Cell adhesion and intracellular calcium signaling in neurons

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Abstract
Cell adhesion molecules (CAMs) play indispensable roles in the developing and mature brain by regulating neuronal migration and differentiation, neurite outgrowth, axonal fasciculation, synapse formation and synaptic plasticity. CAM-mediated changes in neuronal behavior depend on a number of intracellular signaling cascades including changes in various second messengers, among which CAM-dependent changes in intracellular Ca^{2+} levels play a prominent role. Ca^{2+} is an essential secondary intracellular signaling molecule that regulates fundamental cellular functions in various cell types, including neurons. We present a systematic review of the studies reporting changes in intracellular Ca^{2+} levels in response to activation of the immunoglobulin superfamily CAMs, cadherins and integrins in neurons. We also analyze current experimental evidence on the Ca^{2+} sources and channels involved in intracellular Ca^{2+} increases mediated by CAMs of these families, and systematically review the role of the voltage-dependent Ca^{2+} channels (VDCCs) in neurite outgrowth induced by activation of these CAMs. Molecular mechanisms linking CAMs to VDCCs and intracellular Ca^{2+} stores in neurons are discussed.

Keywords: Cell adhesion molecule, Neurons, Calcium, Voltage dependent Ca^{2+} channel, Neurite outgrowth

Review
Cell adhesion molecules (CAMs) expressed on the neuronal cell surface play crucial roles in neuronal migration, axonal fasciculation, and neurite outgrowth during brain development. They also play an important role in regulation of synaptic plasticity in adult brain and axonal regeneration in injured nervous system [1-6].

Functions of CAMs are induced in response to their binding to ligands presented either in the soluble form or on membranes of other cells or on artificial surfaces, a process which is often called CAM activation. CAM activation induces a number of intracellular signaling cascades, which are essential for CAM-mediated functions (for extensive review see [5,7-11]). Among signaling cascades activated by CAMs, changes in intracellular Ca^{2+} levels have been documented to occur in neurons in response to activation of virtually all families of CAMs, including the three main families comprising the immunoglobulin superfamily (IgSF), cadherins and integrins.

Intracellular Ca^{2+} serves as a secondary signaling messenger with Ca^{2+} channels at the neuronal cell surface and internal Ca^{2+} stores regulating intracellular Ca^{2+} concentrations in neurons and other cells. Intracellular Ca^{2+} has critical roles in all aspects of neuronal development including neurite elongation and neuronal growth cone motility in developing neurons [12-17].

Intracellular signaling cascades activated by CAMs in response to ligand binding induce a number of physiologically important responses in neurons, among which changes in neurite outgrowth are probably the best characterized for CAMs of different families [5,7,11,18]. In this review, we systematically analyze studies reporting changes in intracellular Ca^{2+} levels in response to activation of IgSF CAMs, cadherins and integrins in neurons. We also analyze the experimental evidence supporting the involvement of the cell surface Ca^{2+} channels and intracellular Ca^{2+} stores in intracellular Ca^{2+} changes induced by CAMs of these families, and review the data showing the effects of Ca^{2+} channel inhibitors on CAM-induced neurite outgrowth.

Changes in intracellular Ca^{2+} levels induced by activation of CAMs of the immunoglobulin superfamily (IgSF)
CAM-induced increases in intracellular Ca^{2+} levels were first documented in response to activation of the neural
cell adhesion molecule (NCAM) and L1, both members of the IgSF (Table 1). NCAM mediates homophilic adhesion, i.e. extracellular domains of NCAM molecules on cell surface membranes of adjacent cells bind to each other. It functions in the developing nervous system by regulating neuronal migration and differentiation and also plays an important role in adult brain by regulating memory formation and brain plasticity [19,20]. Application of purified NCAM, which binds to NCAM at the cell surface, induced an increase in intracellular Ca$^{2+}$ levels in small cerebellar neurons [21]. Similar results were obtained with artificial ligands of NCAM, such as peptide ligands of NCAM, which bind to the extracellular domain of NCAM and which have been shown to induce an increase in intracellular Ca$^{2+}$ levels in PC12 cells and rat hippocampal neurons loaded with a Ca$^{2+}$ indicator Fura-2 acetoxy-methyl ester (AM) [22,23]. The effects of antibodies against the extracellular domain of NCAM used as an artificial NCAM ligand were analyzed in several studies and have been shown to increase intracellular Ca$^{2+}$ levels in PC12 cells and in small cerebellar neurons as measured using fluorimetry and a Ca$^{2+}$ indicator Quin-2AM [24,25]. In another study, polyclonal but not monoclonal antibodies against the extracellular domain of NCAM have been shown to induce an increase in intracellular Ca$^{2+}$ levels in dorsal root ganglion neurons but not in small cerebellar neurons loaded with Fura-2AM [21], suggesting that the type of the antibodies used can influence the effects of the antibody on intracellular Ca$^{2+}$ levels.

CAMs of the L1 family, including L1, close homolog of L1 (CHL1), neurofascin and Neuron-glia cell adhesion molecule (NgCAM), also mediate homophilic adhesion and play a prominent role in the developing central nervous system (CNS) [26,27]. Fluorimetric observations of Quin-2AM loaded cells and microscopical recording of Fura-2AM loaded neurons showed that purified or recombinant L1 and antibodies against the extracellular domain of L1 induce an increase in intracellular Ca$^{2+}$ levels in PC12 cells [24], small cerebellar neurons [21,25,28], cortical neurons [29,30], and dorsal root ganglion neurons [21]. Ca$^{2+}$ influx in response to L1 activation has also been observed using voltage patch clamp recordings in cortical neurons [30]. In another study, however, activation of L1 had no effect on intracellular Ca$^{2+}$ levels in Fura-2AM loaded growth cones of dorsal root ganglion neurons [31]. Interestingly, the effect of L1 antibodies depended on the epitope recognized, with one study reporting an increase in intracellular Ca$^{2+}$ levels in neurons incubated with a monoclonal antibody against an epitope within fibronectin type III repeats of L1, but not in neurons incubated with the monoclonal antibodies against epitopes within immunoglobulin domains [28], suggesting that differences in L1 ligands used may contribute to differences in the effects on intracellular Ca$^{2+}$ levels. Similar to L1, exposure to immunopurified NgCAM or anti-NgCAM antibodies induced an increase in intracellular Ca$^{2+}$ levels in neurons from the brains of songbird zebra finch loaded with a Ca$^{2+}$ indicator Fluo-3AM and analyzed by confocal microscopy [32]. While the role of Ca$^{2+}$ in signaling induced by another member of this family, CHL1, has been recently suggested [33], the direct evidence that CHL1 can also induce an increase in intracellular Ca$^{2+}$ levels is still missing.

In addition to NCAM and L1 family members, activation of the immunoglobulin superfamily cell adhesion molecules neuroplastin (Np) and limbic system-associated membrane protein (LAMP) has also been shown to induce increases in intracellular Ca$^{2+}$ levels. Np mediates cell-to-cell adhesion via homophilic interactions and is expressed as two isoforms named according to the molecular weight Np55 and Np65. Application of recombinant ectodomains of Np55 and Np65 or a mimicking peptide of Np65 induced an increase in intracellular Ca$^{2+}$ levels in synapses of cultured hippocampal neurons loaded with a Ca$^{2+}$ indicator Fluo-4AM [34,35]. Soluble recombinant LAMP has been shown to induce increases in intracellular Ca$^{2+}$ in hippocampal neurons and neurons from visual cortex loaded with Fluo-3AM [36].

Experiments with inhibitors of various types of Ca$^{2+}$ channels indicate that changes in intracellular Ca$^{2+}$ levels in response to activation of IgSF CAMs can be mediated by several classes of VDCCs. Inhibitors of L- and T-type VDCCs reduced the increase in intracellular Ca$^{2+}$ levels observed in response to NCAM activation in cultured hippocampal neurons [23] and PC12 cells [24]. Pimozide, an inhibitor of T-type VDCCs, was more potent in inhibiting the NCAM-induced Ca$^{2+}$ response when compared to nifedipine, an inhibitor of L-type VDCCs, in cultured hippocampal neurons [23]. Fluorometric Ca$^{2+}$ measurements showed that inhibitors of nonselective cation channels also reduced NCAM-dependent Ca$^{2+}$ influx in Fura-2AM loaded neurons, suggesting that these channels are also activated in response to NCAM ligands [23]. Nifedipine fully blocked and ω-conotoxin, an inhibitor of N-type VDCCs, partially blocked L1-dependent increase in intracellular Ca$^{2+}$ [29,30,37], while in another study inhibitors of L-type VDCCs verapamil and diltiazem failed to block the L1-dependent Ca$^{2+}$ influx in mouse dorsal root ganglion neurons and small cerebellar neurons [21]. Nifedipine and ω-conotoxin blocked NgCAM-induced Ca$^{2+}$ influx [32]. The LAMP-induced intracellular Ca$^{2+}$ increases in hippocampal neurons were fully inhibited by nifedipine but not ω-conotoxin [36]. Altogether, these data indicate that different members of IgSF act at different VDCCs in a tissue specific manner.

Not only inhibitors of cell surface Ca$^{2+}$ channels, but also depletion of the internal Ca$^{2+}$ stores by incubation
| CAMs | Method/Ca$^{2+}$ indicator | Ligand, concentration | Cell types (localization) | Effect on Ca$^{2+}$ | Inhibitors tested (effect on ligand induced Ca$^{2+}$ increase) | References |
|------|---------------------------|-----------------------|--------------------------|---------------------|-------------------------------------------------------------|------------|
| IgSF | Fluorimetry/Quin-2AM      | Polyclonal NCAM antibodies, 0.4-1 mg/ml | PC12 cells               | ↑                   | Verapamil (full inhibition)                                | [24]       |
|      |                           |                       |                          |                     | Diltiazem (full inhibition)                                 |            |
|      |                           |                       |                          |                     | Nifedipine (no effect)                                      |            |
|      | Microscopy/Fura-2AM       | Synthetic peptide ligand of NCAM ectodomain, 50 μM | PC12-E2 cells           | ↑                   | Not tested                                                  | [22]       |
|      | Fluorimetry/Quin-2AM      | Fab fragments of monoclonal NCAM antibodies (H2B), 0.1-0.2 mg/ml | PC12 cells               | No effect           | Not tested                                                  | [24]       |
|      | Microscopy/Fura-2AM       | Polyclonal NCAM antibodies, 0.3-0.5 mg/ml | PC12 cells               | ↑                   | Not tested                                                  | [21]       |
|      | Microscopy/Fura-2AM       | Monoclonal NCAM antibodies, 0.1 mg/ml | PC12 cells               | ↑                   | Not tested                                                  | [21]       |
|      | Microscopy/Fura-2AM       | NCAM antibodies, 0.5 mg/ml | Chick ciliary ganglion neurons | No effect | Not tested                                                  | [39]       |
|      | Microscopy/Fura-2AM       | Polyclonal NCAM antibodies, 0.3-1 mg/ml | Mouse dorsal root ganglion neurons | ↑                   | Not tested                                                  | [21]       |
|      | Microscopy/Fura-2AM       | Monoclonal NCAM antibodies, 0.1 mg/ml | Mouse dorsal root ganglion neurons | No effect | Not tested                                                  | [21]       |
|      | Microscopy/Fura-2AM       | Polyclonal NCAM antibodies, 0.5 mg/ml | Mouse small cerebellar neurons | No effect | Not tested                                                  | [21]       |
|      | Microscopy/Fura-2AM       | Monoclonal NCAM antibodies, 0.1 mg/ml | Mouse small cerebellar neurons | No effect | Not tested                                                  | [21]       |
|      | Microscopy/Fura-2AM       | Purified NCAM from mouse brain, 10 μg/ml | Mouse small cerebellar neurons | ↑                   | Not tested                                                  | [21,25]    |
|      | Microscopy/Fura-2AM       | Recombinant fragments of NCAM ectodomain, 0.8 μM | Mouse small cerebellar neurons | ↑                   | Not tested                                                  | [25]       |
|      | Microscopy/Fura-2AM       | Monoclonal NCAM antibodies, 30 μg/ml | Mouse cortical neurons (soma) | No effect | Not tested                                                  | [29,30]    |
|      | Microscopy/Fura-2AM       | Synthetic peptide ligand of ectodomain NCAM, 54 μM | Rat hippocampal neurons | ↑                   | Not tested                                                  | [22]       |
|      | Microscopy/Fura-2AM or Fluo-4AM | Polyclonal NCAM antibodies, 1 mg/ml | Rat hippocampal neurons | ↑                   | Nifedipine (partial inhibition)                             | [22]       |
|      |                           | Synthetic peptide ligand of NCAM, 12-35 μM | Rat hippocampal neurons (soma) | ↑                   | Nifedipine (partial inhibition)                             | [23]       |
|      |                           |                       |                          |                     | Mibefradil (partial inhibition)                             |            |
|      |                           |                       |                          |                     | Pimozide (full inhibition)                                  |            |
|      |                           |                       |                          |                     | ω-conotoxin (no inhibition)                                 |            |
|      |                           |                       |                          |                     | Agatoxin (no inhibition)                                    |            |
|      |                           |                       |                          |                     | Loe908 (partial inhibition)                                 |            |
|      |                           |                       |                          |                     | SKF-96365 (partial inhibition)                              |            |
| L1   | Fluorimetry/Quin-2AM      | Polyclonal L1 antibodies, 0.4-1 mg/ml | PC12 cells               | ↑                   | Not tested                                                  | [21,24]    |
|      | Microscopy/Fura-2AM       | Polyclonal L1 antibodies, 0.3-1 mg/ml | Mouse dorsal root ganglion neurons | ↑                   | Verapamil (no effect)                                      | [21]       |
|      |                           |                       |                          |                     | Diltiazem (no effect)                                       |            |
|      |                           |                       |                          |                     | Cd$^{2+}$/Ni$^{2+}$ (no effect)                              |            |
### Table 1 An overview of CAMs, activation of which induces an increase in intracellular Ca\(^{2+}\) levels (Continued)

| Method         | Description                                                                 | Cell Type                        | Effect |
|----------------|------------------------------------------------------------------------------|----------------------------------|--------|
| Microscopy/Fura-2AM | Recombinant ectodomain of L1 (L1-Fc), 10 μg/ml                               | Rat dorsal root ganglion neurons (growth cones) | ↑†     |
| Microscopy/Fura-2 dextran | L1 expressed by 3 T3 cells                                                   | Rat dorsal root ganglion neurons (growth cones) | No effect |
| Whole cell patch-clamp | Monoclonal L1 antibodies recognizing glycosylated L1, 7.5-30 μg/ml        | Mouse dorsal root ganglion neurons | ↑†     |
| Microscopy/Fura-2 AM | Polyclonal L1 antibodies, 0.3-0.5 mg/ml                                      | Mouse small cerebellar neurons    | ↑†     |
| Fluorimetry/Quin-2AM; Microscopy/Fura2AM | Purified L1 from mouse brain, 10 μg/ml or 0.8 μM | Mouse small cerebellar neurons    | ↑†     |
| Fluorimetry/Quin-2AM | Monoclonal L1 antibodies recognizing FNIII type repeats, 100 μg/ml         | Mouse small cerebellar neurons    | ↑†     |
| Fluorimetry/Quin-2AM | Monoclonal L1 antibodies recognizing Ig-like domains I-VI, 100 μg/ml        | Mouse small cerebellar neurons    | No effect |
| Microscopy/Fura-2-AM | Monoclonal L1 antibodies recognizing glycosylated L1, 7.5-30 μg/ml         | Mouse cortical neurons (soma)     | ↑†     |
| Whole cell patch-clamp | Soluble recombinant ectodomain of Np55, 15 μM                               | Rat hippocampal neurons (synaptic areas) | ↑†     |
| Np55 Microscopy/Fluo-4AM | Purified chicken NgCAM, 1.2 μg/ml                                           | Neostriatal subependymal zone neurons of adult zebra finch | ↑†     |
| Np65 Microscopy/Fluo-4AM | Purified chicken NgCAM, 1.2 μg/ml                                           | Neostriatal subependymal zone neurons of adult zebra finch | ↑†     |
| NgCAM Microscopy/Fluo-3AM; Fura-2AM | Polyclonal NgCAM antibodies, 100 μg/ml                                      | Neostriatal subependymal zone neurons of adult zebra finch | ↑†     |
| LAMP Microscopy/Fluo-3AM | Soluble recombinant LAMP, 30 μg/ml                                          | Rat hippocampal neurons (synaptic areas) | ↑†     |
| Thy-1 Microscopy /Fura-2AM | Fab fragments of monoclonal Thy-1 antibodies, 10 μg/ml                     | PC12 cells (cytosol)              | No effect |

*Full inhibition:* Nifedipine
*Partial inhibition:* ω-conotoxin
| Cadherins                      | Microscopy/Fura-2AM | Soluble fragments of N-cadherin purified from brain or retina, 10 μg/ml | Chick ciliary ganglion neurons (soma, growth cones) | ↑ | Mixture of diltiazem and ω-conotoxin (no inhibition) [39] |
|-------------------------------|---------------------|------------------------------------------------------------------------|-----------------------------------------------------|---|----------------------------------------------------------|
| Whole cell voltage clamp      |                      | Recombinant ectodomain of N-cadherin (N-cadherin-Fc), 20 μg/ml         | Chick ciliary ganglion neurons                       | ↑ | Not tested [41]                                          |
| Microscopy/Fura-2AM           | Soluble recombinant ectodomain of N-cadherin (N-cadherin-Fc), 50 μg/ml | Chick retinal ganglion cells                                        | No effect                                           |   |                                                          |
| Microscopy/FFP-18-AM          | Soluble recombinant ectodomain of N-cadherin (N-cadherin-Fc), 50 μg/ml | Chick retinal ganglion cells (subplasma membrane of growth cones)   | ↑ | Mixture of nifedipine and ω-conotoxin (partial inhibition) [40] |
| Celsr2/ Celsr3                | Microscopy/Fura-2AM  | Soluble recombinant cadherin repeats of Celsr2/Celsr3, 1 μg/ml        | Rat hippocampal neurons                              | ↑ | Not tested [38]                                          |
| Integrins                     | Microscopy/Fura-2AM,  whole cell voltage clamp | RGD peptide (cGRGDSPA), 1 μM                                          | L. stagnalis CNS motoneurons (soma)                 | ↑ | Not tested [48]                                          |
|                                | High-speed microscopy/ Fluo-4AM | Synthetic RGD peptide (RGDS), 0.5-1 μM                                    | Xenopus spinal neurons (growth cones)                 | ↑ | Not tested [44]                                          |
|                                | Microscopy/Fura-2AM  | Soluble Laminin, 20 μg/ml                                               | Chick ciliary ganglion neurons (soma)               | ↑ | Mixture of diltiazem and ω-conotoxin (no inhibition) [39] |
|                                | Microscopy/Fura-2AM  | Laminin, immobilized to the beads, 50 μg/ml                             | Chick dorsal root ganglion neurons (growth cones)   | No effect | Not tested [43]                                          |
|                                | Microscopy/Fura-2AM  | Soluble laminin, 20 μg/ml                                               | Surgically isolated filopodia from growth cones of chick dorsal root ganglion neurons | ↑ | Not tested [43]                                          |
|                                | Microscopy/Fura-2AM  | RGD peptide (GRGDSP), 10 μM                                             | Mouse cortical neurons (soma and neurites)          | ↑ | Gd<sup>3+</sup> (partial inhibition) [45]     |
| Whole cell voltage clamp      | Polyclonal α5β1 integrin antibodies, 10 μg/ml                          | Rat basal forebrain neurons                                           | ↑ | Not tested [47]                                          |
|                               | Microscopy/Fura-2AM  | Synthetic RGD peptide (GRGDSP), 2.5 mM                                  | Rat cortical neurons                                  | ↑ | Not tested [46]                                          |

*Effect observed in the presence of Ba<sup>2+</sup> in the test solution.

CAMs, methods used to detect changes in intracellular Ca<sup>2+</sup> levels, Ca<sup>2+</sup> sensitive indicator used in optical recordings, ligands used to activate CAMs, cell type analyzed and subcellular localization of Ca<sup>2+</sup> changes (if described in the original publication), the effect of CAMs on intracellular Ca<sup>2+</sup> levels (↑ - indicates an increase), the effect of inhibitors of Ca<sup>2+</sup> channels on CAM-induced intracellular Ca<sup>2+</sup> increases, and respective references are listed in the table.
with thapsigargin, a specific inhibitor of the sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPase, inhibited an increase in intracellular Ca\(^{2+}\) levels in response to activation of NCAM in cultured hippocampal neurons [23, 38] and in response to NgCAM activation in cultured forebrain neurons [32], indicating that Ca\(^{2+}\) influx at the cell surface membrane is accompanied by the release of Ca\(^{2+}\) from the internal stores. Internal Ca\(^{2+}\) stores have also been suggested to contribute to intracellular Ca\(^{2+}\) increases in response to activation of Np55 [35].

Changes in intracellular Ca\(^{2+}\) levels induced by activation of cadherins

Optical recordings of chick ciliary ganglion neurons loaded with Fura-2AM showed that application of soluble fragments of N-cadherin purified from brain resulted in an increase in intracellular Ca\(^{2+}\) levels in growth cones and cell bodies of neurons [39]. Increased steady state levels of intracellular Ca\(^{2+}\) were also recorded in growth cones of chick retinal ganglion cells grown on N-cadherin and loaded with a membrane targeted Ca\(^{2+}\) indicator, FFP-18AM [40]. Interestingly, when the Fura-2AM reporter was used, steady state levels of intracellular Ca\(^{2+}\) were found to be not affected by substrate coated recombinant N-cadherin suggesting that N-cadherin influences predominantly submembrane Ca\(^{2+}\) levels [40]. Whole cell voltage clamp recordings also showed that homoplastic binding of N-cadherin on neuronal membranes to soluble N-cadherin or N-cadherin overexpressed in Chinese hamster ovary (CHO) cells increases amplitudes of Ca\(^{2+}\) currents in ciliary ganglion neurons indicating that homoplastic interactions of N-cadherin are sufficient to activate a cellular mechanism that regulates Ca\(^{2+}\) influx [41].

Similarly to members of IgSF, experiments with inhibitors of various types of Ca\(^{2+}\) channels suggest that VDCCs play an important role in cadherin-induced increases in intracellular Ca\(^{2+}\) levels. In particular, co-application of nifedipine and \(\omega\)-conotoxin partially reduced the increase in intracellular Ca\(^{2+}\) levels in response to N-cadherin activation in retinal ganglion cells [40], suggesting that L- and T-type VDCCs are involved in N-cadherin induced Ca\(^{2+}\) influx in neurons. It should be noted, however, that inhibitors of N- and L-type VDCCs \(\omega\)-conotoxin and diltiazem had no significant effect to N-cadherin-induced Ca\(^{2+}\) response in ciliary ganglion neurons [39].

In addition to N-cadherin, increases in intracellular Ca\(^{2+}\) levels have been shown to occur in response to activation of atypical cadherins Celsr2 and Celsr3, which are highly expressed in hippocampal and cortical neurons [42]. Optical imaging of cultured hippocampal neurons loaded with Fura-2AM showed that intracellular Ca\(^{2+}\) levels increased in response to recombinant cadherin repeats of Celsr3 and Celsr2 with a more pronounced effect observed in response to activation of Celsr2 when compared to Celsr3 [38]. This increase in intracellular Ca\(^{2+}\) levels in response to activation of Celsr2 or Celsr3 was inhibited by thapsigargin, indicating that intracellular Ca\(^{2+}\) stores play also a role in Ca\(^{2+}\) increases mediated by cadherin family members [38].

Changes in intracellular Ca\(^{2+}\) levels induced by activation of integrins

Modest increases in intracellular Ca\(^{2+}\) levels were reported to occur in response to exposure of neurons to natural ligands of integrins. Optical recordings showed that laminin induced an increase in intracellular Ca\(^{2+}\) levels in growing Fura-2AM loaded chick ciliary ganglion neurons [39], and in the surgically isolated filopodia of growth cones of chick dorsal root ganglion neurons [43]. Much larger increases in intracellular Ca\(^{2+}\) levels were observed in response to integrin ligand Arginine-Glycine-Aspartic acid (RGD)-sequence containing peptides. Optical recordings of Fluo-4AM loaded cultured Xenopus spinal neurons showed that incubation with soluble RGD peptides elevated intracellular Ca\(^{2+}\) levels in growth cones and increased filopodial Ca\(^{2+}\) transient frequency [44]. Similar results were obtained with adult cortical neurons, in which fibronectin application has produced moderate increases in intracellular Ca\(^{2+}\) levels while larger responses were observed in neurons treated with RGD-containing peptides [45, 46]. Increased Ca\(^{2+}\) currents induced by activation of integrins using multivalent antibodies against integrins were also observed using whole cell patch clamp recordings in neurons acutely dissociated from the medial septum/diagonal band nucleus of the rat [47]. Both, optical recordings of Fura-2AM loaded cell bodies and whole cell voltage clamp recordings showed that RGD peptides increased depolarization induced increases in intracellular Ca\(^{2+}\) levels in motoneurones isolated from the CNS of the pond snail L. stagnalis [48]. It should be noted, however, that high concentrations of RGD peptides used in some of the previous studies [46] have also been shown to induce integrin-independent increases in intracellular Ca\(^{2+}\) levels, such as via activation the N-methyl-D-aspartate (NMDA) receptors in an integrin-independent manner [49]. Therefore, contribution of integrin-independent sources of Ca\(^{2+}\) to overall increases in intracellular Ca\(^{2+}\) levels in studies using RGD peptides cannot be fully excluded. Integrin \(\beta\)-dependent increases in intracellular Ca\(^{2+}\) levels were partially blocked by nifedipine and gadolinium III (Gd\(^{3+}\)), a broad spectrum VDCC inhibitor, in cortical neurons [45]. However, a mixture of diltiazem and \(\omega\)-conotoxin did not affect the laminin-induced Ca\(^{2+}\) increases in somata of chick ciliary ganglion neurons [39]. Depletion of intracellular Ca\(^{2+}\) stores and inhibitors of the ryanodine receptor (RyR) and inositol 1,4,5-triphosphate gated receptor (IP3R), channels through which Ca\(^{2+}\) in intracellular stores is released into the cytosol,
also reduced but did not eliminate increases in intracellular Ca\(^{2+}\) levels in response to RGD-containing integrin ligand peptides in cortical neurons [45]. Therefore, Ca\(^{2+}\) influx via VDCCs and Ca\(^{2+}\) release from internal stores can both contribute to the elevation of intracellular Ca\(^{2+}\) levels in response to integrin activation.

**Changes in intracellular Ca\(^{2+}\) levels induced by activation of other CAMs**

Changes in intracellular Ca\(^{2+}\) levels have also been reported for other neuronal cell surface molecules involved in neuronal adhesion, notably for amyloid precursor protein (APP) and cellular prion protein (PrP). Optical recordings of B103 rat neuroblastoma cells transfected with APP and loaded with Fluo-4AM showed an increase in intracellular Ca\(^{2+}\) levels in response to incubation with amyloid beta (A\(\beta\)), an APP-derived toxic peptide accumulating in brains of Alzheimer's disease patients. Since no changes in intracellular Ca\(^{2+}\) levels in response to A\(\beta\) occurred in cells non-transfected with APP, it was proposed that binding of A\(\beta\) to APP induced Ca\(^{2+}\) influx in these cells [50]. Dysregulation of Ca\(^{2+}\) signaling has been also found in astrocytes from mice missing APP [51].

An increase in intracellular Ca\(^{2+}\) levels have been observed in synaptosomes incubated with recombinant PrP, while function blocking antibodies against PrP inhibited depolarization induced Ca\(^{2+}\) influx via synaptosomal VDCCs, indicating that PrP also plays a role in regulation of intracellular Ca\(^{2+}\) levels [52]. PrP-dependent Ca\(^{2+}\)-influx has been shown to occur in response to such ligands of PrP as laminin and stress-inducible protein 1 in dorsal root ganglion neurons loaded with Fluo-3AM [53]. Reduced depolarization induced Ca\(^{2+}\) influx has been observed using Fura-2AM and a Ca\(^{2+}\) indicator Calcium Green-5N in cerebellar granule cells and hippocampal CA1 neurons from PrP deficient mice, respectively [54,55]. Both submembrane and intracellular levels of Ca\(^{2+}\) were affected by PrP deficiency [55].

Reduced Ca\(^{2+}\) currents have been also recorded in mice deficient in \(\alpha\)-neurexin [56], indicating that neurexin-neuroligin adhesion complexes are also involved in regulation of intracellular Ca\(^{2+}\) levels in neurons. Whether binding of \(\alpha\)-neurexins to neuroligins stimulates Ca\(^{2+}\) influx into neurons remains to be investigated.

**The effect of VDCC inhibitors on neurite outgrowth induced by activation of IgSF CAMs, cadherins and integrins**

VDCCs have been shown to play a multitude of roles in the developing and adult brain being involved in a number of signaling pathways. The role of different types of VDCCs in various brain functions is beyond the scope of this review and we refer the reader to several recent excellent reviews on this subject [57-63]. Below, we summarise current evidence implicating VDCCs in CAM-induced neurite outgrowth.

Analysis of studies investigating effects of various inhibitors of Ca\(^{2+}\) channels on CAM-induced neurite outgrowth is summarized in Table 2. A study by Doherty and colleagues [64], which demonstrated that inhibitors of L-type and N-type VDCCs inhibit NCAM-mediated neurite outgrowth from PC12 cells in an additive manner, was the first to show the fundamental role of VDCCs in neurite outgrowth mediated by CAMs. NCAM-dependent neurite outgrowth has been also shown to be partially inhibited by the inhibitors of N-, L-, and P/Q-type VDCCs in cultured hippocampal neurons [22,65]. Inhibitors of L-type VDCCs also blocked exocytosis in growth cones induced in response to NCAM activation and required for NCAM-dependent neurite outgrowth [66]. Interestingly and surprisingly, another study showed that inhibitors of T-type VDCCs or inhibitors of nonselective cation channels also completely blocked NCAM-dependent neurite outgrowth in cultured hippocampal neurons [23], suggesting that Ca\(^{2+}\) influx via different Ca\(^{2+}\) channels is necessary to raise the overall levels of intracellular Ca\(^{2+}\) above the threshold required for NCAM-dependent neurite outgrowth. Similarly, L1-dependent neurite outgrowth was blocked by inhibitors of L-type (diltiazem, verapamil, or nifedipine) and N-type (\(\omega\)-conotoxin) VDCCs in rat cerebellar neurons and PC12 cells [31,67], and partially inhibited by nifedipine, verapamil and diltiazem in mouse small cerebellar neurons [28]. Neurite outgrowth induced by another member of L1 family, CHL1, was fully blocked by application of either an inhibitor of L- or T-type VDCCs [33]. Inhibitors of L- and N-type VDCCs have been also shown to block neurite outgrowth induced by activation of IgSF cell adhesion molecule Thy-1 [31,67,68]. In contrast, neurite outgrowth induced in cultured hippocampal neurons grown on CHO cells overexpressing LAMP was inhibited by blockers of L- but not N-type VDCCs [36]. Altogether, these observations suggest that Ca\(^{2+}\) influx via distinct Ca\(^{2+}\) channels at the cell surface is required to induce a complete set of molecular changes and responses required for IgSF CAM-dependent neurite outgrowth. This scenario is consistent with the observations showing that VDCCs can activate several independent signaling pathways in growth cones of growing neurites [69].

Similarly to IgSF CAMs, N-cadherin-mediated neurite outgrowth from PC12 cells has been shown to be inhibited by blockers of L- and N-type VDCCs in an additive manner [64]. However, inhibitors of L- and N-type VDCCs failed to block N-cadherin-dependent neurite outgrowth in ciliary ganglion neurons [39] indicating that other types of Ca\(^{2+}\) channels are involved in cadherin-dependent neurite outgrowth in these cells. Surprisingly, inhibitors of L- and N-type VDCCs diltiazem and \(\omega\)-conotoxin
had no effect on integrin-mediated laminin-induced neurite outgrowth in ciliary ganglion neurons [39]. Therefore, VDCCs required for integrin mediated neurite outgrowth remain to be identified.

Potential mechanisms linking CAMs to Ca\(^{2+}\) channels
While data accumulated over the last two decades indicate that activation of CAMs induces an increase in intracellular Ca\(^{2+}\) levels in neurons, the mechanisms of this increase remain incompletely understood and probably involve a number of signaling cascades, which link CAMs to the sources of extra- and intracellular Ca\(^{2+}\) by changing the permeability of the respective channels.

A possibility that CAMs change permeability of VDCCs is supported by the studies on IgSF CAM L1 showing that Ca\(^{2+}\) influx via VDCCs in response to L1 activation occurs without changes in membrane voltage [37] indicating that L1 promotes Ca\(^{2+}\) influx via changing VDCCs properties. Probably the best characterized signaling pathway activated by IgSF CAMs to induce changes in intracellular Ca\(^{2+}\) levels involves the fibroblast growth factor receptor (FGFR) (Figure 1A). FGFR directly interacts with the
members of the immunoglobulin superfamily NCAM [70,71], Nectin-1 [72], neuroplastin [35] and L1 [73,74]. It was proposed that activation of FGFR in this pathway is followed by activation of phospholipase C (PLC), which generates diacylglycerol (DAG), which is then converted into arachidonic acid (AA), which then activates VDCCs and subsequently induces Ca^{2+} influx [37,75]. In agreement with this model, ion influx through VDCCs in response to L1 activation was inhibited by a DAG lipase inhibitor and blocked in sensory neurons expressing dominant negative FGFR [37]. Further confirming this model, inhibitors of FGFR and PLC also reduced an increase in intracellular Ca^{2+} levels in response to NCAM activation in cultured hippocampal neurons [23].

Inhibitors of FGFR do not fully block NCAM-mediated increases in intracellular Ca^{2+} levels, suggesting that other
factors also contribute to changes in the levels of intracellular Ca^{2+}. Src-family tyrosine kinases were implicated since inhibitors of this family of tyrosine kinases partially reduced an increase in intracellular Ca^{2+} levels in response to activation of NCAM in neurons [23]. Src-family tyrosine kinases associate with and regulate the activity of L-type VDCCs [76], and are activated by different members of the immunoglobulin superfamily including NCAM [77] and L1 [78] (Figure 1A). Interestingly, NCAM and L1 act at different members of the Src-family tyrosine kinase family, fyn and src respectively [78-80]. It remains to be determined whether this influences the ability of NCAM and L1 to activate different VDCCs at the cell surface.

The G protein pathway is another pathway which may contribute to activation of VDCCs [81,82], possibly via inducing PLC activation [83] (Figure 1A). Pertussis toxin (PTX), an inhibitor of the G protein, inhibited Ca^{2+} influx in response to activation of NCAM in PC12 cells [24] and in response to NgCAM in avian forebrain neurons [32].

An increase in intracellular levels of Ca^{2+} in response to activation of IgSF CAMs at the cell surface has also been linked to activation of a number of Ca^{2+} dependent enzymes, such as protein kinase C (PKC) or calcium/calmodulin-dependent kinase II α (CaMKIIα), which are activated by NCAM [84-87]. It is therefore possible that PKC and CaMKIIα provide a positive feedback loop to increase the Ca^{2+} influx via VDCCs in response to activation of NCAM (Figure 1A). Interestingly, however, the long-term exposure of neurons to the PKC activator phorbol 12-myristate 13-acetate inhibited L1-dependent increases in intracellular Ca^{2+} levels [29,88], suggesting that PKC may also play a role in reducing intracellular Ca^{2+} levels following CAM activation.

There is also limited evidence on the interactions between IgSF CAMs and VDCCs in neurons. Both L-type and T-type VDCCs co-immunoprecipitated with NCAM from the mouse brain lysates indicating that NCAM forms a molecular complex with VDCCs [86]. In response to ligand binding, NCAM redistributes to lipid rafts [77,89], where VDCCs are also accumulated [86]. Whether the interactions between NCAM and VDCCs influence the permeability of VDCCs remains to be investigated.

IgSF CAM-activated signaling pathways can also play a central role in inducing Ca^{2+} release from internal stores. Inhibitors of PLC inhibited increases in levels of intracellular Ca^{2+} in response to NCAM activation, suggesting that Ca^{2+} release from internal stores occurs in response to inositol-3-phosphate produced by PLC (Figure 1A). Additionally, activation of RyR via cAMP and PKA pathways may also result in Ca^{2+} release from internal stores [90] (Figure 1A). This CAM-dependent activation of RyR via cAMP and PKA pathways have been shown to have important consequences for neuronal behavior, such as turning of growth cones of growing neurites either towards the source of Ca^{2+} signal, which occurs on the L1- or N-cadherin substrate, or away from the source of Ca^{2+} signal which occurs on the laminin substrate in dorsal root ganglion neurons [90].

Similarly to IgSF members cadherins also interact with FGFR [91-93] (Figure 1B). This observation suggests that cadherins can activate signaling pathways which are similar to or partially overlap with signaling pathways activated by IgSF members to induce Ca^{2+} influx at the cell surface. N-cadherin dependent regulation of VDCCs involves, however, also a small GTPase RhoA [94,95] (Figure 1B).

FGFR also binds to integrins [96], and may be involved in integrin-dependent Ca^{2+} signalling (Figure 1C). Inhibition of Src family tyrosine kinases also partially reduced an increase in intracellular Ca^{2+} levels in response to activation integrins in neurons [47], suggesting that kinases of this family are involved (Figure 1C). Integrin-dependent activation of L-type VDCCs has also been shown to be dependent on PKA [47], which phosphorylates VDCCs and facilitates their function [97] (Figure 1C). Laminin-induced integrin-mediated increases in intracellular Ca^{2+} levels in growth cones were also blocked by inhibitors of PKC and CaMKIIα [43] (Figure 1C).

Integrins have also been found in a complex with VDCCs. Integrins containing α3 subunit are linked to VDCCs by laminin in the Torpedo electric organ synapses [98]. In cerebellar granular neurons, integrin α5β1 associates with short transient receptor potential channel 5 (TrpC5) [99]. TrpC channels are metabolically-activated Ca^{2+} channels, which are widely expressed in different tissues and cell types playing diverse physiological functions [100]. They also play a critical role in neuronal development (for extensive review see [101]) and neurite outgrowth in particular [99,102]. In non-neuronal human embryonic kidney HEK293 cells, α5β1-integrin has been shown to associate with L-type VDCCs [103], and ligand-dependent complex formation between β1-integrin and L-type VDCCs has been described in mouse embryonic stem cells [104]. Whether binding of integrins to VDCCs and TRPCs influences permeability of these channels remains however unknown.

**Conclusion**

In conclusion, a number of studies indicate that CAMs play an important role in regulation of intracellular Ca^{2+} levels in neurons by acting at VDCCs and possibly other types of Ca^{2+} channels in the neuronal cell surface plasma membrane and in the intracellular Ca^{2+} stores. Not only VDCCs, but also other neuronal plasma membrane Ca^{2+} channels such as transient receptor potential channels, stretch-activated channels, and cyclic nucleotide-gated...
channels have been reported to play a role in neuronal development [105-107]. Direct links between CAMs and other types of Ca\textsuperscript{2+} channels in neurons remain however unknown. Current data indicate that CAMs activate Ca\textsuperscript{2+} channels by inducing intracellular signaling cascades which can either activate or remove inhibition of Ca\textsuperscript{2+} channels to induce an increase in intracellular Ca\textsuperscript{2+} levels. It remains to be investigated whether formation of the molecular complexes between CAMs and Ca\textsuperscript{2+} channels directly influences the activity of the latter.

Most of the previous work was performed using artificial stimulation protocols by applying soluble ligands in the culture medium and monitoring bulk changes in intracellular Ca\textsuperscript{2+} levels. Better imaging technologies which appeared recently may help to investigate the dynamics of local CAM-dependent Ca\textsuperscript{2+} changes occurring during cell-to-cell contact formation, and particularly during synapse formation. A combination of such technologies with biochemical analysis and optical imaging of the synapse enriched cytoskeleton components and enzymes may provide valuable information about the mechanisms of the molecular rearrangements accompanying contact maturation. The development of genetically encoded Ca\textsuperscript{2+} reporters [108-111] with a better defined subcellular localization as compared to chemical dyes used in previous studies may also allow monitoring intracellular Ca\textsuperscript{2+} levels in CAM-enriched membrane microdomains in different neuronal compartments, which is a promising new direction for further research.

Abbreviations

AA: Arachidonic acid; AB: Amyloid beta; AM: Acetoxymethyl ester; APP: Amyloid precursor protein; CAMs: Cell adhesion molecules; CaMKII: Calcium/calmodulin-dependent kinase II α; CHL1: Close homolog of L1; CHO: Chinese hamster ovary cells; CNS: Central nervous system; DAG: Dihydyglcerol; ER: Endoplasmic reticulum; FGR: Fibroblast growth factor receptor; HEK 293 cells: Human embryonic kidney 293 cells; IP3R: Inositol 1,4,5-triphosphate receptor; LAMP: Lysosome-associated membrane protein; NCAM: Neural cell adhesion molecule; N-CAM: Neuron-glia cell adhesion molecule; NMDA: N-methyl-D-aspartate; Np: Neuronal; NSCCs: Nonselective cation channels; PLC: Phospholipase C; PKA: Protein kinase A; PKC: Protein kinase C; PTX: Pertussis toxin; PKC: Protein kinase; RGD: Arginine-Glycine-Aspartic acid; RYR: Ryanodine receptor; TPC5: Transient receptor potential channel 5; VOCCs: Voltage-dependent calcium channels.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

LS, IL and VS have performed literature search and analysis and written the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by the National Health and Medical Research Council (to VS).

Received: 22 October 2013 Accepted: 5 December 2013 Published: 13 December 2013

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