Fe65 Interacts with P2X₂ Subunits at Excitatory Synapses and Modulates Receptor Function*

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Ionotropic receptors in the neuronal plasma membrane are organized in macromolecular complexes, which assure their proper localization and regulate signal transduction. P2X receptors, the ionotropic receptors activated by extracellular ATP, have been shown to influence synaptic transmission. Using a yeast two-hybrid approach with the P2X₂ subunit C-terminal domain as bait we isolated the β-amyloid precursor protein-binding proteins Fe65 and Fe65-like 1 as the first identified proteins interacting with neuronal P2X receptors. We confirmed the direct interaction of Fe65 and the P2X₂ C-terminal domain by glutathione S-transferase pull-down experiments. No interaction was observed between Fe65 and the naturally occurring P2X₂ splice variant P2X₂ᵇ, indicating that alternative splicing can regulate the receptor complex assembly. We generated two antibodies to Fe65 to determine its subcellular localization using postembedding immunogold labeling electron microscopy. We found labeling for Fe65 at the pre- and postsynaptic specialization of CA1 hippocampal pyramidal cell/Schaffer collateral synapses. By double immunogold labeling, we determined that Fe65 colocalizes with P2X₂ subunits at the postsynaptic specialization of excitatory synapses. Moreover, P2X₂ and Fe65 could be coimmunoprecipitated from brain membrane extracts, demonstrating that the interaction occurs in vivo. The assembly with Fe65 regulates the functional properties of P2X₂ receptors. Thus, the time- and activation-dependent change in ionic selectivity of P2X₂ receptors was inhibited by coexpression of Fe65, suggesting a novel role for Fe65 in regulating P2X receptor function and ATP-mediated synaptic transmission.

P2X receptors are ligand-gated ion channels activated by extracellular ATP. Seven P2X receptor subunits have been identified (P2X₁–P2X₇) that combine to form trimeric receptors of homomeric or heteromeric composition (1). Each P2X subunit has two transmembrane domains, a long extracellular loop, and intracellular N and C termini (1). Upon binding of ATP, P2X receptors open an intrinsic pore selectively permeable to monovalent cations and Ca²⁺ (1). Two kinds of deviations from these pore properties have been described. First, chicken and human P2X₄ receptors, in addition to cations, allow Cl⁻ to pass (2, 3), and second, P2X₂, P2X₄, and P2X₇ receptors become increasingly permeable to organic cations (e.g. N-methyl-D-glucamine) and fluorescent dyes during prolonged or repeated exposure to extracellular ATP (4–6). The kinetics and extent of these selectivity changes can be influenced by point mutations in the transmembrane segments (5, 7), and by deletions, chimeras, and point mutations in their intracellular C-terminal domain (4, 8–10). The C-terminal domain of P2X subunits is the least conserved part of the protein both in length and amino acid composition, indicating that it might confer subunit specific properties. Indeed, the C-terminal domain of P2X subunits was found to determine desensitization kinetics (11–13), constitutes a target of enzymatic modulation (14), and is differentially involved in receptor trafficking in and out of the plasma membrane (15–17). Also, it mediates the interaction of heterologously expressed P2X receptors with intracellular or membrane proteins that influence their functional properties (18–21). The P2X₂ subunit heterologously expressed in human embryonic kidney 293 cells has been found to associate with a multiprotein complex containing 11 proteins (18). However, although it is likely that some of the interactions occur via the long intracellular C-terminal domain of P2X₂ν, this has not yet been determined. Moreover, using affinity chromatography of brain extracts, P2X₂ subunit C-terminal domains have been shown to associate with myelin basic protein and βIII tubulin (22). However, to our knowledge, the interaction of P2X₂ subunits with these proteins has not been demonstrated to occur in vivo.

P2X₂, P2X₄, and P2X₇ subunits reside at the peripheral portion of the postsynaptic membrane of synapses identified as glutamatergic on the basis of structural characteristics and immunohistochemistry (23). This precise location could appose P2X receptors to distinct neurotransmitter release sites and help to compartmentalize intracellular signaling cascades. Interaction with intracellular proteins regulates the targeting and/or signaling of ionotropic glutamate, acetylcholine, γ-aminobutyric acid, and glycine receptors (24). By analogy, a similar regulation of P2X receptors is expected. In this context, the P2X₂ν subunit is of special interest, because its C-terminal domain contains several proline-rich sequences (25, 26). These sequences constitute protein-protein interaction motifs that can bind, e.g. Src homology 3 (SH3), Enabled/VASP homology (EVH1), or WW (named for two conserved Trp residues) domains. We therefore used the intracellular C-terminal domain of the P2X₂ν subunit as bait in a yeast two-hybrid screening of a rat brain cDNA library, and isolated the β-amyloid precursor protein (APP) interacting protein Fe65. We show here that Fe65 associates with P2X₂ν subunits both in vitro and in vivo, and that both proteins colocalize at the postsynaptic density of excitatory synapses in the hippocampus. This

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³ The abbreviations used are: SH, Src homology domain; APP, β-amyloid precursor protein; PTB, phosphotyrosine binding; PID, phosphotyrosine interacting domain; Y2H, yeast two-hybrid; GST, glutathione S-transferase; NMDG, N-methyl-D-aspartate; Trx, thioredoxin.

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interaction has functional consequences, in that the ionic selectivity changes shown by P2X₂ receptors are drastically reduced upon coexpression with Fe65 providing a novel mechanism for the regulation of synaptic receptor function.

**EXPERIMENTAL PROCEDURES**

**Plasmids**

P2X₂ Constructs—Yeast two-hybrid (Y2H) screening of the cDNA sequence corresponding to the full cytoplasmic domain of P2X₂ comprising amino acids 355–472 (P2X₂CD) was amplified by PCR using full-length P2X₂ cDNA (kindly provided by Dr. D. Julius, University of California, San Francisco, CA) as template. The sequence coding for the P2X₂ splice variant P2X₂(b) cytoplasmic domain comprising amino acids 355–403 (P2X₂(b)CD) was amplified by PCR using the P2X₂(b) cDNA (kindly provided by E. Glowatzki, Johns Hopkins University, Baltimore, MD). The obtained PCR products were cloned in pLexN vector (27) using the EcoRI/BamHI sites inserted in the oligonucleotides. For recombinant protein expression the sequences coding for the P2X₂CD and P2X₂(b)CD were amplified by PCR with oligonucleotides containing BamHI and NotI sites, the digested PCR products were cloned in-frame with glutathione S-transferase (GST; pGEX-4T-1 vector; Amersham Biosciences) or thioredoxin (Trx; pET-32a(+); Novagen).

Fe65 Constructs—The sequences coding for Fe65 fragments comprising amino acids 218–479 (Fe65-202), 218–309, 218–284, 255–284, and 285–479 were amplified by PCR using full-length Fe65 cDNA (kindly provided by Dr. T. Sudhof, University of Texas Southwestern, TX) as template and cloned in pVP16-4 vector using the same strategy as with P2X₂ constructs. For recombinant protein expression the sequence coding for amino acids 218–479, 197–255, and 40–100 were amplified by PCR and cloned in-frame both into pGEX-4T-1 and pET-32a(+) vectors employing the same strategy as with the P2X₂CD.

**Yeast Two-hybrid Assays**

The plasmid P2X₂CD-pLexN was used as bait to isolate putative interacting proteins from the rat brain cDNA library from postnatal day 8 (27) cloned into pVP16-3 vector. The P2X₂CD-pLexN and cDNA library were cotransformed into Saccharomyces cerevisiae reporter strain L40 (MATa trpl1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ) using the lithium acetate/single-stranded carrier DNA/polylethylene glycol method (28). The L40 strain harbors the reporter genes lacZ and his3, therefore cotransformants were plated on −THULL plates (lacking tryptophan, leucine, uracil, lysine, and histidine) to assay for activation of the HIS3 reporter gene, and on −UTL plates (lacking tryptophan, leucine, and uracil) to test β-galactosidase activity as described (29). Positive colonies were selected, amplified by PCR employing vector-specific primers, and sequenced. Identity of the interacting proteins was obtained via BLAST on the NCBI data base. Direct assay of the corresponding overexpressed proteins for 2ha t4 ° Ci n pull-down assays was performed by using the Aminolink resin from Pierce to which the corresponding Trx fusion protein was coupled following the manufacturer’s instructions. Characterization of the purified antibodies was performed using Western blot of brain crude membrane fractions (P21 rats) as described (23). The purified antibodies were used at a final concentration of 2 µg/ml. Preabsorption controls were performed by an overnight incubation of the antibodies with 15 µg/ml of the corresponding Trx fusion protein.

**Generation of Antibodies**

Polyclonal antibodies were raised in rabbits using the GST fusion protein containing amino acids 197–255 or amino acids 40–100 of Fe65 (antibodies Fe65-32 or -35, respectively). Antibody purification was performed by using the Aminolink resin from Pierce to which the corresponding Trx fusion proteins were coupled following the manufacturer’s instructions. Characterization of the purified antibodies was performed using Western blot of brain crude membrane fractions (P21 rats) as described (23). The purified antibodies were used at a final concentration of 2 µg/ml. Preabsorption controls were performed by an overnight incubation of the antibodies with 15 µg/ml of the corresponding Trx fusion protein.

**Light and Electron Microscopy**

Four postnatal day 35 Sprague-Dawley rats were transcardially perfused. Animals were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (6.5 mg/kg). After checking anesthetic depth, animals were perfused with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.12 M phosphate buffer, pH 7.2. After perfusion, brains were removed, fixed for an additional hour at 4 °C, rinsed in buffer, and stored overnight at 4 °C. For the detection of receptor subunits with peroxidase immunostaining a similar protocol to the one already described (23) was used. Briefly, vibratome brain slices from two animals were pre-blocked with serum, after they were incubated in Fe65 primary antibodies (for Fe65-32 and -35 sera: 1:1000; for Fe65-32 and -35 affinity purified antibodies 1 µg/ml) and processed using the avidin-biotin-peroxidase system (Vector Laboratories). Antibody binding was visualized using 3,3-diaminobenzidine tetrahydrochloride. Controls were done by omitting and preabsorption of primary antibody with the corresponding protein for Fe65-32 (20 µg/ml final concentration) and Fe65-35 (10 µg/ml final concentration). Analysis was done with an Olympus upright research microscope.

For the detection of receptor subunits with immunogold labeling, a similar protocol described to the one already described (23) was used. CA1 hippocampal regions from two animals were carefully dissected and processed for freeze substitution and low-temperature embedding. For postembedding immunocytochemistry (23), ultrathin sections (80 nm in thickness) on nickel grids were incubated in sodium borohydride and glycine Tris-buffered saline solution with Triton X-100. After the sections were pre-blocked with serum, they were incubated with affinity purified primary antibodies for Fe65 (Fe65-32, 10 µg/ml; and Fe65-35, 5 µg/ml), and labeled with colloidal gold-coupled secondary antibodies (Amersham Biosciences). The analysis of the localization of Fe65 and P2X₂ receptor subunits was performed as described (23) with double postembedding immunogold labeling using paraformaldehyde vapors between two sequential immunogold labeling procedures. Controls were carried out by omitting the primary antibody in the first and sequential immunogold labeling and preabsorption of primary antibody with the corresponding peptide conjugate for P2X₂ (50 µg/ml final concentration) or protein for Fe65-32 (20 µg/ml final concentration) and Fe65-35 (10 µg/ml final concentration). Electron micrographs were...
Molecular Complex Formed in Vivo by Fe65 and P2X\textsubscript{2} Subunits

taken at \( \times 34,300 \) magnification with a Philips 300M TEM and scanned at a resolution of 1,600 d.p.i. using an Epson Expression 1680 scanner. Image processing was performed with Adobe Photoshop using only the brightness and contrast commands to enhance gold particles.

**Two-electrode Voltage-clamp Measurements**

Rat P2X\textsubscript{2} receptor subunits were isolated by screening a rat lung library (Clontech) under low stringency conditions using the rat P2X\textsubscript{2} as a probe as described (30). P2X\textsubscript{2} cDNA subcloned in pCdnA3 and Fe65 or P2X\textsubscript{7} cDNA subcloned in pgEM vector were used to synthesize capped RNA as previously described (30). Defolliculated oocytes were injected with \( \sim 50 \) nl of cRNA per oocyte at concentrations of 25 ng/\( \mu \)l for P2X\textsubscript{2} or P2X\textsubscript{7} alone or 25 ng/\( \mu \)l for each P2X\textsubscript{2} or P2X\textsubscript{7} and Fe65 cRNA for coexpression experiments. Two electrode voltage-clamp recordings were performed 2–4 days after cRNA injection in SDs sample buffer, analyzed by SDS-PAGE electrophoresis, and probed by Western blot using P2X2 antibody (U. S. Biological Corp.), P2X7 antibody (Calbiochem), or IgG from rabbit (Sigma) in LP buffer (200 mM NaCl, 100 mM HEPES, pH 7.4, 10 mM EDTA, 100 mM NaF, 20 mM NaVO\textsubscript{4}, 2 mM phenylmethylsulfonyl fluoride, and 1.4% Triton X-100) for 3 h at 4 °C. Protein A/G PLUS-agarose was then added to the samples and the mixtures were further incubated 45 min at 4 °C. The protein-bead complexes were washed three times with LP buffer, and proteins were eluted by boiling for 5 min in SDS sample buffer, analyzed by SDS-PAGE electrophoresis, and probed by Western blot using P2X2 antibody (U. S. Biological Corp.), P2X7 antibody (Calbiochem), or the Fe65-32 antibody described above.

**RESULTS**

Identification of the Adaptor Protein Fe65 as an Interaction Partner of the P2X\textsubscript{2} Receptor Subunit—To identify proteins that interact with P2X\textsubscript{2} receptors, we screened a Y2H brain cDNA library (postnatal day 8) (27) with the cytosolic C-terminal domain of the P2X\textsubscript{2} subunit (amino acids 355–472; P2X\textsubscript{2}CD) as bait (Fig. 1A). Approximately one million clones were screened. Of them, 212 induced transcription of LexA-driven reporter genes for both histidine prototrophy and \( \beta \)-galactosidase activity. The most abundant cDNA isolated was represented by 29 clones (5 independent cDNAs; Fig. 1C) with overlapping sequences identical to the adapter protein Fe65 from the rat (31) (Fig. 1C). In the same screening seven clones were isolated (2 independent cDNAs) containing overlapping sequences that were 96% identical to the human Fe65-like 1 (Fe65L1) cDNA (32, 33), indicating that they represent its rat orthologue. The isolation of multiple overlapping clones of two homologous proteins lends additional credibility to their interaction with P2X\textsubscript{2} subunits. One of the isolated Fe65 cDNA clones, coding for amino acids 218–479 (Fe65-202), was used to confirm the interaction with the P2X\textsubscript{2}CD in the Y2H system (Fig. 1B). As evidence of specificity, no interaction was observed when instead of P2X\textsubscript{2} the C-terminal domains of P2X\textsubscript{2} or P2X\textsubscript{7} were used (data not shown). Fe65 is a brain-enriched multidomain protein containing one WW protein interaction domain and two phosphotyrosine binding/interacting domains (PTB/PID). To date, several proteins interacting with the different domains of Fe65 have been identified. Thus, the WW domain interacts with Mena, the mammalian homologue of Drosophila Enabled (34), a protein mediating cell-matrix interaction. The PTB1 domain binds to the transcription factor CF2/LSP/LPB1, to the low-density lipoprotein receptor-related protein, and to the histone acetyltransferase Tip60 (35–37). The PTB2 domain interacts with the NPTY motif located in the intracellular part of the APP (38, 39). All five Fe65 cDNAs isolated in Y2H screening contained the complete WW domain, whereas only three of them also included part of the PTB1 domain, and none the PTB2 domain (Fig. 1C). To further delimit the region of interaction, several deletions of the Fe65-202 cDNA clone were generated and cotransformed in yeast with the P2X\textsubscript{2}CD. Using this approach, we identified the WW domain of Fe65 as both necessary and sufficient for the interaction evidenced by \( \beta \)-galactosidase activity measurements (Fig. 1B). Interestingly, the growth in histidine-deficient medium was accelerated when the sequence N-terminal to the WW domain of Fe65 (amino acids 218–254) was present, indicating that this part of the protein can stabilize the complex. Constructs containing only the PTB1 domain and sequences C-terminal to the WW domain were not able to induce histidine prototrophy or \( \beta \)-galactosidase activity, arguing that this part of the sequence cannot sustain the interaction with P2X\textsubscript{2} (Fig. 1B). In a reciprocal experiment, no interaction was observed between Fe65-202 and the naturally occurring P2X\textsubscript{2} splice variant, P2X\textsubscript{2}(202), missing 69 amino acids (underlined in Fig. 1A) from the C-terminal domain (Fig. 1D), implicating alternative splicing as a regulatory mechanism of this interaction between Fe65 and the P2X\textsubscript{2} subunit.

Fe65 Binds Directly to the P2X\textsubscript{2} Subunit in Vitro and in Vivo—To confirm the interaction between P2X\textsubscript{2} and Fe65, we used affinity chromatography of GST recombinant fusion proteins on GST fusion proteins immobilized on glutathione-agarose beads (GST pull-down assays). Both glutathione-agarose beads alone or bound to GST served as negative controls for the assay (Fig. 2A). Using immobilized GST-fused Fe65-202 (GST-Fe65-202), the C-terminal domain of P2X\textsubscript{2} (P2X\textsubscript{2}CD) was detected bound to the agarose beads by Western blot with a commercial antibody specific to P2X\textsubscript{2} (Fig. 2A). No P2X\textsubscript{2} immunoreactivity was detected in the negative controls, or when the resin was incubated with the C-terminal domain of the P2X\textsubscript{2} splice variant (40, 41) (Fig. 2A). In the pull-down experiment in reverse orientation, Fe65-202 cosedimented with immobilized GST-P2X\textsubscript{2}CD (Fig. 2B). Together,
these findings demonstrate that the interaction between Fe65 and P2X2 is both specific and direct.

To determine whether P2X2 subunits associate with Fe65 in vivo, we performed coimmunoprecipitation experiments on crude rat brain membrane fractions. We found that P2X2 antibodies coimmunoprecipitate Fe65 as determined by Western blot using the Fe65-32 antibody developed in our laboratory (Fig. 3A). Fe65-202 does not interact with the C-terminal domain of the P2X7 receptor subunit in a yeast-two hybrid assay (not shown). Accordingly, Fe65 was not detected when the brain membranes were incubated with a commercially available P2X7 antibody. The co-immunoprecipitation was also performed using a rabbit IgG providing an additional control for the specificity of the assay (Fig. 3A). In Fig. 3, B and C, we show that P2X2 or P2X2 subunits were co-immunoprecipitated with their corresponding antibodies.

Fe65 and P2X2 Colocalize in Excitatory Postsynaptic Specializations of the Hippocampus—To assess where P2X2 and Fe65 colocalize in rat brain, we generated two polyclonal antibodies (Fe65-32 and Fe65-35) directed against different parts of the Fe65 sequence N-terminal to the WW domain. To evaluate the specificity of these antibodies, Western blots of crude rat brain membrane preparations (P21) were performed. Both antibodies detected a thick protein band (∼100 kDa) corresponding to Fe65 and consisting of two subbands (Fig. 4A). The same finding has been reported with different Fe65 antibodies, both in brain and transfected cells, and has been attributed to different phosphorylation states of Fe65 (35). One of the antibodies to Fe65 (Fe65-35) recognized an additional band of ∼68 kDa, which might correspond to the Fe65L2 variant (42). The Fe65-32 antibody did not recognize the 68-kDa protein, but labeled a band of ∼20 kDa, which could be because of protein degradation. No labeling was detected when the primary antibodies were preincubated with the corresponding fusion protein, indicating specificity for the epitope. We found immunoreactivity with identical patterns for both antibodies in a variety of brain areas (Fig. 4 and data not shown). In the hippocampus, pyramidal cells in the CA1 and CA3 regions, as well as granule cells of the dentate gyrus, showed immunostaining for Fe65 (Fig. 4B). In CA1 neurons, labeling extended from the soma to the distal dendrites in the stratum radiatum. Similarly, in mouse brain, immunoreactivity to Fe65 antibodies has been found widely distributed with a high expression level described for the hippocampal region (43). The subcellular localization of Fe65 in the brain has not been described so far, but immunofluorescence labeling of cultured neurons detected Fe65 in both growth cones and in dendrites (42). P2X2 subunits are present in many brain regions, including the hippocampus (23, 44), and have been localized in excitatory postsynaptic specializations of cerebellum and hippocampus (23). To determine whether Fe65

FIGURE 1. The adaptor protein Fe65 interacts with the P2X2 subunit C-terminal domain via its WW domain. A, putative membrane topology of the P2X2 subunit and amino acid sequence of its C-terminal domain used as bait in the Y2H screening. The proline-rich sequences are marked by black boxes, whereas the putative PTB binding domain is marked by a gray box in the P2X2 sequence. The amino acids not present in the P2X2(b) variant are underlined. B, Y2H analysis confirms the interaction of the P2X2 C-terminal domain with the Fe65-202 (amino acid 218–479) and showed that the WW domain of Fe65 (amino acids 255–284) is sufficient for the interaction with the P2X2 C-terminal domain. Yeast colonies growing in –UTL plates indicate transformation of both LexA- and VP16-containing vectors, whereas colonies growing in –THULL plates indicate interaction and subsequent activation of the his3 reporter gene. β-Gal shows the results of the β-galactosidase assay performed on the colonies grown on –UTL plates. C, schematic diagram of the domain structure of Fe65 is depicted for comparison with the Fe65 cDNA clones isolated from the screening (shown below). D, Fe65-202 did not interact with the C-terminal domain of the P2X2(b) splice variant, indicating that molecular determinants of the interaction are present in the missing amino acid sequence.
was present at the synapse, we performed postembedding immunogold labeling. The pattern of immunogold labeling at hippocampal excitatory CA1 synapses apposed to Schaffer collaterals was similar for both Fe65 antibodies (Fig. 5, A–G). Gold particles were present at both the presynaptic terminal (P) and dendritic spines (S). Notably, on spines, immunogold particles were preferentially located at the edge of the postsynaptic specialization (marked by arrows in Fig. 5, A–G), where P2X2 receptor subunit immunoreactivity has previously been detected (23). To determine whether Fe65 and the P2X2 subunits are indeed present at the same synapses, we performed sequential immunogold labeling using two different sizes of gold particles. The corresponding results showed that the two proteins colocalize at the postsynaptic specialization remarkably close to the edge of the postsynaptic density (Fig. 5, H–J, arrows), which argue that Fe65 could regulate synaptic receptor function. In addition, immunogold labeling corresponding to both P2X2 and Fe65 was also found, although less frequently colocalized at endoplasmic reticulum membranes (Fig. 5K, arrow).

**Fe65 Inhibits the ATP-induced Pore Dilation of P2X2 Channels**—To evaluate changes of P2X2 receptor function upon interaction with Fe65, we injected *X. laevis* oocytes with P2X2 cRNA alone (P2X2 receptors) or with cRNAs encoding P2X2 and Fe65 (P2X2-Fe65 receptors), and compared ATP-elicited receptor currents in both cases. The currents obtained for P2X2 or P2X2-Fe65 receptors showed comparable basic properties in extracellular solution containing Na⁺ as the sole permeant ion. Thus, they presented similar current amplitude (p > 0.02; Table 1), kinetics (Fig. 6A), and ATP EC50 values (Fig. 6B; 10.2 ± 0.3 and 15.3 ± 1.2 μM with Hill slopes of 1.9 ± 0.1 and 1.9 ± 0.3, for P2X2 and P2X2-Fe65, respectively (n = 3–15)).

P2X2 receptors display a time- and activation-dependent increase in the permeability to organic cations and fluorescent dyes in a subset of cells (5, 6). Measurements of fluorescence resonance energy transfer have revealed cytosolic channel motions associated with this pore dilation (10), which were prevented by immobilization of the C-terminal domain. We therefore hypothesized that Fe65 binding might restrain the P2X2 C terminus and alter the changes in ionic selectivity following agonist exposure. We measured selectivity changes as shifts in the reversal potential of currents recorded during prolonged exposure to ATP in an extracellular solution containing the organic cation NMDG⁺ as permeant ion. Indeed, these measurements showed drastic differences between P2X2 receptors and P2X2-Fe65 receptors in all of four membrane fractions were additionally processed by Western blot using the P2X2 antibody, or the Fe65-32 antibodies were used for coimmunoprecipitation (2 mg of protein). Fe65 could not be detected when the brain membrane fractions (2 mg of protein) were incubated with a P2X7 antibody, indicating the specificity of the assay. B, the P2X7 antibody-treated membrane fractions were additionally processed by Western blot using the P2X7 antibody, to show coimmunoprecipitation of the corresponding protein. Input lane contains 30 μg of brain protein. C, same as B for the P2X7 antibody. Input lane contains 10 μg of brain protein.

**FIGURE 2. Fe65 directly interacts with the P2X2 subunit C-terminal domain.** A, GST pull-down analysis shows a direct interaction between amino acids 218 and 479 of Fe65 fused to GST (Fe65-202-GST) and the P2X2 C-terminal domain (P2X2-CD). Upper panel, Fe65-202-GST was bound to glutathione-agarose beads and incubated with overexpressed P2X2 or P2X2CD-GST. Glutathione-agarose beads alone or bound to GST were used as negative control. Western blot of the bound proteins using commercial antibodies to P2X2 showed immunoreactivity for the P2X2 C-terminal domain only when Fe65-202-GST was bound to the resin. As determined by the Y2H assay, Fe65-202 did not interact with the C-terminal domain of the P2X2 splice variant. Lower blot, an antibody directed to GST was used to check the amount of GST-fused proteins bound to the beads. B, GST pull-down analysis shows a direct interaction between Fe65-202 and P2X2-CD-GST. Upper panel, the same experiment as A was also performed with P2X2-CD-GST bound to glutathione-agarose beads and incubated with overexpressed Fe65-202. Western blot of the bound proteins using the Fe65-32 antibody (Fig. 3) showed a strong signal when P2X2-CD-GST was bound to the resin. Glutathione-agarose beads alone or bound to GST used as a control also retained a detectable but small amount of Fe65-202, probably because of a direct interaction of Fe65-202 with the resin. Lower blot, an antibody directed to GST was used to check the amount of GST-fused proteins bound to the beads.

**FIGURE 3. Interaction of Fe65 with P2X2 in vivo.** A, brain crude membrane fractions were used for coimmunoprecipitation with P2X2, P2X7, rabbit IgG, or Fe65-32 antibodies (Ab). The detection was performed using the Fe65-32 antibody. A band of ~100 kDa corresponding to Fe65 was detected in the brain membrane fraction (5 μg of protein) as well as when the P2X2 or the Fe65-32 antibodies were used for coimmunoprecipitation (2 μg of protein). Fe65 could not be detected when the brain membrane fractions (2 μg of protein) were incubated with a P2X7 antibody, indicating the specificity of the assay. B, the P2X7 antibody-treated membrane fractions were additionally processed by Western blot using the P2X7 antibody, to show coimmunoprecipitation of the corresponding protein. Input lane contains 30 μg of brain protein. C, same as B for the P2X7 antibody. Input lane contains 10 μg of brain protein.
Figure 4. Immunoreactivity of Fe65 in hippocampus. A, characterization of the antibodies Fe65-32 and Fe65-35 by Western blot of brain homogenates. 5 or 10 μg of protein was loaded per lane. Both polyclonal antibodies recognize a band of ~100 kDa corresponding to Fe65. When the Western blots were performed using antibodies preincubated with the corresponding fusion protein, no signal was detected indicating the specificity of the antibodies for the epitope. B, light microscopy micrographs showing the immunohistochemical reactions for the Fe65-32 and Fe65-35 antibodies in the hippocampus. In both CA1 and CA3 regions, immunohistochemical reaction was observed extending from the cell body to the dendrites. Scale bars: 600 μm (hippocampus); 300 μm (CA1 and CA3).

Figure 5. The adaptor protein Fe65 is present in Schaffer collaterals/CA1 dendritic spine hippocampal synapses where it colocalizes with P2X2 subunits. A–G, immunogold localization of Fe65 using Fe65-32 (A–C) or Fe65-35 (D–G) antibodies. Gold particles were found both at the pre- and postsynaptic specialization of excitatory hippocampal synapses. Arrows in A–J indicate the presence of Fe65 immunogold in the outer portion of postsynaptic density, where P2X2 receptor subunits are predominantly found. The arrow in K indicates the presence of P2X2 and Fe65 at the endoplasmic reticulum. S, dendritic spine; P, presynaptic terminal. Scale bar: 200 nm.
Molecular Complex Formed in Vivo by Fe65 and P2X2 Subunits

TABLE 1
P2X2 receptors show time- and activation-dependent changes in ionic selectivity that are hindered upon coexpression with Fe65

Results obtained after heterologous expression in Xenopus oocytes. Reversal potential (Erev) was measured and P_NMDG/P_Na was calculated 1 s (I1) and 30 s (or 45) (I2) after beginning the 100 μM (or 1 mM) ATP application in an extracellular solution containing NMDG+ as the sole permeant ion. The parameters used for P2X2-injected oocytes are shown in parentheses. Irev refers to the current obtained when the extracellular solution contained Na+ as the sole permeant ion. The number of oocytes measured is represented by n.

|              | Erev mV | P_NMDG/P_Na | Erev mV | P_NMDG/P_Na | Maximal current μA | Erev mV |
|--------------|---------|-------------|---------|-------------|--------------------|---------|
|              | I1      |             | I2      |             |                    |         |
| P2X2         | −72.2 ± 0.6 (n = 47) | 0.057 ± 0.001 | −65.6 ± 0.6 (n = 34) | 0.107 ± 0.004 | −13.2 ± 0.3 (n = 41) | −6.4 ± 1.3 |
| P2X2 + Fe65  | −74.7 ± 0.5 (n = 48) | 0.052 ± 0.001 | −67.8 ± 0.6 (n = 41) | 0.068 ± 0.002 | −12.7 ± 0.4 (n = 12) | −8.2 ± 0.7 |
| P2X7         | −68.4 ± 0.6 (n = 25) | 0.065 ± 0.002 | −48.1 ± 0.9 (n = 25) | 0.141 ± 0.007 | −6.17 ± 0.9 (n = 16) | −6.9 ± 0.4 |
| P2X2 + Fe65  | −69.1 ± 0.7 (n = 22) | 0.065 ± 0.003 | −50.9 ± 1.4 (n = 25) | 0.148 ± 0.006 | −5.0 ± 0.7 (n = 25) | −5.5 ± 0.5 |

|              | Irev mV |              | Irev mV |              |                    |         |
|--------------|---------|--------------|---------|--------------|--------------------|---------|
|              | I1      |              | I2      |              |                    |         |
| P2X2         | −56.5 ± 0.6 |              | −67.8 ± 0.6 |              | −50.9 ± 1.4 |              |         |
| P2X2 + Fe65  | −65.6 ± 0.6 |              | −67.8 ± 0.6 |              | −50.9 ± 1.4 |              |         |
| P2X7         | −67.8 ± 0.6 |              | −67.8 ± 0.6 |              | −50.9 ± 1.4 |              |         |
| P2X2 + Fe65  | −67.8 ± 0.6 |              | −67.8 ± 0.6 |              | −50.9 ± 1.4 |              |         |

FIGURE 6. Fe65 does not substantially change the current kinetics or the ATP sensitivity of P2X2 receptors. A, currents evoked by 10 μM ATP on oocytes expressing P2X2 or P2X2-Fe65 receptors. No apparent changes in current kinetics were observed upon coexpression. B, concentration-response curves for ATP in oocytes expressing P2X2 (open circles) or P2X2-Fe65 (close circles) receptors. A concentration-response curve was fitted to the data using IgGPro software. Values obtained for the EC50 values were 10.2 ± 0.5 and 15.3 ± 1.2 μM for P2X2 and P2X2-Fe65, respectively. Data are mean ± S.E. of 4 to 15 experiments from 2 different batches of oocytes.

batches of oocytes. P2X2 and P2X7-Fe65 receptors presented similar current amplitudes (Table 1), ruling out differences in the expression level as a confounding factor in our analysis (45). Additionally, because of the variability in the permeability changes presented by P2X2 receptors (9) a similar number of oocytes expressing P2X2 or P2X2-Fe65 receptors were measured per batch and all obtained measurements were pooled for the analysis. Representative currents to supramaximal response curves for ATP in oocytes expressing P2X2 receptors show time- and activation-dependent changes in ionic selectivity that are hindered upon coexpression with Fe65. No apparent changes in current kinetics were described (9) changed from 0.057 (at I1) to 0.107 ± 0.004 (at I2) after beginning the 100 μM (or 1 mM) ATP application in an extracellular solution containing NMDG+ as the sole permeant ion. The parameters used for P2X2-injected oocytes are shown in parentheses. Irev refers to the current obtained when the extracellular solution contained Na+ as the sole permeant ion. The number of oocytes measured is represented by n.

(p > 0.02) were observed (Table 1). To determine the specificity of the regulation of P2X2 receptor function by Fe65, we performed the same functional experiments coexpressing Fe65 and P2X7 receptor subunits (three batches of oocytes). Similar to P2X2 receptors, P2X7 receptors undergo changes in permeability upon exposure to agonist (4, 6). However, although they present several proline-rich motifs in the C-terminal domain (18) P2X2 subunits do not interact with Fe65 as we determined by Y2H and communoprecipitation assays. By repeatedly applying ramp pulses, we observed a time-dependent shift in the reversal potential of the P2X2 receptor expressed in Xenopus oocytes. However, in contrast to what we observed for P2X2 receptors, the changes were of the same magnitude in oocytes expressing P2X2-Fe65 receptors (p > 0.02). Thus, the mean reversal potential (Erev) of the current at 1 (I1) and 45 s (I2) after the start of ATP application changed from −68.4 ± 0.6 mV (at I1) to −67.8 ± 0.6 mV (at I2, n = 47) and from −74.7 ± 0.5 mV (at I1) to −66.8 ± 0.6 mV (at I2, n = 48) for P2X7 and P2X2-Fe65 receptors, respectively (Table 1). The corresponding P_NMDG/P_Na permeability ratios calculated as previously described (9) changed from 0.057 ± 0.001 (at I1) to 0.107 ± 0.004 (at I2, n = 47) and from 0.052 ± 0.001 (at I1) to 0.068 ± 0.002 (at I2, n = 48) for P2X2 and P2X2-Fe65 receptors, respectively. The time course of the changes in Erev upon ATP application for the oocytes depicted in Fig. 7, B and D, is shown in Fig. 7E. When currents through P2X2 or P2X2-Fe65 receptors were recorded in extracellular Na+, no significant shifts in Erev were observed (Table 1). To determine the specificity of the regulation of P2X2 receptor function by Fe65, we performed the same functional experiments coexpressing Fe65 and P2X7 receptor subunits (three batches of oocytes). Similar to P2X2 receptors, P2X7 receptors undergo changes in permeability upon exposure to agonist (4, 6). However, although they present several proline-rich motifs in the C-terminal domain (18) P2X2 subunits do not interact with Fe65 as we determined by Y2H and communoprecipitation assays. By repeatedly applying ramp pulses, we observed a time-dependent shift in the reversal potential of the P2X2 receptor expressed in Xenopus oocytes. However, in contrast to what we observed for P2X2 receptors, the changes were of the same magnitude in oocytes expressing P2X2-Fe65 receptors (p > 0.02). Thus, the mean reversal potential (Erev) of the current at 1 (I1) and 45 s (I2) after the start of ATP application changed from −68.4 ± 0.6 mV (at I1) to −67.8 ± 0.6 mV (at I2, n = 25) and from −69.1 ± 0.5 mV (at I1) to −50.9 ± 0.4 mV (at I2, n = 22) for P2X2 and P2X2-Fe65 receptors, respectively (Table 1). The corresponding P_NMDG/P_Na permeability ratios changed from 0.065 ± 0.002 (at I1) to 0.141 ± 0.007 (at I2, n = 25) and from 0.065 ± 0.002 (at I1) to 0.148 ± 0.006 (at I2, n = 22) for P2X7 and P2X7-Fe65 receptors, respectively. Also, no changes were observed in current amplitude or in reversal potential when Na+ was the main permeant ion (Table 1). These data underline the importance of the C-terminal domain for the permeability changes of P2X2 receptors, and present a novel mechanism by which interacting proteins could control the function of synaptic receptors.
Molecular Complex Formed in Vivo by Fe65 and P2X2 Subunits

We have identified the adaptor protein Fe65 as a native binding partner of the P2X2 receptor subunit by Y2H screening of a rat brain cDNA library. Several lines of evidence acquired in vitro and in vivo confirm the association between the P2X2 subunit and Fe65: 1) overlapping partial cDNA sequences coding for Fe65 (Fig. 1C) and the homologous gene Fe65-like 1 were identified in the Y2H screening; 2) the interaction could be verified by co-dedimentation assays using immobilized GST-fused proteins (Fig. 2, A and B); 3) both proteins were co-immunoprecipitated from rat brain crude membrane fractions (Fig. 3A); 4) P2X2 and Fe65 present overlapping distribution in many brain areas including the hippocampus, where they co-localize at the postsynaptic specialization of excitatory synapses (Figs. 4 and 5); and 5) assembly with Fe65 regulates P2X2 receptor function (Figs. 6 and 7). In conclusion, the P2X2 subunit and the adaptor protein Fe65 interact directly and form a molecular complex with distinct functional characteristics at excitatory synapses. Fe65 is a multidomain protein containing two different types of protein-protein interaction domains: one WW domain and two PTB domains. Via its C-terminal PTB domain, Fe65 interacts with APP, increases the proteolytic processing of APP to the β-amyloid peptide (46) and together with a C-terminal fragment of APP translocate to the nucleus, where both associate with the histone acetyltransferase Tip60 to regulate transcription (37). In addition, through its interaction with the mammalian homologue of Drosophila Enabled (34), Fe65 regulates axonal growth cone motility (47). P2X2 subunits contain in their intracellular C-terminal domain a putative PTB binding domain (NKSY; Fig. 1A) similar to the interaction domain of Tip60 with the Fe65 PTB1 domain (NKLY) (37). However, as determined by Y2H deletion analysis, the PTB1 domain of Fe65 is neither sufficient nor necessary for interaction with P2X2. Instead, we could show that the association with the P2X2 subunit is mediated by the WW domain of Fe65. WW domains are short (30–40 amino acids) protein modules characterized by two tryptophan residues located 20–22 amino acids apart (48) that interact with proline-rich or proline-containing ligands. WW domain-containing proteins have been implicated in a number of cellular processes, including regulation and anchoring of membrane channels. Thus, the amiloride-sensitive epithelial sodium channel α and γ subunits bind via a PPIX motif to the WW domains of the ubiquitin protein ligases Nedd4/Nedd4-2. Disruption of this interaction leads to increased activity of epithelial sodium channel and arterial hypertension (Liddell syndrome) (49).

It is noteworthy that, although the P2X2 C-terminal domain presents several proline-containing motifs including a putative SH3 binding domain (PXXP) (48) (Fig. 1A), no additional proteins containing proline-interacting domains were isolated in the Y2H screening, which points to the specificity of the interaction between Fe65 and the P2X2 subunit. Moreover, Fe65 is not binding in vitro to the P2X2 splice variant P2X2Δ20 that is also present in the rat brain (41) (Fig. 1A), indicating that the adaptor protein might differently regulate the function of P2X2 receptors depending on their subunit composition. An analogous situation has been described for the NMDA receptor subunit NR1, where alternative splicing of the C-terminal sequence Cl regulates the association with yotiao and neurofilament 1 (24).

We describe here for the first time the subcellular localization of Fe65 using postembedding immunogold labeling at hippocampal excitatory synapses. Both antibodies used showed immunolabeling at the pre- and postsynaptic specialization. At the presynaptic terminal, Fe65 has been shown by immunofluorescence analysis to colocalize with APP (43). Interestingly for us, Fe65 and P2X2 are colocalized at the postsynaptic density as shown by double immunolabeling studies using two different sizes of gold particles. The spatial restriction found for Fe65 and P2X2 subunits agrees with our recent data showing that P2X2 subunits are not found throughout the postsynaptic specialization but rather confined to its outer portion (23). The putative protein complex around P2X2 subunit-containing receptors seems to be of a different nature than the complex network of proteins so far identified at the excitatory postsynaptic density that regulates targeting, anchoring, and coupling to intracellular signaling machinery of glutamate receptors. Thus, both the amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)- and NMDA-type ionotropic glutamate receptors present at the same synapses as the P2X2 receptor do not assemble with their interacting proteins via proline-rich sequences but rather bind via PDZ binding domains to PDZ-domain-containing adaptor proteins (24). As for many scaffolding proteins at the synapse, the modular structure of Fe65 suggests that it might link the P2X2 subunit containing receptors to additional cytosolic proteins and the cytoskeleton. It remains a challenge for future research to elucidate if previously identified proteins interacting with Fe65 are also coupled to P2X2 subunit-containing receptors. It will be of particular interest to determine whether P2X2 subunits and the β-amyloid precursor protein are present in the same protein complex.
and if this interaction has functional consequences in physiological or pathological conditions.

Upon binding of extracellular ATP, P2X$_{2}$ receptors open an intrinsic pore selectively permeable to monovalent cations and Ca$^{2+}$. With prolonged or repeated exposure to ATP, this pore progressively dilates eventually allowing the passage of large organic cations and fluorescent probes (5, 6). Here we show that P2X$_{2}$-Fe65 receptors do not change their permeability to the same extent as P2X$_{2}$ receptors, whereas other macroscopic properties including current activation, deactivation, the permeability to monovalent cations, and the sensitivity to ATP are not changed by the assembly. The effect of Fe65 on the permeability properties of P2X$_{2}$ receptors is specific because permeability changes of P2X$_{2}$ receptors (4, 6) are not affected by coexpression of Fe65 (Table 1). Two aspects of the time- and activation-dependent changes of ionic selectivity of P2X$_{2}$ receptors are of particular interest with respect to their assembly with Fe65. First, permeability changes were shown to arise from intrinsic conformational changes of the receptors involving both transmembrane segments and the C terminus (4, 5, 8–10). Fe65 could affect these conformational rearrangements and the associated changes in selectivity by tethering the P2X$_{2}$ C-terminal domain. Second, the permeability changes of P2X$_{2}$ receptors depend on the expression level, implicating an interplay between neighboring P2X$_{2}$ receptors in the process (45). The association with Fe65 might interfere with this process, e.g. if it occupies a domain of the P2X$_{2}$ subunits required for the cross-talk between receptors. In conclusion, Fe65 seems capable of regulating the ionic selectivity of neuronal P2X$_{2}$ receptors by influencing either or both of these processes, providing a novel mechanism for functional regulation of synaptic transmission.

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