFP-IRES-GFP-BRAF mutant constructs in the lateral

amygdala of rats and subsequent fear conditioning paradigm (Figure 4a; cf. ref. [30]). During the fear conditioning training, rats expressing shRNA-PalGFP-IRES-GFP-BRaf(Q257R) and -BRaf(A246P) had the same responses to a tone presentation and electrical footshock, and exhibited the same amount of freezing compared to control nonexpressing rats or rats expressing shRNA-PalGFP-IRES-GFP-BRaf(WT) (Figure 4d). During the testing 24 h after fear conditioning, rats expressing BRaf(Q257R) and BRaf(A246P) mutants displayed reduced amounts of freezing in cued fear conditioning tests (Figure 4d), indicating impairments of cued fear memory by BRaf(Q257R) and BRaf(A246P) mutants. As a control, rats expressing BRaf(WT) exhibited the same amount of freezing compared to nonexpressing rats (Figure 4d). A full analysis of all BRaf mutant constructs showed the amounts of freezing in expressing animals correlated well with the altered rupture forces required to unbind Ras and BRaf pairs (Figure 4e). Collectively, these results indicate that the magnitude of altered strengths in Ras–BRaf intermolecular bonds predicts the extent of learning impairments in BRaf-expressing animals.

In this study, we have demonstrated that single-molecule force spectroscopy is a powerful approach for functionally assessing intermolecular interactions of signaling effectors at the piconewton level. In particular, the method allows us to delineate two intermolecular interaction sites of signaling effectors Ras and BRaf in the canonical MAPK pathway. By precisely quantifying the strength of Ras–BRaf intermolecular bonds, single-molecule force spectroscopy provides an effective way to evaluate the efficacy of physiological and pathological Ras–BRaf signal transduction, and to predict the behavioral consequence of human disease-linked BRaf mutations.

Intermolecular interactions of signaling molecules predict the behavior of signal transduction mediating various biological functions. Because Ras–Raf signaling regulates the signal transduction pathways essential for the growth, proliferation, differentiation, oncogenesis, cognition, and mental illness,^[2,17,18] the intermolecular interaction between Ras and Raf has been the focus of intensive research for many years. Accumulating data have ascertained that Ras directly interacts with RBD and CRD domains of Raf with high- and low-affinity molecular bonds, respectively, and both the interactions seem to be necessary for Ras–Raf signal transduction.^[3,6,40] Crystal structural analysis of the complex between Rap1 and Raf RBD predicts that Ras switch I region binds with RBD, whereas Ras II region binds with CRD of Raf.^[41] More recently, solved crystal structure of the Ras–Raf RBD complex,^[42] together with the previous biochemical studies,^[3–7] confirm quantitatively that the interaction of Ras and Raf RBD governs Ras–Raf signaling to the downstream MAPK pathway. However, attempts to crystallize the Ras–Raf CRD complex and to quantify the low-affinity binding between Ras and Raf CRD to correlate with the functional Ras–Raf signal transduction have not been successful.^[2,5,6] Thus, the nature and functional impact of Ras–Raf CRD interaction remain unclear. Here, we report the single-molecule force spectroscopic assessment of intermolecular interaction between Ras and BRaf, which reveals two interacting sites forming ≈ 35 pN and ≈ 90 pN molecular bonds, respectively. Quantitative analysis of the intermolecular binding forces between single Ras and wild-type or mutant BRaf proteins indicates that the amount of Ras–BRaf binding forces matches to the efficacy of synaptic Ras–BRaf signaling. In particular, the magnitude of increase or decrease in Ras–BRaf binding forces of both the low- and high-affinity sites

predicts the amount of increase or decrease in synaptic Ras–BRAF signaling calibrated as the size of synaptic transmission. Consistent with the previous finding that both down- and upregulation of synaptic Ras–BRAF signaling may force it to deviate from its “happy-medium” point, shrink its signaling dynamic range, and reduce the capacity for synaptic plasticity,^[43] and impair cognition,^[44–46] our results suggest that the increase or decrease in Ras–BRAF intermolecular binding forces leads to the predicted learning impairments.

Because signaling molecules relay signal transduction through complex, carefully orchestrated intermolecular interactions,^[15,17–20] characterizing intermolecular interactions between signaling effectors, particularly with high-throughput approaches, become central to understanding physiological and pathological cell processes, as well as advancing precision medicine and synthetic biology.^[1,2,9,47] However, the available methods for assessing the interactions, including biochemical assays and structure-based computational algorithms, all have limitations that prevent them from accurately and/or efficiently calculating intermolecular interactions of signaling effectors.^[2,6,9] Single-molecule force analysis has been widely used to characterize biochemical interactions of single molecules, including cytoskeletal motors, ligand–receptor pairs, DNAs, RNAs, and viruses.^[10–14] However, whether the technique may be adapted to examine functional intermolecular interactions of signaling molecules has not yet been validated. Here, we show a single-molecule force spectroscopic examination of the intermolecular interaction between Ras and BRAF, two key signaling molecules in the canonical MAPK pathway. Our optical tweezers’ analysis reveals that Ras and wild-type and mutant BRAF form multiple intermolecular bonds, and the strengths of rupture force required to rupture the Ras–BRAF bonds are within the optimal operation range (0–150 pN) of single-molecule force spectroscopy.^[10] Importantly, the method allows characterization of Ras–BRAF intermolecular interactions and detects alteration in Ras–BRAF intermolecular binding forces at the piconewton level. While the binding affinity of Ras and wild-type and mutant BRAF measured with the biochemical pull-down assay shows an overall good correlation with Ras–BRAF binding force, the experiments also indicate that the single-molecule force spectroscopy has a far greater resolving power than the biochemical assay. We have previously demonstrated that the picoampere resolution patch-clamp recordings (of synaptic transmission) and robust learning tests (i.e., fear conditioning and Y-maze tasks) provide high-resolution measurements that can determine the efficacy and outcome of signal transduction in neuronal cells and behaving animals.^[30,35,43] Indeed, the amount of changes in Ras–BRAF signaling in BRAF mutant expressing neurons quantified with patch-clamp recordings and the extent of cognitive impairments in BRAF mutant expressing animals evaluated with learning tests match almost perfectly to the magnitude of changes in Ras–BRAF binding force. These results indicate that the intermolecular Ras–BRAF binding force makes strong prediction of the efficacy and outcome of Ras–BRAF signaling, and that single-molecule force spectroscopy is an effective tool to dissect intermolecular interactions of signaling molecules. Given the simplicity and flexibility of optical tweezers’ technique,^[11,14,48] we expect the method to be broadly employed to study inter-molecular interactions of hundreds of other signaling molecules and to provide high-throughput analysis when combined with autonomous algorithms and devices in the near future.

Experimental Section

Animal Preparation:

Male and female Sprague Dawley rats were used to prepare cultured neurons, cultured slices and behavior experiments in this study. Animals were maintained in the animal facility at the University of Virginia and family or pair housed in the temperature-controlled animal room with 12 h light/12 h dark cycle. All procedures for animal surgery and maintenance were performed following protocols approved by the Animal Care and Use Committee of the University of Virginia and in accordance with US National Institutes of Health guidelines.

Constructs and Protein Purification:

GST-fused RBD and CRD expression constructs were generated by subcloning the two domains (amino acids, 136–298) of wild-type or mutant human BRAf into pGEX4T-1 (Glutathione S-transferase (GST)-fusion vector, GE Healthcare). GST–RBD–CRD fusion proteins were induced in XL10-Gold *Escherichia coli* strain (#200314, Stratagene), purified using Glutathione Sepharose 4B (#17-0756-01, GE Healthcare), and concentrated with Amicon Ultra centrifugal filters (Ultracel-30K, Millipore). Hexahistidine (His)-tagged Ras(WT) expression constructs were made by subcloning polymerase chain reaction (PCR)-amplified full-length human H-Ras into pET28a(+) (His-tag vector, Novagen). His-tagged Ras(WT) proteins were induced in BL21-CodonPlus (DE3)-RILP strain (#230280, Agilent Technologies), purified using *Ni-NTA* His-Bind Purification Kit (#70751–3, EMD Millipore Novagen), and quantified by Bradford assay kit (Bio-Rad).

Single-Molecule Force Spectroscopy:

The force spectroscopic measurement was made with a custom-made optical tweezers' system built around an inverted Zeiss Axiovert 200 microscope as previously reported (Figure S1a, Supporting Information).^[39,49] Optical trapping polystyrene beads were prepared in a similar way as previously described.^[39,50] In brief, His-tagged Ras was linked to polystyrene beads (5 μm in diameter) covalently, coated with anti-His-tag antibody, and truncated wild-type or mutant GST-fused BRAf containing RBD and CRD domains was linked to the beads covalently, coated with anti-GST antibody. To reduce the probability of binding of multiple molecules, the protein densities on the bead were kept <10 molecules μm^{-2} . Initially, ≈ 1000 BRAf-linked beads were suspended in 1×10^{-3} M Tris–Cl buffer at pH 7.4 and injected into a Petri dish hold with a 3D piezoelectric transducer (3D-PZT) sample stage (Nanonics Imaging LTD, Israel) controlled by a custom-written labview software program. Some of the beads were trapped and attached to the vertical surface of a polylysine-coated coverglass using a 1064 nm, 2 W diode-pumped neodymium yttrium aluminum garnet (Nd:YAG) trapping laser (Coherent COMPASS 1.064 2000N; Coherent, Santa Clara, CA, USA) focused with a 100×1.3 numerical aperture (NA) objective. The remaining unattached BRAf-linked beads were removed by washing with the Tris–Cl buffer for three times. Then, ≈ 1000 Ras-linked beads suspended in the Tris–Cl buffer containing additional 10×10^{-9} M GTP were added in the Petri dish. During the measurement, a coverslip-attached, BRAf-linked bead was steered to contact an optically trapped Ras-linked bead for 1 s, and then retracted at a constant velocity of $5 \mu\text{m s}^{-1}$ (equivalent to a loading rate of 10^3 pN s^{-1}) by PZT. The displacement of Ras-coated beads from the center of optical

tweezers was captured by a custom-made quadrant photodiodes detector and converted to the unbinding force. The calibrated stiffness and spatial resolution of the optical tweezers' system were ≈ 0.2 pN nm⁻¹ and ≈ 7 nm, respectively. Although the system can measure rupture forces up to ≈ 200 pN,^[39] the measurements were typically analyzed within the linear force–displacement range or up to ≈ 150 pN (cf. ref. [10]), which were sufficient to capture the forces required to delineate multiple intermolecular bonds between Ras and gain- or loss-of-function of BRAf mutants. Replacing GST-fused BRAf-linked beads with GST-linked beads revealed a nonspecific low-rupture force distribution peaked at ≈ 15 pN (Figure S1b, Supporting Information), which was subtracted from the rupture force distributions of Ras and BRAf pairs. The adhesion between beads occurred in <30% cases and unbinding of beads predominantly occurred in isolated steps, indicating total rupture of intermolecular bonds in these force traces.

Cultured Neuron Preparation:

Hippocampal cultured neurons were prepared from rat embryos at embryonic day 18 (E18 pregnant females) as previously described^[51] with minor modifications. Cells were dissociated mechanically after trypsin treatment, and plated onto poly-D-lysine (Sigma, P1149)-coated plastic culture dishes at the density of $\approx 5 \times 10^4$ cells cm⁻². Plated cells were recovered in the plating media (Dulbecco modified eagle medium (DMEM) with 2×10^{-3} M glutamine, 10% fetal bovine serum (FBS), 0.45% glucose, and 0.11 mg mL⁻¹ sodium pyruvate) for 3–4 h, and then maintained in the Neurobasal media (Invitrogen, 21103–049) supplemented with B27 (Invitrogen, 17504–044) and Glutamax (Invitrogen, 35050–061) before use.

Biochemistry:

Protein-binding assay was performed in a TNM buffer containing 50×10^{-3} M Tris–Cl, 100×10^{-3} M NaCl, 5×10^{-3} M MgCl₂, 0.1% Triton X-100, and a protease inhibitor cocktail (complete, ethylenediaminetetraacetic acid (EDTA)-free, Roche) at pH 7.4. His-Ras(WT)-bound agarose beads (5 μ g) were mixed with various amount of purified GST–BRAf(RBD–CRD) mutant proteins (up to 5 μ g), and incubated at 4 °C for 45 min. The beads were then washed 3 $\times$ with TNM buffer containing additional 40×10^{-3} M imidazole and 1 $\times$ with TNM buffer without imidazole. GST–BRAf(RBD–CRD) proteins retained on the beads were immunoblotted using anti-GST antibodies (26H1, Cell Signaling Tech). The amounts of bound GST–BRAf(RBD–CRD) proteins were quantified by densitometry and then calculated by comparing the pure GST–BRAf(RBD–CRD) inputs blotted simultaneously with nonspecific binding to plain agarose beads adjusted.

Hippocampal Cultured Slice Preparation:

Cultured slices were prepared from postnatal 6–7 d old rats following the previous studies.^[24,30] In brief, the hippocampi were dissected out in ice-cold N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES)-buffered Hanks' solution (pH 7.35) under sterile conditions, sectioned into 400 μ m slices on a tissue chopper, and explanted onto a Millicell-CM membrane (0.4 μ m pore size; Millipore). The membranes were then placed in 750 μ L of MEM culture medium, contained (in $\times 10^{-3}$ M): HEPES 30, heat-inactivated horse serum 20%, glutamine 1.4, D-glucose 16.25, NaHCO₃ 5, CaCl₂ 1, MgSO₄ 2, insulin 1 mg mL⁻¹,

ascorbic acid 0.012% at pH 7.28 and osmolarity 320. Cultured slices were maintained at 35 °C, in a humidified incubator (ambient air enriched with 5% CO₂).

Recombinant Protein Expression:

Wild-type and mutant shRNAPalGFP-IRES-GFP-BRaf constructs were prepared and expressed in hippocampal CA1 pyramidal and amygdala neurons in cultured slices and/or intact brains using lentivirus as previously reported.^[30,35,43] Briefly, for in vitro expression, cultured slices were infected after 7 d in vitro and then incubated on culture media and 5% CO₂ for 3.5 d before experiments. For behavioral tests, animals were initially anesthetized by an intraperitoneal injection of ketamine and xylazine (10 and 2 mg kg⁻¹, respectively), and then placed in a stereotaxic frame. A glass pipette was used to make pressure injections of viral solution in the amygdala or hippocampal CA1 region according to stereotaxic coordinates. After injection, animals were allowed to recover from the anesthesia and returned to their cages.

Electrophysiology:

Simultaneous whole-cell recordings were obtained from nearby infected and noninfected neuron pairs, under visual guidance using fluorescence and transmitted light illumination with two Axopatch-200B amplifiers (Molecular Devices, Sunnyvale, CA, USA) as previously described.^[30,52,53] Cultured rat organotypic slices exhibited relatively high spontaneous activity comparable to intact brains. Thus, high calcium and magnesium bath solution (29 ± 1.5 °C), containing (in × 10⁻³ M): NaCl 119, KCl 2.5, CaCl₂ 4, MgCl₂ 4, NaHCO₃ 26, NaH₂PO₄ 1, glucose 11, picrotoxin 0.1, and 2-chloroadenosine 0.002, at pH 7.4, was used to prevent epileptic activity after blocking inhibition. The bath solutions were gassed with 5% CO₂/95% O₂. Patch recording pipettes (3–6 MΩ) contained (in × 10⁻³ M) cesium methanesulfonate 115, CsCl 20, HEPES 10, MgCl₂ 2.5, Na₂ATP 4, Na₃GTP 0.4, sodium phosphocreatine 10, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 0.6, and spermine 0.1, at pH 7.25. Synaptic responses were evoked by bipolar electrodes with single voltage pulses (200 μs, up to 20 V) placed in hippocampal *Sesamum radiatum* ≈300 μm away from the recorded hippocampal CA1 pyramidal neurons. To minimize the effect from AMPA responses, the peak NMDA responses at +40 mV were measured after digital subtraction of estimated AMPA responses at +40 mV.

Behavioral Analysis:

Fear conditioning tests and Y-maze tests, started at ≈5 pm, followed the previous reports.^[30,35,54] Briefly, Y-maze learning tests were carried out using an elevated Y-maze with one start arm and two goal arms (56.5 × 12 cm each), surrounded by an opaque 26 cm high wall (San Diego Instruments, San Diego, CA, USA). A food well, located 5 cm from the end of each goal arm, was filled with 0.1 mL sucrose-sweetened nonfat dry milk (12.5%) as a reward. Animals were initially habituated to the Y-maze and milk reward. Once all the animals were running freely on the Y-maze and readily consuming the milk rewards, testing began. Animals were trained and tested on a conditional learning task, in which interchangeable floor inserts, either plain white or black wire mesh, covering the whole of the start arm, were used as a conditional cue. The presence of the plain white insert in the start arm indicated that the 0.1 mL milk reward was available in the right goal arm, whereas

the presence of the black wire-mesh insert was associated with the reward in the left arm. Animals received 14 training–testing sessions consisting of 10 trials per session with an intertrial interval of 5 min. Each session consisted of five trials with each of the two floor inserts, and no more than three consecutive trials with the same floor insert, according to a pseudorandom sequence. The percentage of correct responses, or entering the floor-matching goal arm and consuming the milk reward, was calculated for each session.

Fear conditioning was performed in an isolated shock chamber (Coulbourn Instruments, Wilmette, IL, USA). The training consisted of a 3 min exposure of mice to the conditioning box followed by a foot shock (2 s, 1 mA) after a preceding tone (30 s, 3 kHz, 80 dB) three times (Interstimulus interval = 120 s) and allowed to stay in chamber for 1 min after the third foot shock. The memory test was performed 24 h later by exposing the animals to a novel box for 3 min followed by a tone (3 min, 3 kHz, 80 dB). All data were recorded using a video-based FreezeFrame system (Coulbourn Instruments). Freezing, defined as a lack of movement except for heart beat and respiration associated with a crouching posture, and locomotor activity were analyzed offline with the Acti-metrics software (Coulbourn Instruments).

Statistical Analysis:

For single-molecule force spectroscopy experiments, the data were fitted with multinormal functions, and the fittings were statistically tested. For biochemistry, electrophysiology, and behavior experiments, mutant proteins, cells, and animals were randomly assigned into control or experimental groups and investigators were blinded to experiment treatments in cultured slices and animals. Statistical results were reported as mean $\pm$ standard error of the mean. Given the negative correlation between the variation and square root of sample number, n , the group sample size was typically set to be ≈ 10 –36 to optimize the efficiency and power of statistical tests. Statistical significances of the means ($p < 0.05$; two sides) were determined using Wilcoxon and Mann–Whitney Rank Sum nonparametric tests for paired and unpaired samples, respectively, and statistical significances of the linear relationships of two data groups were determined using linear regression t -tests provided that the normality and constant variance tests passed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

BRaf mutations alter Ras–BRaf intermolecular bonds. a) Schematic drawing for the design of force-spectroscopy experiments. b) Upper, force-spectroscopic recording traces for rupturing of wild-type Ras from wild-type or mutant BRaf linked to optically trapped bead, and coverslip-attached bead, respectively. Lower, histograms of unbinding forces for rupturing of Ras(WT) from BRaf(WT) ($n = 500$ out of 1811 trials), BRaf(Q257R) ($n = 497$ out of 2261 trials), BRaf(Q257E) ($n = 478$ out of 2069 trials), BRaf(Q257A) ($n = 456$ out of 2267 trials), BRaf(A246P) ($n = 487$ out of 2527 trials), BRaf(A246F) ($n = 499$ out of 2011 trials), BRaf(A246G) ($n = 441$ out of 1849 trials), or BRaf(A246S) ($n = 500$ out of 2212 trials) linked to optically trapped bead and coverslip-attached bead, respectively. The

histograms are fitted with multinormal function $f(x, \mu_i = 1, 2, 3, \sigma, k_i = 1, 2, 3) = \sum_i k_i \cdot e^{-\frac{(x - \mu_i)^2}{2\sigma^2}}$ (where e is the base of the natural logarithm, k_i is the experimentally calculated coefficient, μ_i is the mean rupture force under the i th peak, and σ is the standard deviation of the rupture

force around the peak; see Table S1 (Supporting Information) for peak values). The fitting values for BRaf(WT) ($k_1 = 8.6$, $k_2 = 5.6$, $k_3 = 7.5$, $\mu_1 = 36$, $\mu_2 = 88$, $\mu_3 = 128$, $\sigma = 8.5$, $R^2 = 0.75$, $p < 0.01$), BRaf(Q257R) ($k_1 = 6.4$, $k_2 = 11.0$, $k_3 = 5.2$, $\mu_1 = 64$, $\mu_2 = 93$, $\mu_3 = 157$, $\sigma = 8.5$, $R^2 = 0.70$, $p < 0.01$), BRaf(Q257E) ($k_1 = 4.1$, $k_2 = 5.8$, $k_3 = 5.8$, $\mu_1 = 28$, $\mu_2 = 66$, $\mu_3 = 99$, $\sigma = 8.5$, $R^2 = 0.58$, $p < 0.01$), BRaf(Q257A) ($k_1 = 5.3$, $k_2 = 5.7$, $k_3 = 4.3$, $\mu_1 = 35$, $\mu_2 = 60$, $\mu_3 = 96$, $\sigma = 8.5$, $R^2 = 0.59$, $p < 0.01$), BRaf(A246P) ($k_1 = 8.0$, $k_2 = 8.3$, $k_3 = 11.6$, $\mu_1 = 48$, $\mu_2 = 110$, $\mu_3 = 150$, $\sigma = 8.5$, $R^2 = 0.78$, $p < 0.01$), BRaf(A246F) ($k_1 = 6.0$, $k_2 = 8.1$, $k_3 = 6.2$, $\mu_1 = 36$, $\mu_2 = 93$, $\mu_3 = 122$, $\sigma = 8.5$, $R^2 = 0.84$, $p < 0.01$), BRaf(A246G) ($k_1 = 3.0$, $k_2 = 5.9$, $k_3 = 6.0$, $\mu_1 = 26$, $\mu_2 = 68$, $\mu_3 = 98$, $\sigma = 8.5$, $R^2 = 0.79$, $p < 0.01$), and BRaf(A246S) ($k_1 = 4.7$, $k_2 = 5.8$, $k_3 = 7.0$, $\mu_1 = 33$, $\mu_2 = 74$, $\mu_3 = 101$, $\sigma = 8.5$, $R^2 = 0.73$, $p < 0.01$).

Figure 2.

BRaf mutations increase or decrease Ras–BRaf binding affinity. a) Schematic drawing for the design of biochemistry experiments. b) Western blots of in vitro GST-BRaf(RBD–CRD) mutant proteins bound to Ras(WT)-bound His-tag beads. Note that the plain agarose beads (5 μ g) and pure GST-BRaf(RBD–CRD) proteins (80 ng) loaded in the last two lanes were used for nonspecific binding adjustment and normalization. c) Plots of amounts of the bound GST-BRaf(RBD–CRD) wild-type and mutant proteins against amounts of the loaded GST-BRaf(RBD–CRD) proteins (see Table S2 (Supporting Information) for values). Note the significant shift or no change (BRaf(Q257R): $Z = 2.366$, $p < 0.05$; BRaf(Q257E): $Z = -2.028$, $p < 0.05$; BRaf(Q257A): $Z = -2.366$, $p < 0.05$; BRaf(A246P): $Z = 2.366$, $p < 0.05$; BRaf(A246F): $Z = -1.183$, $p = 0.24$; BRaf(A246G): $Z = -2.366$, $p < 0.05$; BRaf(A246S): $Z = -2.366$, $p < 0.05$; Wilcoxon tests) in binding curves of BRaf mutants compared to wild-type of BRaf. d) Plot shows correlation between binding affinities of Ras(WT) with BRaf proteins (K_d) and unbinding forces for rupturing of Ras(WT) from corresponding BRaf proteins ($n = 8$; normality test: $p = 0.15$; constant variance test: $p = 0.71$; $r = 0.794$; $p < 0.05$).

Figure 3.

BRaf mutations alter synaptic AMPA-R-mediated transmission. **a**) Schematic drawing outlines design of electrophysiology experiments. **b**) Upper, evoked AMPA-R-mediated (-60 mV), and NMDA-R-mediated ($+40$ mV) currents recorded from control nonexpressing (Ctrl) CA1 neurons and nearby CA1 neurons expressing replacement wild-type BRaf or BRaf mutants. **c**) Left, relative amplitudes of synaptic AMPA responses in CA1 neurons expressing replacement BRaf(WT) (Ctrl: -28.4 ± 2.0 pA; Exp: -27.8 ± 1.5 pA; $n = 28$; $Z = 0.023$, $p = 0.98$), BRaf(Q257R) (Ctrl: -23.8 ± 1.7 pA; Exp: -30.6 ± 2.1 pA; $n = 33$; $Z = 2.609$, $p < 0.01$), BRaf(Q257E) (Ctrl: -23.0 ± 1.4 pA; Exp: -18.4 ± 1.3 pA; $n = 33$; $Z = -2.489$, $p < 0.05$), BRaf(Q257A) (Ctrl: -20.4 ± 1.6 pA; Exp: -16.0 ± 1.4 pA; $n = 25$; $Z = -2.974$, $p < 0.005$), BRaf(A246P) (Ctrl: -24.3 ± 1.9 pA; Exp: -32.1 ± 2.4 pA; $n = 30$; $Z = 3.034$, $p < 0.005$), BRaf(A246F) (Ctrl: -25.9 ± 1.9 pA; Exp: -25.5 ± 1.9 pA; $n = 34$; $Z = -0.063$, $p = 0.95$), BRaf(A246G) (Ctrl: -23.3 ± 2.0 pA; Exp: -19.9 ± 1.9 pA; $n = 34$; $Z = -3.633$, $p < 0.001$), and BRaf(A246S) (Ctrl: -20.4 ± 1.6 pA; Exp: -16.0 ± 1.4 pA; $n = 25$; $Z = -2.974$, $p < 0.005$). **d**) Relative AMPA and NMDA currents as a percentage of control versus combined rupture force.

<0.005), or BRaf(A246S) (Ctrl: -29.7 ± 2.4 pA; Exp: -24.7 ± 2.5 pA; $n = 24$; $Z = -2.433$, $p < 0.05$) compared to nearby control nonexpressing neurons. Right, relative amplitudes of synaptic NMDA responses in CA1 neurons expressing replacement BRaf(WT) (Ctrl: 63.2 ± 4.1 pA; Exp: 61.2 ± 4.2 pA; $n = 28$; $Z = -0.068$, $p = 0.95$), BRaf(Q257R) (Ctrl: 55.4 ± 4.1 pA; Exp: 53.2 ± 4.5 pA; $n = 33$; $Z = -0.572$, $p = 0.57$), BRaf(Q257E) (Ctrl: 53.7 ± 4.0 pA; Exp: 48.7 ± 3.2 pA; $n = 33$; $Z = -1.045$, $p = 0.30$), BRaf(Q257A) (Ctrl: 50.0 ± 4.2 pA; Exp: 47.1 ± 4.6 pA; $n = 25$; $Z = -1.063$, $p = 0.29$), BRaf(A246P) (Ctrl: 49.6 ± 4.6 pA; Exp: 53.0 ± 4.0 pA; $n = 30$; $Z = 1.121$, $p = 0.26$), BRaf(A246F) (Ctrl: 55.1 ± 4.3 pA; Exp: 56.9 ± 4.4 pA; $n = 34$; $Z = -0.453$, $p = 0.65$), BRaf(A246G) (Ctrl: 47.0 ± 4.8 pA; Exp: 45.0 ± 2.8 pA; $n = 34$; $Z = -0.331$, $p = 0.74$), or BRaf(A246S) (Ctrl: 66.7 ± 6.4 pA; Exp: 64.6 ± 6.2 pA; $n = 24$; $Z = -0.229$, $p = 0.82$) compared to nearby control nonexpressing neurons. The relative values and standard errors were normalized to average amounts of AMPA and NMDA responses from control CA1 cells. Asterisks indicate $p < 0.05$ (Wilcoxon tests). d) Plots show correlations between synaptic AMPA ($n = 8$; normality test: $p = 0.71$; constant variance test: $p = 0.42$; $r = 0.984$; $p < 0.001$) and NMDA ($n = 8$; normality test: $p = 0.13$; constant variance test: $p = 0.75$; $r = 0.691$; $p = 0.06$) responses of CA1 neurons expressing replacement BRaf proteins and unbinding forces for rupturing of Ras(WT) from corresponding BRaf proteins.

Figure 4.

BRAF mutations impair learning. a) Schematic drawing outlines the design of behavioral tests. b) Upper, during the Y-maze learning test, animals expressing shRNA-PalGFP-IRES-GFP-BRaf(Q257R) or shRNA-PalGFP-IRES-GFP-BRaf(A246P) exhibited a significant reduction in percentage of correct choices compared to control nonexpressing animals and animals expressing shRNA-PalGFP-IRES-GFP-BRaf(WT). Lower, percentages of correct choices in animals expressing shRNA-PalGFP-IRES-GFP-BRaf(WT), -BRaf(Q257R), or -BRaf(A246P). (BRaf(WT): $79.2 \pm 1.6\%$, $n = 24$; $U = 241.0$; $p = 0.34$; BRaf(Q257R): $64.2 \pm 1.4\%$, $n = 24$; $U = 39.5$; $p < 0.001$; BRaf(A246P): $61.9 \pm 1.4\%$, $n = 24$; $U = 32.5$; $p < 0.001$) compared to control nonexpressing animals ($80.8 \pm 1.9\%$, $n = 24$). c) The plot shows correlation between correct choices of animals expressing BRaf proteins and altered unbinding forces for rupturing of Ras(WT) from corresponding BRaf proteins ($n = 8$; normality test: $p = 0.77$; constant variance test: $p = 0.71$; $r = 0.979$; $p < 0.001$). Values for

percentages of correct choices in animals expressing shRNA-PalGFP-IRES-GFP-BRaf(Q257E), -BRaf(Q257A), -BRaf(A246F), -BRaf(A246G), or -BRaf(A246S) (BRaf(Q257E): $67.0 \pm 1.9\%$, $n = 24$; $U = 86.0$; $p < 0.001$; BRaf(Q257A): $67.4 \pm 2.2\%$, $n = 24$; $U = 94.0$; $p < 0.001$; BRaf(A246F): $75.3 \pm 2.9\%$, $n = 23$; $U = 328.0$; $p = 0.27$; BRaf(A246G): $65.3 \pm 1.8\%$, $n = 23$; $U = 490.0$; $p < 0.001$; BRaf(A246S): $70.4 \pm 2.3\%$, $n = 23$; $U = 417.5$; $p < 0.005$) compared to control nonexpressing animals. d) Upper left, distances traveled during the initial exposure for animals expressing shRNA-PalGFP-IRES-GFP-BRaf(WT), -BRaf(Q257R), or -BRaf(A246P) (BRaf(WT): 329.3 ± 17.6 cm, $n = 25$; $U = 3890.5$; $p = 0.81$; BRaf(Q257R): 326.0 ± 16.6 cm, $n = 23$; $U = 360.0$; $p = 0.80$; BRaf(A246P): 329.1 ± 16.3 cm, $n = 23$; $U = 363.5$; $p = 0.75$) compared to control nonexpressing animals (Ctrl: 329.7 ± 19.7 cm, $n = 30$). Upper right: moving speeds for animals expressing shRNA-PalGFP-IRES-GFP-BRaf(WT), -BRaf(Q257R), or -BRaf(A246P) during the tone presentation (BRaf(WT): 1.79 ± 0.17 cm s⁻¹, $n = 25$; $U = 353.5$; $p = 0.72$; BRaf(Q257R): 1.82 ± 0.17 cm s⁻¹, $n = 23$; $U = 312.0$; $p = 0.56$; BRaf(A246P): 1.76 ± 0.16 cm s⁻¹, $n = 23$; $U = 328.0$; $p = 0.77$) compared to control nonexpressing animals (Ctrl: 1.73 ± 0.15 cm s⁻¹, $n = 30$), and those during electric foot shock (BRaf(WT): 11.8 ± 1.0 cm s⁻¹, $n = 25$; $U = 369.0$; $p = 0.93$; BRaf(Q257R): 12.0 ± 1.2 cm s⁻¹, $n = 23$; $U = 326.0$; $p = 0.74$; BRaf(A246P): 12.0 ± 1.0 cm s⁻¹, $n = 23$; $U = 331.5$; $p = 0.82$) compared to control nonexpressing animals (Ctrl: 11.5 ± 0.9 cm s⁻¹, $n = 30$). Lower, amounts of freezing for animals expressing shRNA-PalGFP-IRES-GFP-BRaf(WT), -BRaf(Q257R), or -BRaf(A246P) during training (BRaf(WT): $17.9 \pm 1.9\%$, $n = 25$; $U = 371.5$; $p = 0.96$; BRaf(Q257R): $19.0 \pm 2.0\%$, $n = 23$; $U = 328.5$; $p = 0.77$; BRaf(A246P): $18.4 \pm 2.1\%$, $n = 23$; $U = 330.5$; $p = 0.80$) compared to control nonexpressing animals (Ctrl: $17.3 \pm 1.3\%$, $n = 30$), and during contextual fear conditioning test (BRaf(WT): $49.3 \pm 4.5\%$, $n = 25$; $U = 373.5$; $p = 0.99$; BRaf(Q257R): $29.7 \pm 3.3\%$, $n = 23$; $U = 513.0$; $p < 0.005$; BRaf(A246P): $27.7 \pm 3.4\%$, $n = 23$; $U = 538.0$; $p < 0.001$) compared to control nonexpressing animals (Ctrl: $48.8 \pm 4.4\%$, $n = 30$). e) Plot shows correlation between amounts of freezing for animals expressing BRaf proteins and altered unbinding forces for rupturing of Ras(WT) from corresponding BRaf proteins ($n = 8$; normality test: $p = 0.28$; constant variance test: $p = 0.10$; $r = 0.956$; $p < 0.001$). Values for amounts of freezing for animals expressing shRNA-PalGFP-IRES-GFP-BRaf(Q257E), -BRaf(Q257A), -BRaf(A246F), -BRaf(A246G), or -BRaf(A246S) during contextual fear conditioning test (BRaf(Q257E): $32.6 \pm 3.2\%$, $n = 22$; $U = 466.0$; $p < 0.05$; BRaf(Q257A): $30.6 \pm 2.9\%$, $n = 22$; $U = 491.5$; $p < 0.005$; BRaf(A246F): $48.2 \pm 4.6\%$, $n = 23$; $U = 347.0$; $p = 0.98$; BRaf(A246G): $34.5 \pm 2.9\%$, $n = 23$; $U = 472.0$; $p < 0.05$; BRaf(A246S): $34.4 \pm 3.2\%$, $n = 23$; $U = 458.0$; $p < 0.05$) compared to control nonexpressing animals. The large pink dots with error bars indicate the averages and standard errors. Asterisk indicates $p < 0.05$ (Mann-Whitney rank sum test).