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CLIP Identifies Nova-Regulated RNA Networks in the Brain

Jernej Ule,[1,2*] Kirk B. Jensen,[1,2*] Matteo Ruggiu,[1,2] Aldo Mele,[1,2] Aljaž Ule,[3] Robert B. Darnell[1,2†]

Nova proteins are neuron-specific antigens targeted in paraneoplastic opsoclonus myoclonus ataxia (POMA), an autoimmune neurologic disease characterized by abnormal motor inhibition. Nova proteins regulate neuronal pre-messenger RNA splicing by directly binding to RNA. To identify Nova RNA targets, we developed a method to purify protein-RNA complexes from mouse brain with the use of ultraviolet cross-linking and immunoprecipitation (CLIP). Thirty-four transcripts were identified multiple times by Nova CLIP. Three-quarters of these encode proteins that function at the neuronal synapse, and one-third are involved in neuronal inhibition. Splicing targets confirmed in Nova$^{-/-}$ mice include c-Jun N-terminal kinase 2, neogenin, and gephyrin; the latter encodes a protein that clusters inhibitory γ-aminobutyric acid and glycine receptors, two previously identified Nova splicing targets. Thus, CLIP reveals that Nova coordinately regulates a biologically coherent set of RNAs encoding multiple components of the inhibitory synapse, an observation that may relate to the cause of abnormal motor inhibition in POMA.

RNA binding proteins are associated with a large number of human disorders, particularly autoimmune and neurologic diseases. Notable examples include the small nuclear ribonucleoproteins (snRNPs) in systemic lupus erythematosis (*1*, *2*), Nova and Hu in the paraneoplastic neurologic degenerations, FMRp in fragile X mental retardation syndrome, and ataxin-1 in spinocerebellar ataxia type 1 (*3*–*6*). Understanding the role these proteins play in normal and in diseased brain requires methods to identify the set of RNAs to which they bind in vivo and the use of mouse models of these disorders for RNA target validation.

Identification of bona fide disease-related sets of RNA targets for RNA binding proteins has only been accomplished in a few instances. Because of their high abundance, the RNA targets of lupus autoantigens were directly identified with the use of autoimmune sera to immunoprecipitate snRNPs (*3*). However, the RNA targets of a number of other diseases, particularly those involving the nervous system, appear to have considerable complexity and have been correspondingly difficult to identify. RNA selection (*7*–*9*), combined with coimmunoprecipitation of RNA-protein complexes and analysis on gene chips or in knock-out cells (*8*, *10*–*13*), has been used to identify candidate target RNAs, but with important limitations, including low signal/noise, difficulty differentiating direct from indirect protein-RNA interactions, and the inability to identify regions on RNA to which proteins bind.

To identify transcripts that Nova interacts with in vivo, we developed an ultraviolet (UV) cross-linking and immunoprecipitation (CLIP) method (Fig. 1A). Brain tissue was directly irradiated with UV-B light, which forms covalent bonds between protein and RNA that are in direct contact (within angstroms); the relatively low efficiency of this reaction is compensated for by polymerase chain reaction (PCR) amplification. Covalent binding allows rigorous purification schemes for obtaining highly purified protein-RNA complexes, including stringent washing of immunoprecipitates, boiling in SDS, separating complexes on SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and transferring them to nitrocellulose, which retains protein-RNA complexes but not free RNA. Also, covalent cross-linking allowed us to partially digest the RNA while retaining the

[1]Howard Hughes Medical Institute, [2]Laboratory of Molecular Neuro-Oncology, Rockefeller University, New York, NY 10021, USA. [3]Center for Research in Experimental Economics and Political Decision-Making, University of Amsterdam, 1018 WB Amsterdam, Netherlands.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: darnelr@mail.rockefeller.edu

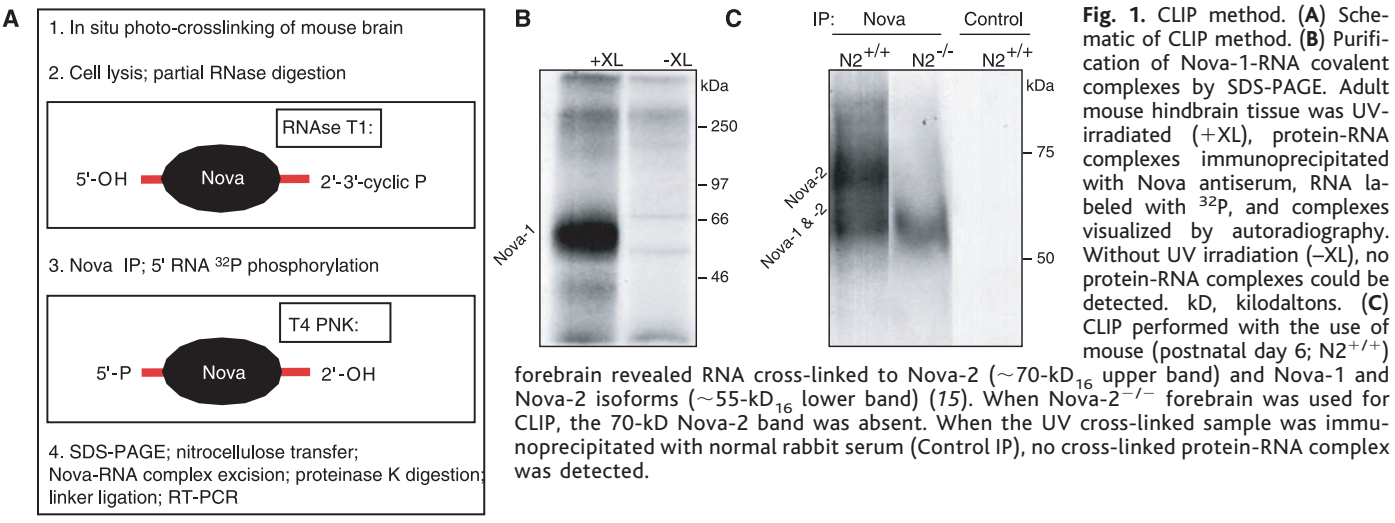


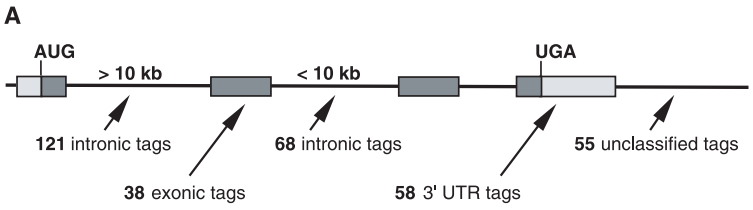
Fig. 1. CLIP method. (**A**) Schematic of CLIP method. (**B**) Purification of Nova-1-RNA covalent complexes by SDS-PAGE. Adult mouse hindbrain tissue was UV-irradiated (+XL), protein-RNA complexes immunoprecipitated with Nova antiserum, RNA labeled with ^{32}P, and complexes visualized by autoradiography. Without UV irradiation (–XL), no protein-RNA complexes could be detected. kD, kilodaltons. (**C**) CLIP performed with the use of mouse (postnatal day 6; N2$^{+/+}$) forebrain revealed RNA cross-linked to Nova-2 (~70-kD$_{16}$ upper band) and Nova-1 and Nova-2 isoforms (~55-kD$_{16}$ lower band) (*15*). When Nova-2$^{-/-}$ forebrain was used for CLIP, the 70-kD Nova-2 band was absent. When the UV cross-linked sample was immunoprecipitated with normal rabbit serum (Control IP), no cross-linked protein-RNA complex was detected.

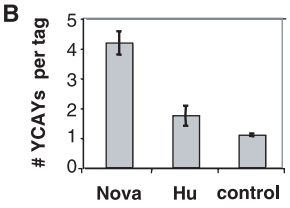
core element involved in protein binding such that short (60- to 100-nucleotide) RNA tags can be purified, allowing both the identification of the bound RNA species and location of the binding site. Protein was removed with proteinase K, and RNA cloned with the use of linker ligation and reverse transcription (RT)–PCR. After identification of candidate RNAs, we verified targets with the use of several stringent tests: sequence comparisons with known Nova binding sites, demonstration of direct RNA-protein interactions, and quantification of changes in alternative splicing in Nova null mouse brain.

When we used CLIP to identify Nova-RNA complexes from mouse brain, RNA was copurified with Nova only after UV-B irradiation; in the absence of cross-linking or when preimmune rabbit serum was used for immunoprecipitation, no RNA copurified with Nova (Fig. 1, B and C). As an additional control to assess the specificity of the interaction, we performed CLIP with the use of wild-type versus Nova-2$^{-/-}$ brain with an antibody that recognizes both Nova-1 and Nova-2 proteins. In wild-type brain, cross-linked bands migrating with both Nova-1 and Nova-2 proteins were evident, but in Nova-2$^{-/-}$ brain RNA cross-linked specifically to protein corresponding to Nova-1, whereas the band migrating at the molecular weight of 70 kdaltons, corresponding to Nova-2 isoform, was lost (Fig. 1C). Thus, the ability of CLIP to isolate RNA is dependent on photocross-linking and on the presence of immunoprecipitated Nova protein.

We sequenced 340 Nova CLIP tags (Fig. 2A and table S1), which had an average length of 71 nucleotides. The largest set of CLIP tags (121) were within long introns; 99 (82%) of these were within the first three introns. Sixty-eight were found within introns shorter than 10 kb, and 18 (26%) of these flanked alternative exons. Fifty-eight were within 3′ untranslated regions and 38 within exons, 2 of which are reported to be alternatively spliced. We compared the sequence of the Nova CLIP tags with the known Nova binding element, multimers of the YCAY (where Y indicates a pyrimidine, U or C) tetramer (*8*, *14*–*16*). Tetramer frequency analysis revealed that, on average, each tag harbored 4.2 YCAY tetramers, compared to an expected frequency of 1.1 YCAY tetramers in random sequences and the observed frequencies of 1.1 and 1.7 in random genomic sequences and CLIP tags obtained with an unrelated RNA binding protein, respectively (Fig. 2B and fig. S1). Moreover, analysis of nucleotide frequencies flanking all CA dimers present in Nova CLIP tags shows an overrepresentation of YCAU tetramers flanked by pyrimidines (Fig. 2C), which is also evident in the five most frequent hexamers, which are overrepresented 15 to 30 times; no such increase was evident in analysis of control tags (Fig. 2D). We performed filter binding assays with the use of purified Nova-2 protein and RNA transcribed from four different Nova CLIP tags (Fig. 2, E and F). All four RNAs bound Nova-2 with high-affinity (Fig. 2E; 23 to ~400 nM). Control tags of genomic sequence immediately 5′ to the CLIP tags did not bind, nor did CLIP tags in which CA dinucleotides were mutated to AA (*17*). Thus, Nova protein selectively cross-links to high affinity RNA targets harboring Nova binding sites. The CLIP tags identified reflect the $(UCAUY)_3$ and YCAYC motifs observed in RNA selection experiments performed with Nova (*8*) and in functional studies demonstrating that Nova regulates alternative splicing by binding clusters of at least three intronic UCAU tetramers in target RNAs (*11*, *18*).

We used Nova$^{-/-}$ mouse brain (*11*, *19*) to validate candidate RNAs by assessing the use





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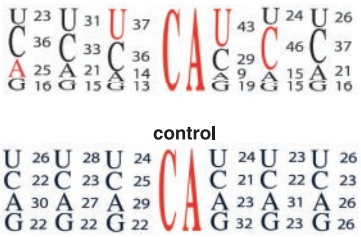
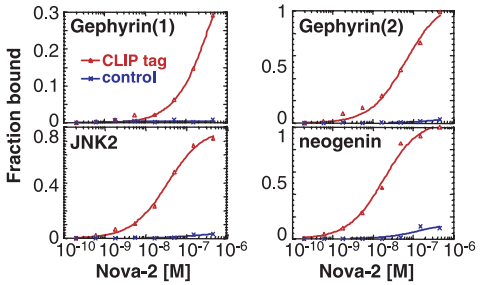


D

hexamer	Nova tags obs/exp	control tags obs/exp
UCCAUC	30.1	1.3
CCAUCC	27.3	1.2
AUCCAU	25.0	0.9
CAUCCA	23.7	1.7
UCAUCC	14.7	0.9



F

RNA tag	transcribed sequence	Kd [nM]
gephyrin1	GGGCCAGUUCAACCACAGGUCCCCAGCUUCCAUCCAUUGGUUGGGUGCUAGUAUCUGCAUCUGACUCUUUCAGCU	~400
control	GGGAGCUGAUGUAUGCUGCCUGGUUGGUGGCUCAGUGUCCUAGAGAUCUCGGGAUCCAGGUCAGUUGAGACUGCC	>10^3
gephyrin2	GGGCCAACCACAAAUGCCAGCACCUCUUAAUAACAAUCAGCAUGACCUCUGCCUAAGUCUUGGCUUCUUCCUCAGAA	64
control	GGGGAGGAUGCCCUGCACCAGUACAGUAAAUGAGUGCUUGGAGGAAGAUCAGUGUGGCACUGUAAAGCCUAGUGUGC	>10^3
JNK2	GGGGUGUUCUUCCAUUUUCCACAUUCUUCACACUAACAUGCGUCUUCAUGCU	34
control	GGGGUUUUGUAAAGGGAGCCGACGGCAUAGGGCACUAGCUCUGUCCCCUCGU	>10^3
neogenin	GGGCUGUGAUUAGUGCCCAUCCCAUCCAUUCCCUCGAUAACCCUCACCAUCAUUUCCACUCCAG	23
control	GGGGUCUACACUGGCUGGAAGGAGGGGAAUGAGACCAAAAAUGAUGAUGCCCUUUGACUCUCAG	>10^3

Fig. 2. Analysis of 340 Nova CLIP tags. (**A**) Genomic location of the tags. Tags belonging to genomic regions with no annotated transcripts are labeled as "unclassified." (**B**) YCAY tetramer abundance in Nova and Hu CLIP tags in comparison to control tags. The average number of YCAY tetramers per Nova CLIP tag is 4.18 (99% confidence interval ± 0.39, average tag length 71 ± 18 nucleotides, n = 340) compared with 1.7 per CLIP tag of an unrelated RNA binding protein, Hu (99% confidence interval ± 0.21, average tag length 62 ± 16 nucleotides, n = 94), and 1.1 per random fragment of transcribed genomic sequence (Control; 99% confidence interval ± 0.03, average tag length 71 ± 18 nucleotides, n = 3400). (**C**) Frequency of nucleotides flanking all CA dimers in Nova CLIP and genomic tags. Nucleotides in red have a frequency 25% higher than that expected on the basis of total nucleotide composition of 29% U, 35% C, 20% A, and 17% G in Nova CLIP tags. (**D**) The five most frequent hexamers in Nova CLIP tags and the ratio of the average observed and expected (obs/exp) abundance compared to control tags; even the least abundant of these, UCAUCC, is in 10-fold excess relative to the average control tag (P = 0.013, z test). (**E**) Filter binding assay results using Nova-2 fusion protein at the indicated concentrations and synthetic RNAs shown in (F). (**F**) Sequences of transcribed CLIP tag RNAs and control RNAs corresponding to genomic sequences immediately 5′ to the CLIP tags.

of alternatively spliced exons present near Nova CLIP tags. Of 18 RNAs assayed, 7 showed changes in alternative splicing in Nova-null mouse brain ranging from 1.6-fold to 60-fold (Fig. 3) (*17*). C-Jun N-terminal kinase 2 (JNK2) is a cytoplasmic signaling protein that translocates to the nucleus to phosphorylate and activate several transcription factors, including activating transcription factor 2 (ATF2) and c-Jun. The alternatively spliced exons 6a and exon 6b encode isoforms that preferentially bind ATF2 and c-Jun, respectively (*20*), and are preferentially included in brain and nonneuronal tissues, respectively (*17*, *21*). A Nova CLIP tag was identified in JNK2 pre-mRNA, near exon 6b (Fig. 3A). RT-PCR analysis revealed a net sixfold change in exon use in Nova-$2^{-/-}$ relative to Nova-$2^{+/+}$ cortex: a threefold decrease in use of the exon 6a isoform and a twofold increase in exon 6b in JNK2 RNA (Fig. 3A).

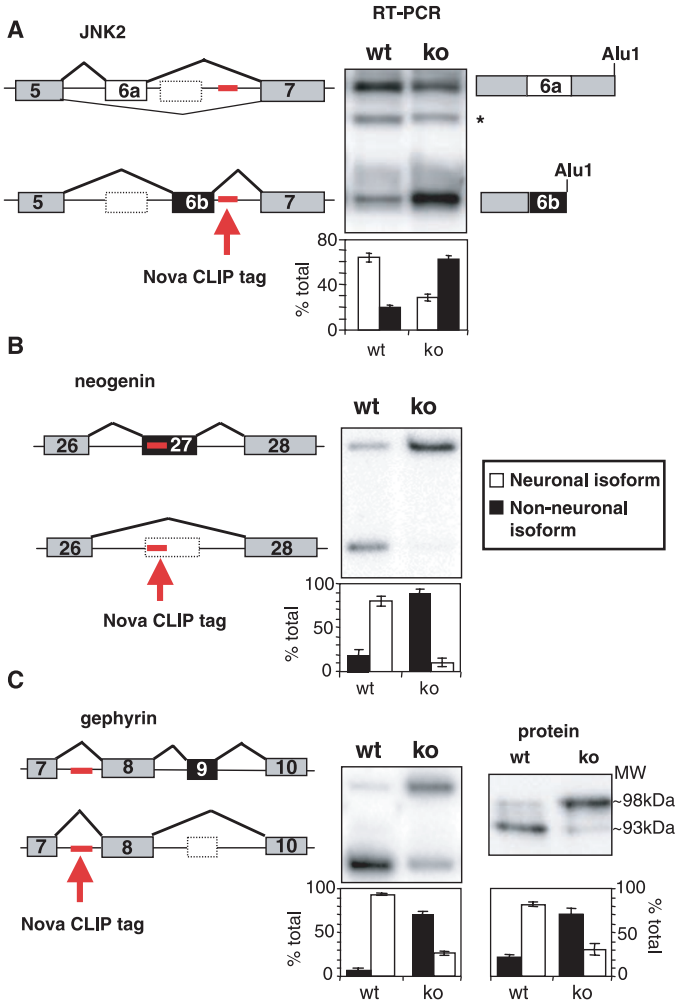


Fig. 3. Nova-dependent regulation of JNK2 (**A**), neogenin (**B**), and gephyrin (**C**) alternative splicing. Schematics of pre-mRNA alternative splicing in the vicinity of the Nova CLIP tags are shown on left. For each transcript [(A) to (C)], neuronal (white) and nonneuronal (black) isoforms are indicated in schematic form and quantified in bar graphs. Autoradiograms (center) of RT-PCR products for each transcript, generated with the use of RNA isolated from Nova $2^{+/+}$ [wild-type (wt)] and Nova $2^{-/-}$ [knock-out (ko)] P6 and P7 brain cortex, show the migration of bands corresponding to specific spliced isoforms. Each autoradiagram was quantitated and is plotted with the standard error from three litters. In (A), an asterisk marks a minor splice variant (isoform IV), and JNK2 PCR products present after digestion with Alu 1 are illustrated. In (C), a Western blot analysis of gephyrin protein in Nova 2 P1 wt and ko cortex is shown.

Neogenin, a homolog of DCC (deleted in colorectal cancer), has been reported to bind netrin-1 (*22*), although its role in axon guidance has not yet been fully elucidated. Neogenin is expressed in all adult mouse tissues and has four alternative exons (*23*), one of which, exon 27, contains a Nova CLIP tag. We assayed splicing of all four alternative exons in RNA obtained from the cortex of Nova-2 null mice and found that alternative splicing of exon 27 was drastically altered relative to wild-type brain, such that there was ~36-fold increase in the use of exon 27 in RNA from Nova-$2^{-/-}$ relative that in to Nova-$2^{+/+}$ cortex (Fig. 3B). In contrast, there was no change in use of the other three alternatively spliced neogenin exons in Nova-$2^{-/-}$ relative to Nova-$2^{+/+}$ cortex (*17*), consistent with previous observations that Nova regulates splicing in only a subset of regulated exons (*11*, *18*).

Two Nova CLIP tags were identified in gephyrin, one in intron 7, near the alternatively spliced exon 9, and a second in intron 14 (Fig. 3C and table S2). In wild-type mouse brain, gephyrin transcripts preferentially exclude exon 9 (96%). In Nova-$2^{-/-}$ mouse brain, only 27% of gephyrin transcripts exclude exon 9, and there is a compensatory increase in exon 9 inclusion (73% compared with 4% in wild-type cortex; Fig. 3C). We examined each of the seven gephyrin exons reported to be alternatively spliced (*24*) and found that only exon 9 was regulated by Nova (Fig. 3C) (*17*). Thus, the presence of Nova in neurons correlates with alternative exon skipping in gephyrin and neogenin transcripts.

We also detected changes in gephyrin protein isoform expression in Nova-$2^{-/-}$ cortex by Western blot (Fig. 3C), and these were consistent in magnitude with the changes seen in gephyrin transcripts. We surveyed gephyrin exon 9 use in different tissues by RT-PCR analysis, which revealed that gephyrin transcripts in nonneuronal tissues include 22 (testis) to 115 (heart) times more exon 9 than those in brain (fig. S2), which is similar in scale to the 60-fold change seen in Nova-$2^{-/-}$ versus wild-type cortex (Fig. 3C).

Nova was the first mammalian tissue-specific splicing factor identified (*11*, *18*). We previously found that Nova binds to glycine receptor α2 subunit (GlyRα2) and γ-aminobutyric acid type A 2 γ-aminobutyric acid type A receptor γ2 subunit ($GABA_A$γ2) intronic sequences to mediate a two- to threefold increase in inclusion of exons 3A and γ2L, respectively (*11*, *19*), although other factors are likely to play important roles in regulating their splicing (*25*, *26*). Here, we used an unbiased screen to identify Nova RNA targets in vivo and found that JNK2, neogenin, and gephyrin pre-mRNAs

Table 1. Twenty-one multiple-hit Nova CLIP-tags organized by primary encoded function.

Function	Proteins
Inhibitory	Gephyrin, microtubule-associated protein 1b, $GABA_B$ receptor 2, heterotrimericguanine nucleotide–binding protein–coupled inwardly rectifying K^+ channel, nicotinic acetylcholine receptor β2 subunit (Acrb2)
Postsynaptic	Flamingo 1, plasma membrane Ca^{2+} adenosine triphosphatase 2, Shank1, brain sodium channel 1 αsubunit, protein kinase C ζ, calneuron 1
Presynaptic	Neurexin II, H/T-cadherin, Teneurin 2, rabaptin-5
Signaling, protein synthesis, other	Ataxin 2-binding protein, ribosomal S6 kinase 3, ribosomal protein L13a, parathymosin, diacylglycerol kinase ι, thymocyte cyclic adenosine monophosphate regulated phosphoprotein

show 6- to 60-fold Nova-dependent effects on alternative splicing. The magnitude of Nova's effect on alternative splicing of these transcripts is unprecedented in mammals (*26*). Moreover, each of these genes are expressed both within and outside the brain, and previous work has identified exons preferentially used (or excluded) in the brain. Our results indicate that Nova is the primary, if not sole, determinant of brain-specific alternative splicing of these and perhaps other transcripts.

In addition to gephyrin RNA, a number of RNAs were identified multiple times within the set of 340 Nova CLIP tags, suggesting that these might compose a particularly robust subset of RNA targets. Seventy-seven CLIP tags (23%) mapped to only 34 transcripts, each of which contained two or more tags. Twenty-one of these 34 transcripts correspond to characterized genes (Table 1); 15 (71%) of these encode proteins that function in the synapse, which, together with previous studies of Nova action on GlyRα2 and $GABA_A\gamma2$ pre-mRNAs (*11*, *18*, *25*), indicates that Nova coordinately regulates a biologically coherent set of RNAs.

An intriguing subset of Nova target RNAs involved in synaptic biology includes those involved in neuronal inhibition (table S2). These include the microtubule-associated protein MAP1b, which anchors $GABA_C$ receptors to the cytoskeleton and modulates their sensitivity (*27*); $GABA_B$ 2 receptor and GIRK2, which mediate slow inhibitory postsynaptic potentials (*28*); the K^+ voltage-gated channel KCNQ3, which mediates inhibition of repetitive action potentials (*29*); the nicotinic acetylcholine receptors β2 and α2, which contain CLIP tags at homologous positions in exon 5 and are, together as α4β2 heteropentamers, highly expressed on GABAergic interneurons, thus influencing inhibitory activity (*30*); and the JNK proteins (Fig. 3A), which are essential for neuronal microtubule integrity by controlling phosphorylation of MAP1b and MAP2 and for the regulation of GABA action in *Caenorhabditis elegans* inhibitory motor neurons (*31*).

Gephyrin RNA is a particularly interesting target for Nova action. Gephyrin is essential for the correct localization of $GABA_A\gamma2$ (*32*) and GlyRα2 (*33*) subunits to the inhibitory synapse, and Nova regulates alternative splicing of transcripts encoding both of those receptors (*11*, *18*). Like Nova, gephyrin has been reported to be the target of a cancer-associated neurologic disorder manifest by excess motor activity (*34*). Finally, Nova-dependent regulation of a network of RNAs encoding proteins that mediate neuronal inhibition correlates with the defective motor inhibition in $Nova^{-/-}$ mice (*11*) and in POMA patients (*4*).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/302/5648/1212/DC1
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Nonsteroidal Anti-Inflammatory Drugs Can Lower Amyloidogenic $A\beta_{42}$ by Inhibiting Rho

Yan Zhou,* Yuan Su, Baolin Li, Feng Liu, John W. Ryder, Xin Wu, Patricia A. Gonzalez-DeWhitt, Valentina Gelfanova, John E. Hale, Patrick C. May, Steven M. Paul,* Binhui Ni

A subset of nonsteroidal anti-inflammatory drugs (NSAIDs) has been shown to preferentially reduce the secretion of the highly amyloidogenic, 42-residue amyloid-β peptide $A\beta_{42}$. We found that Rho and its effector, Rho-associated kinase, preferentially regulated the amount of $A\beta_{42}$ produced in vitro and that only those NSAIDs effective as Rho inhibitors lowered $A\beta_{42}$. Administration of Y-27632, a selective Rock inhibitor, also preferentially lowered brain levels of $A\beta_{42}$ in a transgenic mouse model of Alzheimer's disease. Thus, the Rho-Rock pathway may regulate amyloid precursor protein processing, and a subset of NSAIDs can reduce $A\beta_{42}$ through inhibition of Rho activity.

Increased $A\beta_{42}$ accumulation, aggregation, and deposition in brain are key events in the pathogenesis of Alzheimer's disease (AD) (*1*). Accordingly, the reported beneficial effect of certain NSAIDs in reducing the risk of developing AD (*2*) may be due to their ability to reduce the amount of $A\beta_{42}$ in the brain. Indeed, a subset of NSAIDs lowers $A\beta_{42}$ in a variety of cultured cells independent of their effects on cyclooxygenase (COX) activity (*3*). However, the mechanism(s) by which this subset of NSAIDs preferentially lowers $A\beta_{42}$ is unclear. In addition to inhibiting COX activity, sulindac sulfide, a prototypic $A\beta_{42}$-lowering NSAID (*3*), also inhibits Ras-mediated signal transduction (*4*, *5*). Ras and Ras-like small GTP-binding proteins (small G proteins) regulate a wide variety of cellular functions such as gene expression, cytoskeletal reorganization, and vesicle trafficking. Because NSAIDs may regulate the activity of certain small G proteins, we investigated the possible role of several small G proteins in regulating

Neuroscience Discovery Research and Bioresearch Technologies and Proteins, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285, USA.

*To whom correspondence should be addressed. E-mail: paul_steven_m@lilly.com; zhou_yan_yz@lilly.com