

Immunomodulator CD200 Promotes Neurotrophic Activity by Interacting with and Activating the Fibroblast Growth Factor Receptor

Stanislava Pankratova · Halla Bjornsdottir ·
Claus Christensen · Lanjun Zhang · Shizhong Li ·
Oksana Dmytriyeva · Elisabeth Bock · Vladimir Berezin

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Abstract The CD200 ligand is expressed by a variety of cell types, including vascular endothelia, kidney glomeruli, some subsets of T and B cells, and neurons in the brain and periphery. In contrast, the receptor of CD200, CD200R, has a limited expression pattern and is mainly expressed by cells of myeloid origin. A recently solved crystal structure of the CD200–CD200R ectodomain complex suggests involvement of the first immunoglobulin (Ig)-like modules in ligand-receptor binding, resulting in the inhibition of myeloid cell function. In the central nervous system, CD200 has been implicated in the suppression of microglia activation. We for the first time demonstrated that CD200 can interact with and transduce signaling through activation of the fibroblast growth factor receptor (FGFR), thereby inducing neuritogenesis and promoting neuronal survival in primary neurons. CD200-induced FGFR phosphorylation was abrogated by CD200R, whereas FGF2-induced FGFR activation was inhibited by CD200. We also identified a sequence motif located in the first Ig-like module of CD200, likely representing the minimal CD200 binding site for FGFR. The FGFR binding motif overlaps with the CD200R binding site, suggesting that they can compete for CD200 binding in cells that express both receptors. We propose that CD200 in neurons functions as a ligand of FGFR.

Keywords Neurite outgrowth · CD200 · FGF receptor · Neuronal survival

Introduction

Under normal physiological conditions, the brain is an immunologically privileged site and keeps glial cells, particularly microglia, in a quiescent state. Many inhibitory factors have been implicated in the suppression of microglia activation, including the CD200 receptor (CD200R) and CD200/OX2 system [1, 2]. The CD200 ligand is widely expressed by various cell types, including vascular endothelia, kidney glomeruli, some subsets of T and B cells, and neurons in the brain and periphery [3]. CD200R is mainly expressed by cells of myeloid origin, including leukocytes and microglia [4, 5]. Both CD200 and its receptor are members of the immunoglobulin (Ig) superfamily (IgSF). The proteins contain two Ig modules and a single transmembrane region. The extracellular part of both CD200 and its receptor have similar topology. Phylogenetic analysis suggests that the proteins evolved from a common ancestral protein [4]. CD200 has a short cytoplasmic tail that is devoid of any known signaling motifs. CD200R has a considerably larger cytoplasmic tail with three conserved tyrosine residues, one of which is located within a phosphotyrosine-binding (PTB) domain recognition motif (NPxY). Upon engagement with CD200, the receptor recruits the PTB-bearing proteins Dok1 and/or Dok2, which subsequently inhibit the Ras/mitogen-activated protein kinase (MAPK) pathway, thereby inhibiting macrophage activation [6, 7].

Thus, CD200R is an inhibitory receptor. Structurally related activating receptors, CD200La, also exist and

S.P. and H.B. contributed equally to this work.

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S. Pankratova (✉) · H. Bjornsdottir · C. Christensen · L. Zhang ·
S. Li · O. Dmytriyeva · E. Bock · V. Berezin
Laboratory of Neural Plasticity, Department of Neuroscience and
Pharmacology, Panum Institute, Blegdamsvej 3, 2200 Copenhagen,
Denmark
e-mail: stasya@sund.ku.dk

provide an activating signal [8]. The X-ray structures of ectodomains of the CD200–CD200R complex have recently been reported, implicating the first Ig module of both proteins in protein–protein interactions [8].

The functional role of the interaction between CD200 and CD200R has been demonstrated by *in vivo* and *in vitro* experiments. *In vivo* studies that used CD200-deficient mice showed that the CD200–CD200R interaction constitutively inhibits myeloid function. Mice that lack CD200 exhibited an increased number of activated macrophages in the spleen and lymph nodes, and the animals were more susceptible to autoimmune disease [9]. *In vitro* studies showed that the interaction between CD200 and CD200R inhibits the activation of macrophages, mast cells, and basophils [10, 11].

In the central nervous system, CD200 has been implicated in the suppression of microglia activity. CD200 is broadly expressed by neurons [3, 12, 13], and its receptor is expressed by microglial cells and perivascular macrophages [13]. Furthermore, CD200-deficient mice exhibited a more activated microglia phenotype; they are more sensitive to neural lesions and more susceptible to experimental autoimmune encephalomyelitis, a rodent model of multiple sclerosis [9]. Conversely, in the *Wld^S* mouse strain, which is a spontaneously occurring mutant that expresses higher levels of CD200 than normal mice, the elevated neuronal expression of CD200 protects against inflammation-induced neurodegeneration [14]. Mice that lack CD200R expression exhibit an increase in the production of tumor necrosis factor α (TNF- α) in response to lipopolysaccharides (LPS) [15]. Additionally, blocking CD200R with antibody injected into the striatum has been shown to aggravate microglial activation and the degeneration of dopaminergic neurons in a rat model of Parkinson's disease [16]. The neuroinflammatory changes that result from CD200 deficiency have a negative impact on synaptic plasticity and impair long-term potentiation (LTP) [17]. Intrahippocampal infusion of soluble CD200 protein has been shown to decrease LPS-induced microglial activation in the hippocampus and ameliorate impairments in LTP [18].

To date, the only identified function of CD200 is its interaction with CD200R to activate antiinflammatory signaling in CD200R-expressing mononuclear inflammatory cells. In the present study, we for the first time demonstrate that CD200 induces neurite outgrowth and promotes neuronal survival by interacting with and activating the fibroblast growth factor receptor (FGFR). We also identified a sequence motif located in the first Ig module of CD200, likely representing the CD200 binding site for the FGFR. We propose that CD200 in neurons functions as a ligand of FGFR.

Results

CD200 Induces Neurite Outgrowth in Cultures of Primary Neurons, and This Effect Is Inhibited by the Pharmacological FGFR Inhibitor SU5402 or by Expression of Dominant-Negative FGFR

In the central nervous system, CD200 is constitutively expressed on the surface of neurons and endothelia, whereas its receptor, CD200R (which acts as an inhibitory immune receptor), is primarily expressed by microglial cells [4]. Moreover, an electron microscopy study of the rat hippocampal CA3 region showed that CD200 is selectively expressed on boutons, axons, spines, dendrites, and astrocyte processes [19]. CD200 is also expressed in immature neurons even before they migrate, suggesting its role in neuronal migration and differentiation [12]. Therefore, we investigated whether the CD200 ectodomain affects the differentiation (reflected by neurite outgrowth) of various primary neurons, namely, hippocampal neurons, cortical neurons, motor neurons, and cerebellar granule neurons (CGNs). Cells were seeded in Permanox Lab-Tek chamber slides coated by preincubation with various concentrations of recombinant CD200 protein. Untreated control cells had a basal neurite length of $7 \pm 0.5 \mu\text{m}$ (hippocampal neurons). CD200 dose-dependently induced neurite outgrowth in all of the tested neuronal cultures, with the highest efficacy in hippocampal cultures, in which maximal induction with $4.3 \mu\text{M}$ CD200 resulted in a mean neurite length of $38 \pm 6 \mu\text{m}$ (Fig. 1a, b). The specificity of CD200-induced neurite outgrowth was further confirmed by co-application with CD200 inhibitory antibodies. The CD200-coated wells were treated with increasing concentrations of anti-CD200 antibodies, and then, neurons were grown for 24 h. For controls, either the CD200 antibodies alone or BSA-coated wells were used. Obtained data showed that incubation with the anti-CD200 antibodies significantly decreased CD200-induced neurite outgrowth (Supplementary Fig. 1).

We recently showed that both hippocampal neuron and CGN cultures contain virtually no microglial cells [20]. Thus, the effect of CD200 could not be attributed to indirect effects of CD200 through CD200R, which are only expressed on microglia cells. Numerous cell-surface receptors that belong to the IgSF, including the neuronal cell adhesion molecules L1, NCAM, neuropilin, neurofascin, and necin-1, are also known to be able to induce neurite outgrowth in immature neurons by interacting with and activating FGFR [21–24]. Therefore, we explored whether the neuritogenic effect of CD200, which also belongs to the IgSF, depends on FGFR activation. Primary hippocampal neurons and CGNs, which are known to express FGFRs [25], were grown on immobilized CD200 and treated with various concentrations of the pharmacological FGFR inhibitor SU5402 for 24 h. Untreated neurons or neurons incubated with SU5402 alone

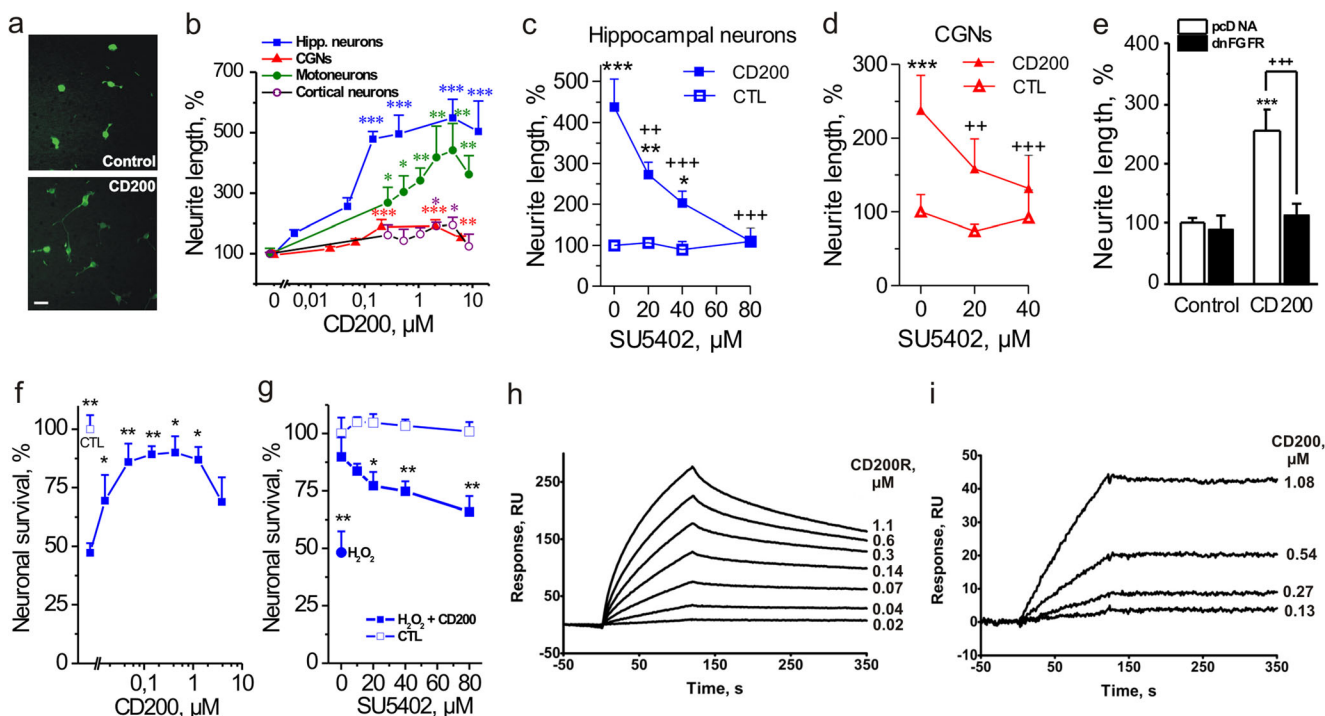


Fig. 1 Effects of CD200 on neurite outgrowth from different types of primary neurons and the inhibitory effect of SU5402 on CD200-induced neurite outgrowth. Recombinant CD200 was immobilized in culturing plates 2 h prior to seeding the neurons. The cells were cultured for 24 h and then fixed and immunostained for GAP-43. **a** Representative fluorescence micrographs of untreated (control) and CD200-stimulated hippocampal neurons (4 μM). Scale bar is 25 μm . **b** Quantification of the effect of various concentrations of CD200 on neurite outgrowth from different types of primary neurons. **c, d** Effect of the FGFR inhibitor SU5402 on CD200-induced neurite outgrowth. Wells were either uncoated (open labels) or coated with CD200 (filled labels), and then, SU5402 was added directly after seeding hippocampal neurons (**c**) or CGNs (**d**). The results are from four independent experiments and expressed as a percentage of control \pm SEM, with untreated control set to 100 %, which corresponds to $7.4 \pm 0.9 \mu\text{m}$ in **c**, and $13.7 \pm 2.6 \mu\text{m}$ in **d**. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with untreated control; ++ $p < 0.01$, +++ $p < 0.001$, compared with CD200-stimulated cells. **e** Impaired FGFR abrogates CD200-induced neurite outgrowth. Hippocampal cells were transfected with either empty vector (pcDNA) or dominant-negative FGFR-encoded vector (dnFGFR) and plated on

uncoated (control) or CD200-coated plates for 24 h. The results are from four independent experiments and expressed as a percentage of control \pm SEM, with untreated control set to 100 %. *** $p < 0.001$, compared with untreated control transfected with empty vector; +++ $p < 0.001$, compared with CD200-treated neurons transfected with empty vector. **f** CD200 protects neurons from H_2O_2 -induced death. Hippocampal neurons [7 days in vitro (DIV)] were treated with 60 μM H_2O_2 alone or in the presence of various concentrations of CD200 for 24 h. CTL, survival rate in untreated control set to 100 %. * $p < 0.05$, ** $p < 0.01$, compared with H_2O_2 -only treated cells. **g** The pharmacological FGFR inhibitor SU5402 decreased the pro-survival effect of CD200 (0.4 μM) in H_2O_2 -treated hippocampal neurons. SU5402 applied alone had no effect on survival. * $p < 0.05$, ** $p < 0.01$, compared with CD200-stimulated and SU5402-untreated cells. **h, i** Binding of CD200 to CD200R1 and FGFR evaluated by SPR analysis. **h** Serially diluted CD200R1 bound to the covalently immobilized ectodomain of CD200 on a CM5 sensor chip with $K_D = 0.1 \mu\text{M}$. **i** Representative binding curves show real-time interaction between the immobilized CD200 ectodomain and serially diluted FGFR1(IIIc). The apparent K_D of this binding was 0.014 μM

were used as controls (Fig. 1c, d). Treatment with SU5402 dose-dependently reduced CD200-induced neurite outgrowth in both neuronal culture types, suggesting that FGFR activation is necessary for the neuritogenic effect of CD200. Additionally, hippocampal neurons that were transfected with dominant-negative FGFR plasmid showed complete abrogation of CD200-induced neurite outgrowth (Fig. 1e).

CD200 Promotes the Survival of Hippocampal Neurons, and This Effect Is Inhibited by SU5402

FGFR is a known promoter of neuronal survival [26]. If it is involved in CD200-induced signaling to lead to a

neuritogenic response, then it could be that CD200 is also a neuronal survival factor. To test this hypothesis, 7-day in vitro hippocampal cultures were treated with CD200 followed by H_2O_2 (60 μM), further cultured for 24 h, stained with Hoechst, and analyzed for the number of surviving neurons. Treatment with H_2O_2 alone resulted in 50 % neuronal death (Fig. 1f). Incubation with the CD200 ectodomain partially rescued cell death at a broad range of concentrations (0.01–1 μM ; Fig. 1f), and this effect was inhibited by the FGFR inhibitor SU5402 (Fig. 1g), suggesting that the activation of FGFR is associated with the pro-survival effect of CD200.

CD200 Interacts with FGFR

It has previously been demonstrated that CD200–CD200R binding affinity for the human proteins and mouse proteins lie within K_D values of 0.5 μM [5] and 4 μM , respectively [27]. In control experiments, we verified that our constructs (CD200 and CD200R1) interact. Recombinant CD200 bound to immobilized CD200R1 with K_D value of $0.07 \pm 0.13 \mu\text{M}$ ($k_a = 2.87 \pm 0.05 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $k_d = 1.91 \pm 0.02 \times 10^{-3} \text{ s}^{-1}$). In the reverse experiment, serially diluted CD200R1 bound to immobilized CD200 with approximately the same affinity ($K_D = 0.1 \pm 0.02 \mu\text{M}$; $k_a = 1.31 \pm 0.03 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $k_d = 1.46 \pm 0.01 \times 10^{-3} \text{ s}^{-1}$; Fig. 1h).

We further investigated whether CD200 interacts with FGFRs directly using an SPR assay. The ectodomain of FGFR1 (Ig2-3) was then covalently immobilized on a sensor chip, and various concentrations of soluble CD200 were injected. CD200 bound to FGFR1 with an apparent K_D of $0.014 \pm 0.24 \mu\text{M}$ ($k_a = 4.02 \pm 0.23 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, $k_d = 5.57 \pm 0.90 \times 10^{-5} \text{ s}^{-1}$; Fig. 1i). The K_D values of the binding of CD200 to FGFR1 were somewhat lower than the binding of CD200 to CD200R1. Thus, the affinity of CD200 toward FGFR1 might be higher than its affinity toward CD200R.

CD200 Induces FGFR1 Phosphorylation, Which Is Abrogated by CD200R

We further investigated whether the interaction between CD200 and FGFR results in FGFR phosphorylation. We used T-REx-293 cells that were stably transfected with human full-length FGFR1 splice variant IIIc tagged by StrepII at its C-terminus [22]. The CD200 ectodomain applied to the cells in the nanomolar range of concentrations for 20 min increased FGFR phosphorylation compared with unstimulated cells (Fig. 2a). This activation was abrogated when the cells were treated with CD200 together with its receptor, CD200R (Fig. 2b). This indicates that the CD200 binding sites for FGFR and CD200R overlap.

CD200 Is a Partial Agonist of FGFR1

We also tested whether CD200 interferes with FGFR phosphorylation induced by one of its cognate ligands, FGF2. T-REx-293 cells were first treated with CD200 for 5 min, and 0.3 nM FGF2 was then added for 20 min. CD200 protein applied at a concentration of 20 nM, which when used alone did not affect FGFR activation, inhibited FGF2-induced receptor activation, suggesting that CD200 is a partial agonist of FGFR (Fig. 2c).

Identification of a Minimal Binding Site for FGFR Within CD200

We then localized the FGFR binding site in CD200. We found that a sequence in the first Ig module of CD200, 63-TWQKKKAVSPEN-74 (UniProtKB/Swiss-Prot accession no. P41217; termed Oxifin1), contains motifs that are homologous to the motifs of several cognate ligands of FGFR, such as FGF3, FGF4, FGF5, FGF10, FGF11, FGF12, FGF13, and FGF14 (Fig. 3a). The X-ray structure of the CD200 ectodomain in complex with inhibitory CD200R was recently solved [8]. We mapped the Oxifin1 motif to an area located within the CC' β strands and CC' loop in the first Ig module of CD200 (Fig. 3b, marked in red and cyan). Amino acid residues within the Oxifin1 motif involved in the CD200 interaction with CD200R are shown in cyan.

We designed a peptide that encompasses the Oxifin1 motif and synthesized it as a tetramer, in which four peptide chains were linked to a lysine backbone via their carboxyl-terminal ends (Fig. 3c). Like CD200, the Oxifin1 peptide directly interacted with both CD200R and FGFR1, with $K_D = 0.07$ and $0.03 \mu\text{M}$, respectively (Fig. 3d, e; Supplementary Fig. 2, Table 1). The binding affinity of Oxifin1 to FGFR1 was within the same order of magnitude as the binding affinity of CD200 to CD200R1 (Table 1). Similar to the CD200 protein, the peptide strongly stimulated FGFR1 phosphorylation in the nanomolar–micromolar range (Fig. 4), and it induced neurite outgrowth in primary neurons in an FGFR activation-dependent manner (Fig. 5a–c). The neuritogenic effect of Oxifin1 was sequence-specific, in which the peptides with scrambled sequences did not induce neurite outgrowth (Fig. 5d). Oxifin1 also displayed neuroprotective properties, and its survival-promoting effect in hippocampal neurons was inhibited by SU5402 (Fig. 5e, f). Thus, we identified a peptide that mimics the interaction between CD200 and FGFR and the biological responses that result from this interaction.

To identify the residues that are important for the neurite outgrowth-promoting effect of Oxifin1, CGNs were grown for 24 h and stimulated with 4.45 μM of the original Oxifin1 peptide or one of six truncated versions of Oxifin1 (Fig. 5g). Truncations of the Oxifin1 sequence from the N-terminal end by two residues (Thr1-Trp2) completely abrogated the peptide's effect on neurite outgrowth, despite the fact that the three lysine residues were left intact. Further truncations (Gln3-Lys4 and Lys5-Lys6) inhibited neurite outgrowth below control levels. In contrast, truncations of the Oxifin1 sequence from the C-terminal end (Glu11-Asn12, Ser9-Pro10, and Ala7-Val8) gradually and strongly increased the neuritogenic response (Fig. 5g), likely because of removal of the inhibitory effect of the Oxifin1 C-terminal part.

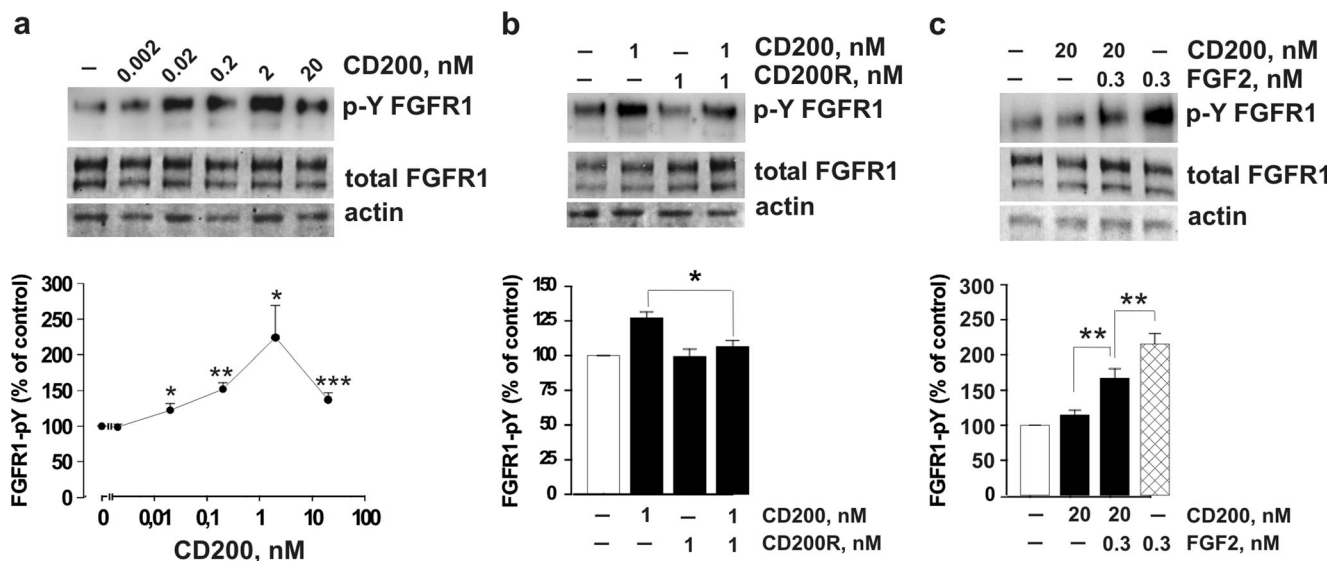


Fig. 2 Treatment with CD200 induced FGFR phosphorylation. T-Rex-293 cells that were stably transfected with StrepII-tagged FGFR1(IIIc) were treated with the CD200 ectodomain co-treated with **a** CD200 and CD200R or **b** CD200 and FGF2 at the indicated concentrations for 20 min. FGFR1c was immunopurified with anti-phosphotyrosine antibodies and analyzed by Western blot using anti-StrepII tag antibodies. **a–c Top** Representative immunoblots of phosphorylated FGFR1 (p-Y FGFR1) and total levels of StrepII-tagged FGFR1 and actin are shown. **Bottom** The results from four independent experiments

were quantified and are expressed as mean±SEM, with FGFR phosphorylation of the unstimulated control set to 100 %. **a** * p <0.05, compared with unstimulated control. **b** Cells stimulated with 1 nM of CD200 or CD200R alone or co-stimulated with two proteins for 20 min. * p <0.05, compared with CD200-stimulated cells. **c** Cells treated with either FGF2 or CD200 alone or pretreated for 5 min with FGF2, followed by the addition of CD200 for 20 min. ** p <0.01, compared with CD200- and FGF2-co-stimulated cells

Discussion

In the human brain, CD200 is robustly expressed in gray matter in the cerebral cortex, hippocampus, striatum, cerebellum, and spinal cord, and it is also detected in oligodendrocytes and astrocytes but not in microglia. CD200R is expressed in perivascular macrophages and microglia. The distribution of CD200 and its receptor suggests their involvement in neuron–glia and glia–glia interactions under normal conditions and in chronic inflammation (e.g., multiple sclerosis) [13]. Neurons constitutively express CD200, and this expression is found not only on neuronal cell bodies but also in axons and dendrites and at the synapse (e.g., boutons and spines) [19]. The function of CD200 expression on neurons has so far been linked to the inhibition of microglia activation through inhibitory CD200R under physiological conditions. However, CD200 was previously suggested to have multiple ligands [4].

Within the IgSF, a distinct group of glycoproteins is expressed both in neurons and in the immune system. This includes CD200, NCAM (CD56), Thy-1 (CD90) [28], CD47 [29], OX 47 (CD147) [30], CD166 [31, 32], and L1 [33]. Moreover, several members of this group have previously been shown to interact with and signal through FGFR (i.e., NCAM, L1) [21, 22, 34]. In the present study, we demonstrated that CD200 is a neurotogenic and survival-promoting factor for primary neurons. We found

that the effects of CD200 in neurons are specific and require FGFR activation.

FGFR is a relatively promiscuous receptor. Four genes in mammals express FGFR4 and various splice variants of FGFR1–3. The cognate ligands, 22 FGFs, display diverse binding affinity toward different FGFR isoforms [35]. Various CAMs have been demonstrated to signal through FGFR, including N-cadherin, neuroplastin, neurofascin, nectin-1, neurexin-1 β [23, 24, 36–38], NCAM, and L1. The promiscuity can be explained by the fact that some CAM modules are homologous with the FGFR-binding motif in FGFs. The interaction between several CAMs and FGFR show certain selectivity to FGFR isoforms [24]. In our experiments, CD200 bound and activated FGFR1 splice variant “c,” which is probably the major form of FGFRs that are expressed by neurons [25].

For many CAMs, signaling through FGFR occurs as a result of *trans*-homophilic/heterophilic interactions of CAMs that are expressed on opposite cells, leading to clustering of neuronal CAMs followed by FGFR dimerization and activation [36, 39]. Thus, CAM–CAM interactions usually precede FGFR activation, and CAM–FGFR interactions in *cis* are responsible for receptor activation. Because neurons do not express the cognate receptor for CD200 (CD200R) [14], *trans* interactions between CD200 and FGFR appear to induce its activation.

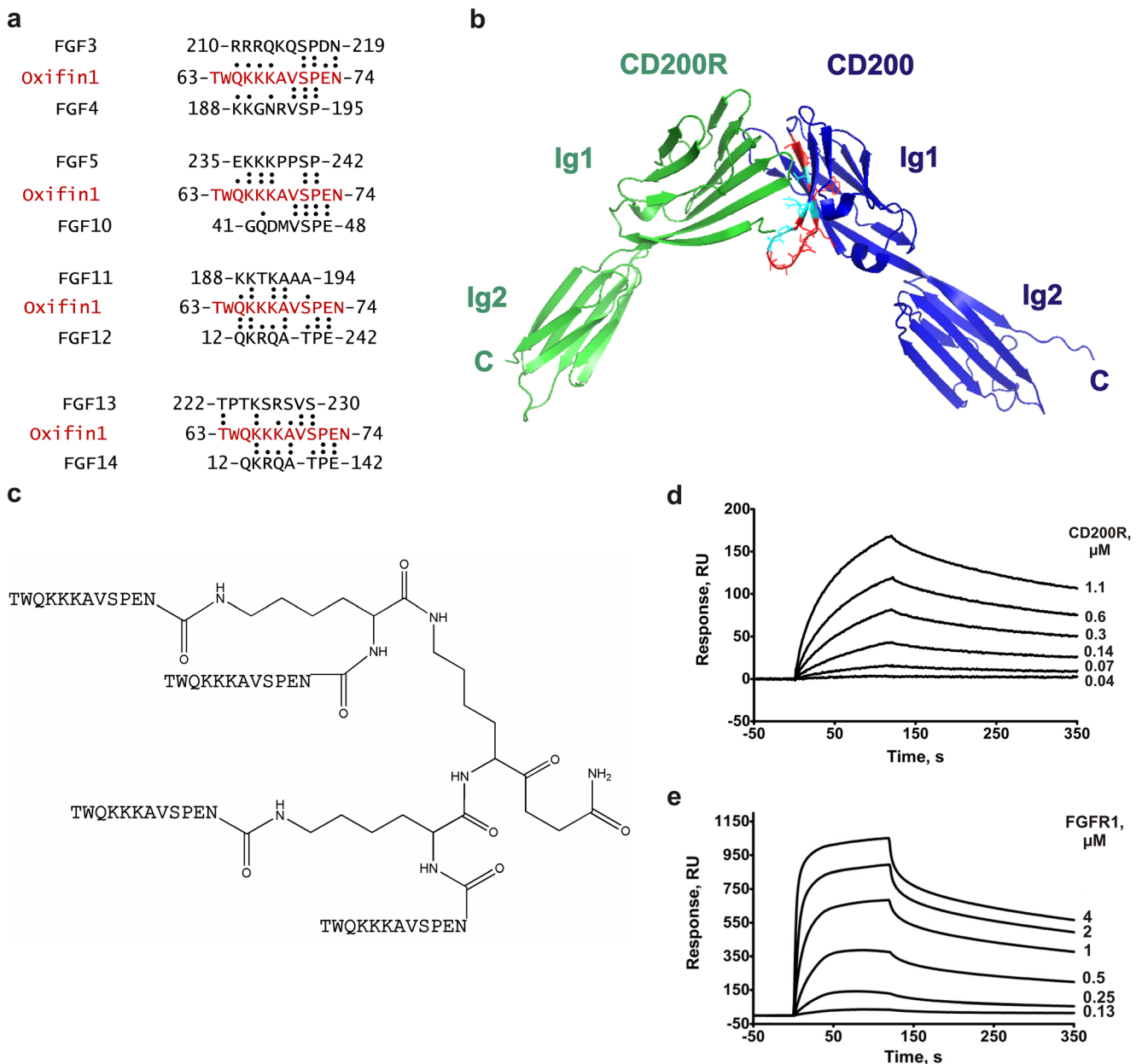


Fig. 3 Sequence motifs of Oxifin1 homologous to various FGFs and position on CD200. **a** Sequence alignment of Oxifin1 to eight different FGFs. **b** The simulated extracellular domain of CD200 consisted of two Ig modules. Oxifin1, highlighted in *cyan* and *red*, is from the C loop of the Ig1 domain. **c** Tetrameric Oxifin1 peptide scheme. **d, e** Representative

binding curves for the binding of CD200R1 (**d**) and FGFR1 (**e**) to the Oxifin1 peptide. Oxifin1 was immobilized on a CM5 sensor chip, and the serially diluted ectodomain of CD200R1 (**d**) or FGFR1 Ig2-3 (**e**) was injected

Table 1 Affinity and rate constants for interaction of CD200R and Ig2–Ig3 module of FGFR with the immobilized Oxifin 1 peptide

Injected protein	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	K_D (M)
CD200R1	$2.20 \pm 0.02 \times 10^4$	$1.60 \pm 0.01 \times 10^{-3}$	$7.27 \pm 0.07 \times 10^{-8}$
FGFR	$5.54 \pm 0.05 \times 10^4$	$1.87 \pm 0.01 \times 10^{-3}$	$3.38 \pm 0.04 \times 10^{-8}$

k_a association rate constant, k_d dissociation rate constant, K_D equilibrium dissociation constant

We also found that CD200 has a higher binding affinity for CD200R and FGFR (Fig. 1h, i). However we suggest that inhibitory CD200R can compete with FGFR for binding to CD200. Indeed, we found that CD200R abrogated FGFR phosphorylation induced by CD200 (Fig. 2b). Because CD200R is not expressed by neurons, this competition may occur on microglial cells that express both CD200R and FGFR. We also found a competitive relationship between FGF2- and CD200-induced FGFR activation, in which CD200 acted as a partial agonist of FGFR (Fig. 2c). Thus,

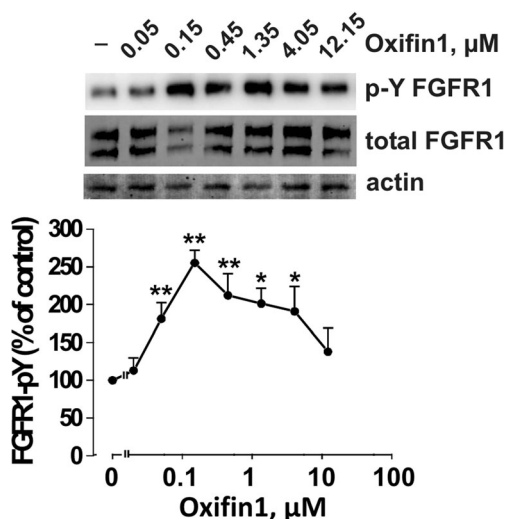


Fig. 4 Oxifin1 induces FGFR phosphorylation. T-Rex-293 cells, transfected with StrepII-tagged FGFR1(IIIc), were treated with the indicated concentrations of Oxifin1 for 20 min. Phosphorylated FGFR was then immunopurified and analyzed by Western blot. *Top* Representative immunoblots of phosphorylated FGFR (p-Y FGFR1), total FGFR1, and actin are shown. *Bottom* Quantification of FGFR1 phosphorylation from four independent experiments expressed as mean \pm SEM, with FGFR phosphorylation of the unstimulated control set to 100 %. * $p < 0.05$, ** $p < 0.01$, compared with unstimulated control

we suggest that neuronal CD200, through its interaction with FGFR, might act as an attenuator of signaling that is locally induced by various FGFs in the brain.

The shape of the dissociation curve for CD200-FGFR binding may however indicate that the FGFR1 recombinant protein contains some aggregates, and the binding is not entirely specific (Fig. 1i). However, all our cell culture results clearly indicate that interaction and activation of the FGFR is crucial for CD200 functioning as an inducer of neurite outgrowth and neuronal survival.

We identified a motif located in the CC' strand-loop-strand region of the first Ig module of CD200, Oxifin1, which mimicked the neuritogenic and survival-promoting activity of CD200. When synthesized as a tetramer, the peptide bound FGFR1 with high affinity and induced receptor phosphorylation at nanomolar concentrations. In the tertiary structure of the CD200 ectodomain, the Oxifin1 motif is part of the CD200-CD200R binding interface, which includes Thr1, Gln3, Ser9, Pro10, and Asn12, corresponding to Thr33, Gln35, Ser41, Pro42, and Asn44 of the CD200 protein (Fig. 3b) [8].

We found that the efficacy of the neuritogenic response was gradually increased with truncations from the C-terminus of the Oxifin1 peptide. Thus, the minimal sequence with the highest neuritogenic response efficacy (TWQKKK) included only two amino acids (Thr33 and Gln35, located in the second half of C' β -strand) involved in the binding of CD200R by CD200 protein (Fig. 3b, g). The three lysines in the N-terminus of the Oxifin1 motif that were not involved in the

interaction between CD200 and CD200R were crucial for the interaction between CD200 and FGFR (Fig. 5g). The mutation of Asp44 in CD200 has been shown to abrogate the binding of CD200 to CD200R [8]. However, Asp12 in Oxifin1 was not important for the neuritogenic activity of the peptide, indicating that the CD200 binding sites for CD200R and FGFR overlap but are not identical. However, there has to be warning signs when the optimal peptide is much shorter and only has a couple of residues in common with the contact residues. The key feature of this peptide is the KKK sequence. One needs to be cautious when using peptides. There is at least one quite well-studied system where peptides from thrombospondin, quite widely used, have finally been refuted recently due to nonspecific effects [40]. Nevertheless, in our truncation experiments, removal of the two residues (Thr1-Trp2) from the N-terminus completely abrogated the peptide's effect on neurite outgrowth, despite the fact that the three lysine residues were left intact, arguing against unspecific effects of the KKK sequence.

Because Oxifin1 is part of the CD200 binding site for CD200R, it represents a valuable pharmacological tool for modulating the CD200-CD200R interaction in the brain and for blocking this interaction in order to inhibit tumor progression [41].

In conclusion, we identified new functions of CD200 that are unrelated to its immunomodulatory mode of action, including the induction of neurite outgrowth and promotion of neuronal survival. We found that CD200 interacts with and activates FGFR, thereby triggering signaling pathways that mediate neuritogenic and neuroprotective responses. We also identified the minimal FGFR binding site, which is part of the CD200 binding site for CD200R and found that the corresponding peptide, Oxifin1, mimicked the effects of CD200 in neurons.

Materials and Methods

Materials

All of the peptides were synthesized as dendrimers composed of four monomers coupled to a lysine backbone using the solid-phase Fmoc protection strategy, and purity was estimated to be ≥ 80 % by high-performance liquid chromatography (Schafer-N, Copenhagen, Denmark). The Oxifin1 peptide (TWQKKKAVSPEN), its scrambled versions (QEKTWNWKPSAK and NTKSVEKPAKQW), and its truncated forms (TWQKKK, TWQKKKAV, TWQKKKAVSP, QKKKAVSPEN, KKA VSPEN, and AVSPEN) were reconstituted in sterile distilled water, and the concentrations were determined by measuring absorbance at 205 nm. The FGFR kinase inhibitor 3-(3-[2-carboxyethyl]-4-methylpyrrol-2-methylidene)-2-indolinone (SU5402) and phosphatase

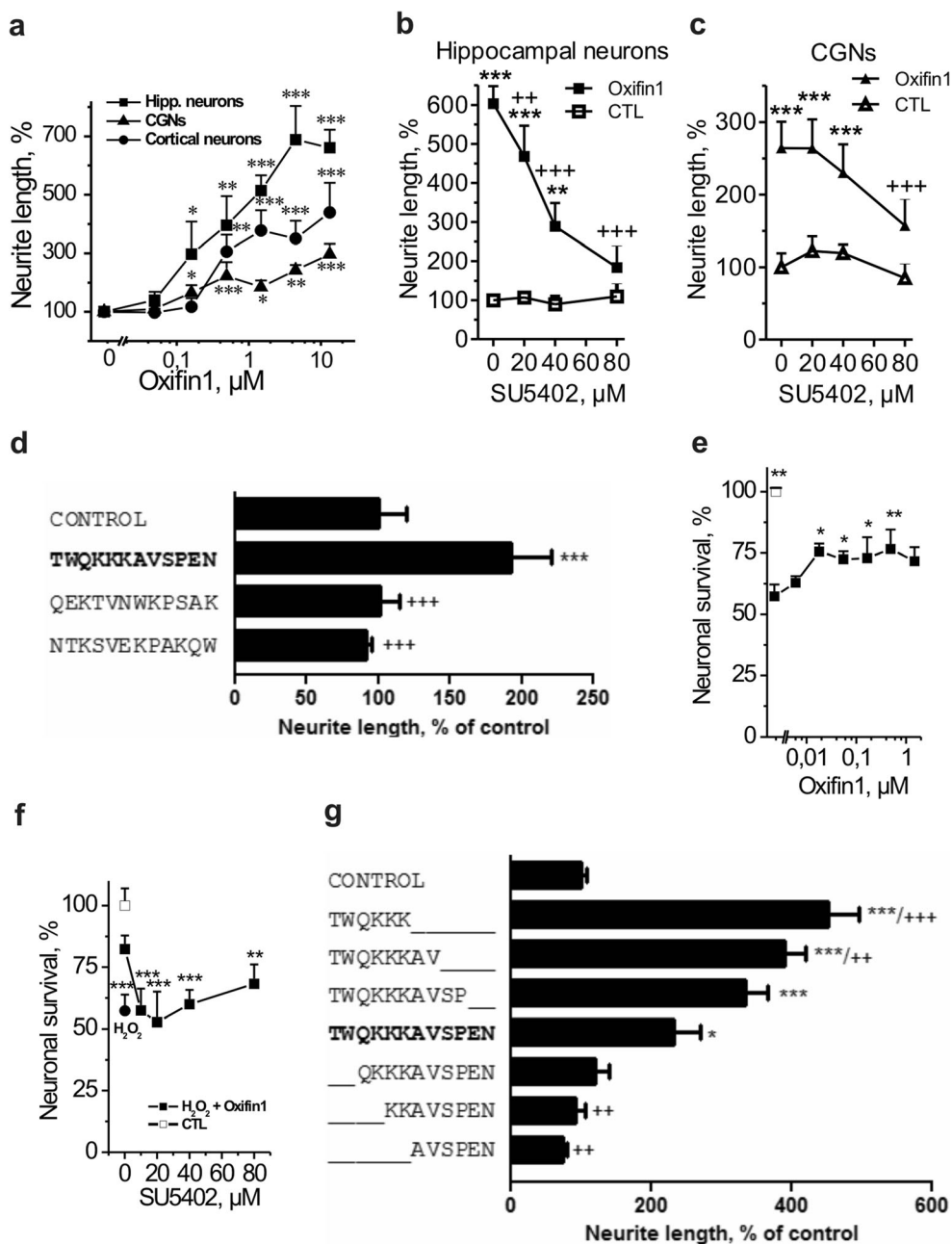


Fig. 5 The neurotrophic effect of the CD200-mimetic peptide Oxifin1 is mediated by FGFR. **a** Quantification of the effect of Oxifin1 on neurite outgrowth from different types for primary neurons. Isolated CGNs, hippocampal neurons, and cortical neurons were plated on eight-well Permax slides and treated with serially diluted Oxifin1 peptide for 24 h. **b, c** Effect of SU5402 on Oxifin1-induced neurite outgrowth. The FGFR1 inhibitor SU5402 was added directly after seeding hippocampal neurons (**b**) or CGNs (**c**). The results are from four independent experiments and expressed as a percentage of control \pm SEM, with untreated control set to 100 %. * p <0.05, ** p <0.01, *** p <0.001, compared with untreated control; ++ p <0.01, +++ p <0.001, compared with Oxifin1-stimulated control. **d** Effect of scrambled versions of Oxifin1 on neurite outgrowth. Cerebellar granule neurons were left to differentiate for 24 h in the presence of the indicated peptide (4.45 μM). The control (*top bar*, peptide-untreated cells) was set to 100 %, and treatment with Oxifin1 peptide (sequence in *bold*) induced twofold

higher neurite outgrowth. *** p <0.001, compared with untreated control. The two scrambled versions of Oxifin1 had no effect on neurite outgrowth. +++ p <0.001, compared with Oxifin1-stimulated cells. **e** Oxifin1 promoted neuronal survival in vitro. Hippocampal neurons (7 DIV) were pretreated with Oxifin1 for 1 h, and H_2O_2 (60 μM) was then added for 24 h. CTL, survival rate in untreated control set to 100 %. * p <0.05, ** p <0.01, compared with H_2O_2 -only treated cells. **f** Treatment with the pharmacological FGFR inhibitor SU5402 decreased the pro-survival effect of Oxifin1 (1.5 μM) in H_2O_2 -treated hippocampal neurons. **g** Evaluation of neuritogenic effect of successive C- and N-terminally truncated derivatives of Oxifin1 (sequence in *bold*). The results are from four independent experiments and expressed as a percentage of control \pm SEM, with untreated control set to 100 %. * p <0.05, *** p <0.001, compared with untreated control; ++ p <0.01, +++ p <0.001, compared with Oxifin1-stimulated control

inhibitor mixture set III were purchased from Calbiochem. B27 supplement, fetal calf serum, hygromycin, GlutaMax, HEPES, Neurobasal medium, and penicillin/streptomycin were obtained from Invitrogen. Trypsin, DNase 1, soybean trypsin inhibitor, bovine serum albumin (BSA), saponin, Nonidet P-40, phenylphosphate, anti-actin antibody, and Tween 20 were obtained from Sigma-Aldrich. The complete EDTA-free protease inhibitor mixture was purchased from Roche. Acetate solution (pH 5.5), ethanolamine-HCl (1-ethyl-3-[3-dimethylaminopropyl] carbodimide), and *N*-hydroxysuccinimide were obtained from GE Healthcare.

Cell Cultures

Wistar rats were obtained from either Taconic (Ejby, Denmark) or Charles River (Sulzfeld, Germany) and handled in accordance with European Union legislation. Animals were treated in accordance with the Danish Animal Welfare Act, and the study was approved by the department of Experimental Medicine at the University of Copenhagen.

Cerebellar granule neurons (CGNs) were prepared from 7-day-old pups essentially as described previously [25]. Hippocampal and cortical neurons were isolated from embryonic day 18–19 rat embryos. Briefly, the cerebella, hippocampi, or cortex were cleared of meninges and vessels and roughly homogenized by chopping in ice-cold modified Krebs–Ringer buffer with trypsin. The neurons were washed in the presence of Dnase 1 and soybean trypsin inhibitor, and cellular debris was pelleted by centrifugation. The neurons were resuspended in neurobasal medium supplemented with 2 % (v/v) B27, 1 % (v/v) GlutaMax, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 % (v/v) HEPES (1 M). Motoneurons were obtained from the ventral horns of the lumbar spinal cord of embryonic day 15 rat embryos as described previously [42].

Neurite Outgrowth Assay

The neurons were plated in eight-well Permanox Lab-Tek chamber slides (Nunc) at a density of 10,000 cells/well in Neurobasal medium. Various concentrations of peptides were added to the cultures either directly after plating the cells or 15 min later after the addition of SU5402. For coating with CD200, chamber slides were preincubated with various concentrations of the protein diluted in phosphate-buffered saline (PBS) for 2–4 h at 37 °C. For coating, control BSA (0.3 µM) was used. In the experiments with CD200 inhibitory antibodies, wells were additionally treated with increasing concentrations of anti-CD200 antibodies (0.25, 0.5, and 1 µg/ml), obtained from either Sino Biologicals (abs1, rabbit polyclonal, number 50074-RP01) or from MyBiosource (abs2, mouse monoclonal, number MBS520069). For control, wells treated with antibodies alone without CD200 were used. Then,

the slides were washed twice with PBS before cell seeding. Twenty-four hours later, the cells were fixed in 4 % v/v formaldehyde in PBS and immunostained with polyclonal rabbit anti-rat growth-associated protein (GAP)-43 antibodies (1:1000; Millipore Billerica) overnight at 4 °C, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit antibody (1:1000; Molecular Probes) for 1 h at room temperature. The evaluation of neurite outgrowth was performed as described previously [20]. To inhibit FGFR function in neurons, hippocampal neurons were transfected with either a kinase-deficient dominant-negative FGFR-encoded vector (dnFGFR) or empty vector (pcDNA3.1) as described previously [36], and then, transfected neurons were plated on either uncoated or CD200-treated wells.

Neuronal Survival Assay

Hippocampal neurons were plated at a density of 50,000 cells/well in poly-L-lysine-coated eight-well LabTek Permanox slides in Neurobasal medium and grown for 7 days. Neurobasal medium was then changed, and the cells were treated with various concentrations of recombinant CD200 or the Oxifin1 peptide for 1 h, followed by freshly diluted H₂O₂ (60 µM; Sigma). For the experiments that involved the pharmacological inhibitor, SU5402 was added 30 min before the protein or peptide. The cells were further cultured for 24 h, fixed in 4 % v/v formaldehyde, and stained with 5 µg/ml Hoechst 33258 (Invitrogen). The analysis of neuronal survival was performed as described previously [20].

Cloning, Protein Expression, and Purification

DNA fragments for cloning the ectodomains of CD200 and CD200R were generated by PCR using the primers indicated in Supplementary Table 1. The DNA fragments were cut with specific restriction enzymes before ligation in a prepared pPICZalphaC plasmid. The *Pichia pastoris* expression system (SMD1168H strain; Invitrogen, Carlsbad, CA, USA) was used for transfection of the cloned plasmids as described previously [43]. The ectodomain of mouse CD200R1 consisted of Ig modules 1 and 2 (residues 33–235, Swiss-Prot accession no. Q9ES57), and the ectodomain of mouse CD200 consisted of Ig modules 1 and 2 (residues 21–233; Swiss-Prot accession no. Q80VX2). Recombinant proteins were purified by ion exchange chromatography and gel filtration as described previously [43] and concentrated using a Centricon-10 (Millipore) centrifugal microultrafiltration tube. The purity of the protein was at least 95 % as estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel. The Ig2–Ig3 FGFR1 fragment was prepared as previously described [43].

Surface Plasmon Resonance

Real-time biomolecular interaction [surface plasmon resonance (SPR)] analysis was performed using a BIAcore 2000 instrument (BIAcore AB) at 25 °C with 10 mM sodium phosphate (pH 7.4) and 150 mM NaCl as running buffer. The recombinant human CD200Fc chimera [1477 resonance units (RU); R&D Systems], CD200RFc (994 RU; R&D Systems), FGFR1 (1221 RU), and Oxifin1 (558 RU) were immobilized on a CM5 chip activated by an activation solution, followed by the injection of protein or peptide in 10 mM sodium acetate, pH 5.0, at a flow rate of 5 μ l/min. Flow cells were inactivated by injecting ethanolamine–HCl. Binding between immobilized components and analytes at the reported concentrations was measured at 25 °C in running buffer at a flow rate of 10–30 μ l/min. For the analysis, the curves of the binding to a blank chip were subtracted from the protein/peptide binding curves and further referenced by subtracting corresponding control curves obtained by injecting running buffer alone. Sensograms were then analyzed by nonlinear curve fitting using BIAevaluation 4.1 software (BIAcore). Curves were fitted to a 1:1 Langmuir interaction model, and the equilibrium constant (K_D) was calculated as k_d/k_a , where k_a and k_d are the association and dissociation rate constants, respectively.

FGFR Phosphorylation Assay

TREX-293 cells (Invitrogen) were stably transfected with human full-length FGFR1, splice variant IIIc, with a C-terminal Strep II tag (IBA Biotec). The cells were maintained in Dulbecco's modified Eagle medium with 200 μ g/ml hygromycin, 10 % fetal calf serum, 1 % (v/v) Glutamax, 100 U/ml penicillin, and 100 μ g/ml streptomycin. To determine the phosphorylation of FGFR1c, 2×10^6 cells were first starved overnight in medium without serum. The cells were then treated with the protein or peptide at various concentrations for 20 min and subsequently lysed in 300 μ l lysis buffer [1 % (v/v) NP-40, 2 % (v/v) complete EDTA-free protease inhibitor mixture, and 1 % (v/v) phosphatase inhibitor mixture set III in PBS]. FGF2 (0.3 nM) was used in each experiment as a positive control. The protein concentration was determined using the bicinchoninic acid assay (Pierce), and 500 μ g protein from each lysate was incubated with 15 μ l agarose-coupled anti-phosphotyrosine antibodies (4G10-AC; Upstate Biotechnologies) for 6 h at 4 °C. The bound protein was washed, eluted with 180 mM phenylphosphate, separated by SDS-PAGE, and transferred to a polyvinylidene fluoride membrane (Millipore). Immunoblotting was performed using rabbit anti-Strep II tag antibodies (1:1000; IBA Biotec) and swine anti-rabbit IgG horseradish peroxidase conjugate (diluted 1:2000; DakoCytomation) in PBS with 5 % (w/v) non-fat milk and 0.1 % (v/v) Tween 20. The immune complexes

were developed by SuperSignal West Dura extended-duration substrate (Pierce) and visualized and quantified using SynGene Gene Tool image analysis software (Synoptics, Cambridge, UK). Four independent experiments were performed.

For the analysis of total FGFR expression, 30 μ g protein from each original lysate was separated by SDS-PAGE and then analyzed by Western blot using antibodies against the StrepII tag. The amount of β -actin (loading control) was visualized using rabbit polyclonal anti-actin antibody (1:1000; Sigma).

Statistics

The data were analyzed using Student's *t* test or one-way repeated-measures analysis of variance (ANOVA) followed by the Newman–Keuls (neuritogenesis) or Dunnett (survival) post hoc test using Prism 4.02 (GraphPad, San Diego, CA, USA). Graphs were plotted with either Prism 4.02 or OriginPro 9.0 (OriginLab, Northampton, MA, USA). The results are expressed as mean \pm SEM. Values of $p < 0.05$ were considered significant.

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