The Role of ATP in the Regulation of NCAM Function

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Introduction

The neural cell adhesion molecule (NCAM) is a membrane glycoprotein that mediates both cell-cell and cell-matrix adhesion through homophilic and heterophilic interactions. It is widely expressed in the nervous system, both during development and in the adult organism. In addition to mediating cell adhesion, NCAM extracellular binding can initiate signaling processes and thereby affect diverse biological processes such as axonal growth and fasciculation, cell migration, and synaptic plasticity (reviewed in [1, 2]). Early studies showed that activation of the fibroblast growth factor receptor (FGFR) is necessary for the neuritogenic signaling of NCAM. FGFR antibodies, as well as a peptide derived from FGFR can inhibit NCAM-induced neurite outgrowth [3]. It has also been shown that stimulating the PC12 neuronal cell line with soluble NCAM-Fc chimeras leads to an increased FGFR phosphorylation, and cerebellar neurons expressing a dominant negative form of FGFR are unable to respond to NCAM stimulation [4]. More recently, a direct interaction between NCAM and FGFR has been demonstrated and an NCAM-derived peptide corresponding to the FGFR binding site of NCAM also promotes the activation of FGFR [5].

NCAM belongs to the immunoglobulin superfamily of cell adhesion molecules, characterized by the immunoglobulin (Ig) module. Alternative splicing and post-translational modifications lead to the generation of a number of distinct NCAM forms, all arising from a single gene. Three main isoforms, NCAM-120, NCAM-140 and NCAM-180 that have been named after their apparent molecular weight, are produced by alternative splicing. NCAM-140 and NCAM-180 are single span transmembrane proteins while NCAM-120 is attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. For all three main isoforms, the extra-

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cellular part of NCAM consists of five N-terminal Ig modules followed by two fibronectin type III (F3) modules. The second (membrane proximal) F3 module contains the sequence AENQQGKS, homologous to the Walker motif A (G/AXXXXGKT/S, where X denotes any amino acid), which is a common nucleotide binding motif [6].

ATP as a Signaling Molecule in the Nervous System

ATP acts as an extracellular signaling molecule both in the central and the peripheral nervous system. It is often released from neural cells as a co-transmitter together with other transmitters such as acetylcholine, noradrenaline, GABA or glutamate [7–10], but can also be released alone [11]. In addition, glial cells have been shown to release ATP by channel-mediated mechanisms [12, 13]. Once released, extracellular ATP is rapidly degraded by a number of ecto-nucleotidases. Ecto-nucleotidases constitute a heterogeneous group of nucleotide-hydrolyzing enzymes that are generally membrane bound, but some ecto-nucleotidases may be cleaved and released extracellularly giving rise to soluble forms [14]. Extracellular ATP is initially converted by ecto-nucleotidases to ADP or AMP, which subsequently is further hydrolyzed to adenosine that is finally cleared from the extracellular space by cellular reuptake. The ecto-nucleotidase activity in rat hippocampus is found to be regulated in different learning paradigms, and has thus been suggested to play an active role in memory formation [15–17].

Extracellular ATP activates purinergic receptors, also called purinoceptors, which are classified as P1 receptors or P2 receptors based on their ligand specificity. P1 receptors are activated by the nucleoside adenosine and P2 receptors by ATP, ADP, UTP, UDP or nucleotide sugars [18]. The P2 receptors are further subdivided in two types, namely P2X receptors that are ligand gated ion channels and P2Y receptors that are G-protein coupled receptors. By activation of P2X receptors, ATP causes postsynaptic influx of Ca^{2+} and Na^{+}, thus acting as a fast excitatory neurotransmitter. In addition to mediating fast neurotransmission, ATP can induce long term structural and functional changes in the nervous system. Neural stem cell proliferation can be stimulated by ATP [19, 20], and ATP enhances neurite outgrowth from PC12 cells, neuroblastoma cells and hippocampal neurons [21–23], but has also been shown to inhibit motor axon outgrowth from rat neural tube explant cultures [24]. In the hippocampus, ATP has further been implicated in both long-term potentiation (LTP) and long-term depression (LTD) [25–27], two principal forms of synaptic plasticity that are considered to be essential in learning and memory.

In addition to activating purinoceptors, extracellular ATP is the substrate of ecto-protein kinases. Inhibiting extracellular protein kinase activity has been shown to block neurite outgrowth from PC12 cells, to inhibit synapse formation between cerebral cortical neurons and to block ATP-induced LTP in guinea-pig hippocampal slices [28–30]. NCAM antibodies inhibit phosphorylation of specific proteins on

the external surface of NG108-15 cells, a neuronal cell line [31]. This implies that the extracellular part of NCAM is a direct target of ecto-protein kinases, but the functional significance of this phosphorylation remains unclear.

NCAM ecto-ATPase Activity

Ecto-nucleotidases are characterized by being activated by high concentrations of either Ca^{2+} or Mg^{2+}. A major fraction of the (Ca^{2+}–Mg^{2+})-dependent ATPase activity of rat brain microsomes can be solubilized with the detergent CHAPS, retaining 85% of the initial enzyme activity. Similarly, the majority of microsomal NCAM is solubilized by this procedure, and immunoisolation of the solubilized NCAM leads to co-isolation of ATPase activity, indicating that NCAM either possesses intrinsic ATPase activity or is tightly associated with an ATPase [32]. Contrary to these findings, the majority of rat brain microsomal Mg^{2+}-ATPase activity does not coelute with NCAM when purified by anion exchange chromatography [33]. Furthermore, an anti-peptide antibody targeting a sequence in the 67 kDa rabbit skeletal muscle transverse tubule ecto-Mg^{2+}-ATPase detects a band of ~67 kDa in immunoblots of solubilized rat brain microsomes, and this band appears in the same anion exchange chromatography fractions at the peak Mg^{2+}-ATPase activity, demonstrating that NCAM is not the major ecto-ATPase in the brain [33].

Further experiments studying the association of a minor fraction of total brain ecto-ATPase activity with NCAM were performed using fibroblasts transfected with either the transmembrane NCAM-140 isoform or the lipid-anchored NCAM-120 isoform. In both cases, immunoisolation of NCAM led to co-isolation of ATPase activity [34]. These findings indicated that the ATPase activity was associated with the extracellular part of NCAM, which was further verified by experiments examining soluble forms of NCAM released from rat brain synaptosomes by either spontaneous proteolytic cleavage or by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC). In these experiments, NCAM spontaneously released from synaptosomes by proteolysis co-eluted with the major peak of ATPase activity when subjected to three different chromatographic procedures. Analysis of the ATPase activity released from synaptosomes by PI-PLC showed that almost 90% of this activity could be removed from the supernatant by NCAM immunoadsorption. Covalent modification of ATP-binding sites on synaptosomes with the ATP analog 5′-fluorosulfonylbenzoyl adenosine (FSBA) followed by NCAM immunoisolation inhibited the NCAM immunoisolated ATPase activity, and subsequent immunoblotting showed FSBA-modification of all three major NCAM isoforms [34]. In conclusion, these data indicate that NCAM has an intrinsic ATPase activity, located in the extracellular part of the protein. The functional significance of this NCAM ecto-ATPase activity is not well understood. Obviously, it contributes to lowering the ATP concentration in the extracellular compartment, which is a prerequisite for ATP functioning as an extracellular signaling molecule, but the rate of ATP hydrolysis catalyzed by NCAM is relatively low, suggesting that

ATP removal by NCAM might not be physiologically important. The enzymatic activity might also cause cyclic conformational changes in NCAM that affect other aspects of NCAM function. Both heterophilic and homophilic NCAM interactions could be influenced by such conformational changes, possibly resulting in activity-dependent modifications of NCAM-mediated adhesion and signaling processes.

NCAM–FGFR Interaction and ATP

The FGFR consists of an extracellular region containing three Ig modules, a single transmembrane helix and an intracellular tyrosine kinase domain. A recombinant protein consisting of FGFR Ig modules 2 and 3 has been shown to interact directly with NCAM F3 modules 1 and 2 by surface plasmon resonance (SPR) [5]. Using nuclear magnetic resonance (NMR), the structure of the NCAM F3 module 2 was determined and by ^{15}N-labeling this module, perturbation of specific amino acid residues could be detected when adding FGFR Ig module 3. The perturbed residues were N^{688}, Q^{690}, G^{691} and K^{692}, indicating that these residues either take part in, or are located in the vicinity of the binding site between these two modules [5]. The perturbed residues overlap with the Walker motif A in NCAM, making it plausible that ATP could interfere with the NCAM-FGFR interaction. Indeed, addition of ATP completely abolished the above described binding of NCAM F3 module 1 and 2 to FGFR Ig module, as observed by SPR [5].

In the presence of ATP, perturbation of two residues in the NCAM F3 module 2, Y^{683} and V^{684}, was also detected by NMR, verifying the suggested direct binding of ATP to NCAM [5]. In a binding model proposed by Kiselyov et al. [5], the side chain of Y^{683} interacts hydrophobically with the adenosine moiety of ATPwhile the K^{692} of the Walker motif A together with the adjacent K^{694} form ionic bonds with the triphosphate moiety of ATP (Fig. 1). Further evidence that the NCAM extracellular binding sites for ATP and for FGFR overlap came from SPR experiments using a 15 amino acid peptide derived from the NCAM sequence comprising the Walker motif A. The peptide, E^{681}-A^{695}, corresponds to the loop between the F and G β strands of the NCAM F3 module 2 and is thus termed the FG loop peptide. A dendrimeric form of the FG loop peptide was shown to directly bind immobilized FGFR Ig modules 2 and 3, and addition of ATP inhibited the binding by 70%, showing that ATP interferes with NCAM–FGFR interaction by binding to the same site on NCAM as FGFR [5].

ATP and NCAM Ectodomain Shedding

It has long been known that NCAM exists both as membrane bound and soluble protein. Several NCAM forms have been detected as soluble proteins in brain, cerebrospinal fluid (CSF), plasma, and in conditioned medium of cultured neurons

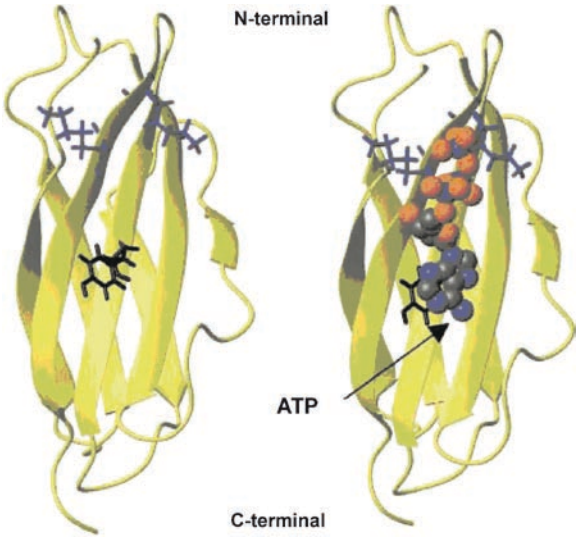

Fig. 1 Ribbon representation of NCAM F3 module 2 illustrating the ATP binding model proposed by Kiselyov et al. [5]. K^{692} and K^{694} are colored blue, Y^{683} is colored black. On the right, the module is shown in complex with ATP

[35–37]. An indication that soluble NCAM plays an important role in development came from a study by Rabinowitz et al. [38]. In this study, all membrane-associated forms of NCAM were replaced with a soluble, secreted form consisting of the extracellular region of NCAM. This mutation resulted in dominant embryonic lethality with abnormalities in neural tube and somite formation apparent from embryonic day 8.5. The same lethal phenotype was observed in embryos derived from embryonic stem cells homozygous for the targeted NCAM mutation, demonstrating that the phenotype is not caused by a homophilic NCAM interaction with endogenous membrane bound NCAM. A number of heterophilic binding partners of NCAM have been identified including the closely related adhesion molecule L1 [39], the glial cell line-derived neurotrophic factor (GDNF) [40], FGFR [5] and extracellular matrix components such as heparin and heparan sulfate proteoglycans [41, 42]. Soluble NCAM interacting with one or more of these binding partners might have a profound impact on essential cell adhesion and/or signaling processes, thus affecting both development and function of the mature nervous system.

Abnormal levels of soluble NCAM have been linked to neuropsychiatric diseases including recurrent unipolar major depression, bipolar disorder and schizophrenia [43, 44]. In schizophrenic patients, increased levels of soluble NCAM have been detected in hippocampus, prefrontal cortex and in CSF [45–48]. A key morphological characteristic of schizophrenia is ventricular enlargement [49] and this characteristic was found to be positively correlated with soluble NCAM levels in CSF [48]. To study the effects of soluble NCAM overproduction in the brain, transgenic mice have been generated that express the soluble extracellular region of

NCAM under control of the neuron-specific enolase promoter [50]. This promoter is inactive until late in neuronal differentiation, thus circumventing the embryonic malformations observed by Rabinowitz et al. [38]. These transgenic mice show a reduced synaptic connectivity of GABAergic interneurons and behavioral deficits similar to those observed in other rodent schizophrenia models [50]. Aberrant GABAergic neurotransmission has been implicated in schizophrenia by both animal models and clinical studies (reviewed in [51, 52]), and it is tempting to speculate that deregulation of NCAM shedding plays a causative role in schizophrenia.

Several mechanisms for generating soluble NCAM have been described, including the secretion of a truncated NCAM protein generated by alternative splicing [53], cleavage of the NCAM-120 GPI anchor [37] and extracellular proteolytic cleavage of membrane bound NCAM by the tissue plasminogen activator-plasmin system [54] or by metalloproteinases [55–57]. Metalloproteinase-mediated NCAM ectodomain release can be induced by ATP, both in primary rat hippocampal neurons and in fibroblastoid L-cells stably transfected with any of the three main NCAM isoforms. ATP-induced NCAM shedding occurs even in L-cells transfected with a mutated NCAM construct in which the three residues (Y^{683}, K^{692} and K^{694} in NCAM F3 module 2) implicated in ATP binding are substituted with alanine [56]. This indicates that the observed shedding does not involve direct ATP-NCAM binding. In contrast to the ATP-induced NCAM shedding observed in cultures of L-cells and hippocampal neurons, spontaneous release of NCAM from rat brain synaptosomes was shown to be inhibited by ATP [34]. However, spontaneous NCAM release from synaptosomes was observed in an EDTA-containing buffer, making it likely that the involved release mechanism is metalloproteinase independent as metalloproteinases are inhibited by EDTA.

NCAM ectodomain shedding has not only been studied in relation to mental and neurological disorders. Cell migration is known to be affected by NCAM expression in several neuronal cell types, and metalloproteinase-dependent shedding of NCAM-140 has been shown to promote cell migration toward extracellular matrix proteins [55]. NCAM-dependent neurite outgrowth can be both promoted and obstructed by NCAM shedding, depending on experimental conditions [56–58]. The significance of NCAM shedding in the regulation of neural plasticity has also been studied in vivo. After induction of LTP in the dentate gyrus of anesthetized rats, a significant increase in soluble NCAM concentration has been detected in hippocampal perfusates [59]. ATP is released after LTP induction in rat hippocampal slices [60] and ATP-induced NCAM shedding could be one of the mechanisms that facilitate the structural remodeling taking place after LTP induction.

ATP and NCAM-Mediated Neurite Outgrowth

The wiring of the nervous system during embryonic development is a highly coordinated process that requires accurate guidance of migrating cells and extending axons. Cell adhesion molecules, including NCAM, have been shown to play an

essential role in these events (reviewed in [61–63]). A key feature of neuronal differentiation is neurite sprouting. Upon homophilic NCAM binding, intracellular signaling is triggered that ultimately leads to neurite extension. This has been shown for a number of neuronal cell types co-cultured with fibroblasts genetically engineered to express NCAM. In this model system, the neuronal cells grown in co-culture with fibroblasts expressing NCAM exhibit significantly enhanced neurite outgrowth in comparison with neuronal cells grown in co-culture with fibroblasts that do not express NCAM. Addition of ATP in the co-culture medium results in a dose-dependent reduction of NCAM-induced neurite outgrowth to the level of control neurite outgrowth (Fig. 2). The inhibitory effect of ATP can be abrogated by adding the ATP-hydrolyzing enzyme apyrase together with ATP. Interestingly, the non-hydrolysable ATP analog AMP-PCP also inhibits NCAM-induced neurite outgrowth with a dose response similar to ATP [23]. These results indicate that ATP itself, not breakdown products hereof, affects NCAM-induced neurite outgrowth, and that ATP hydrolysis is not necessary for the observed inhibition.

As discussed above, NCAM binds directly to and signals through FGFR. The neuritogenic effect of NCAM can be mimicked by adding soluble NCAM F3 module 2, which contains the FGFR binding site, to hippocampal neurons in culture

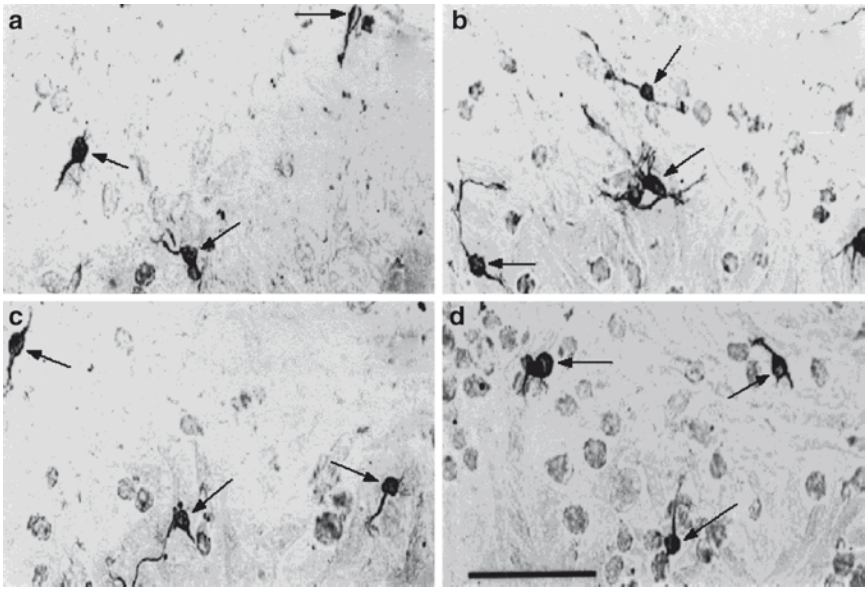

Fig. 2 ATP blocks NCAM-induced neurite outgrowth. Rat hippocampal neurons (indicated by *arrows*) grown on a monolayer of control fibroblasts (**a**, **c**) or fibroblasts expressing NCAM (**b**, **d**), immunostained for GAP-43. NCAM promotes neuritogenesis in the absence (**a**, **b**) but not in the presence (**c**, **d**) of 2.5 mM ATP. Scale bar = 50 μm. From Skladchikova et al. [23]

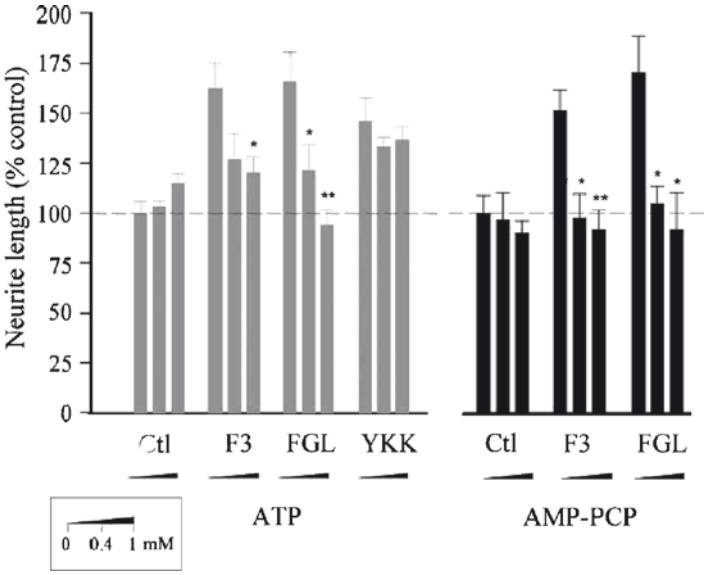

Fig. 3 Effects of ATP and AMP-PCP on NCAM-specific neurite outgrowth. Rat hippocampal neurons were stimulated with either recombinant NCAM F3 module 2 (F3), FG loop peptide (FGL), or FG loop peptide with alanine substitutions at Y^{683}, K^{692} and K^{694} (YKK). After 24 h stimulation in the presence or absence of ATP/AMP-PCP, neurons were fixed and stained in order to quantify neurite outgrowth. ATP dose dependently inhibits the neuritogenic effect of NCAM F3 module 2, and of the FG loop peptide. In contrast, ATP has no inhibitory effect on neurite outgrowth induced by the FG loop peptide having alanine substitutions at the residues involved in ATP binding. *$p<0.05$, ** $p<0.01$. From Kiselyov et al. [5]

(Fig. 3). The neurite outgrowth induced by the F3 module 2 can be inhibited by FGFR antibodies, by ATP, and by AMP-PCP [5]. Similarly, the FG loop peptide also induces neurite outgrowth from hippocampal neurons and this effect can also be inhibited by ATP and AMP-PCP. When the three residues in the FG loop peptide implicated in ATP binding (Y^{683}, K^{692} and K^{694}) are substituted with alanine, the peptide retains neuritogenic potential, but the inhibitory effect of ATP can no longer be detected (Fig. 3). Taken together, these results suggest that ATP inhibits NCAM-induced neuritogenesis by interfering with the direct NCAM-FGFR interaction.

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