Exosomes as a novel way of interneuronal communication

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Abstract
Exosomes are small extracellular vesicles which stem from endosomes fusing with the plasma membrane; they contain lipids, proteins and RNAs that are able to modify receiving cells. Functioning of the brain relies on synapses, and certain patterns of synaptic activity can change the strength of responses at sparse groups of synapses, to modulate circuits underlying associations and memory. These local changes of the synaptic physiology in one neuron driven by another have, so far, been explained by classical signal transduction mechanisms, transcription and post-translational modifications. We have accumulated in vitro evidence that exosomes released by neurons in a way depending on synaptic activity can be recaptured by other neurons. Some lipids, proteins and RNAs contained in exosomes secreted by emitting neurons could directly modify signal transduction and protein expression in receiving cells. Exosomes may be an ideal mechanism for anterograde and retrograde information transfer across synapses underlying local changes in synaptic plasticity. Exosomes might also participate in the spreading across the nervous system of pathological proteins such as PrPSc (abnormal disease-specific conformation of prion protein), APP (amyloid precursor protein) fragments, phosphorylated tau or α-synuclein.

Introduction
The function of the brain relies on the capacity of billions of synapses to transmit patterns of electrical information through neuronal circuits. Reinforcement of circuits underlying associations and memory is based on the ability of chemical synapses to be individually modified after specific patterns of stimulation. The increase or decrease in the strength of synaptic responses requires structural modifications of both pre- and post-synaptic parts, which must be sustained in the long term through modifications in gene expression. Changes in one neuron driven by another have, so far, been explained by classical signal transduction: neurotransmitters released presynaptically bind to postsynaptic receptors allowing calcium entry and activating other second messengers in the postsynaptic cell. This also leads to the release by cell bodies and dendrites of retrograde signals acting presynaptically [1]. Second messengers and enzymatic activities influence, both transiently and durably, effectors of synaptic physiology (adhesion molecules, neurotransmitter receptors, cytoskeleton anchors, etc.) through post-translational modifications and changes in gene expression [2]. The efficacy of such processes must deal with the extreme compartmentalization of the nervous parenchyma. Indeed, control of transcription occurs in the nucleus sometimes several millimetres away from synapses undergoing plastic changes. Some of the transcripts are then transported along dendrites and translated at specific synapses to sustain changes in activity, among thousands of others that remain unchanged. Regulation of translation is another aspect of synaptic plasticity, and numerous demonstrations show that miRNAs (microRNAs), which are expressed within dendrites, regulate translation of targets mediating dendritic growth [3,4].

Our hypothesis is that exosomes may constitute a novel mechanism of interneuronal transfer of molecules used during plastic changes in the brain. For the sake of clarity, the term exosome refers to microvesicles formed through budding of endosomal membranes inside MVBs (multivesicular bodies), and released in the extracellular milieu after fusion of these endosomal membranes into the plasma membrane. In non-neuronal cells, exosomes were shown to transfer membrane and cytoplasmic proteins [5,6], as well as lipids involved in signal transduction [7,8]. Exosomes also contain RNAs; once in the receiving cells, exosomal mRNAs can be translated [9] and small RNAs, including miRNAs, mediate gene silencing [10–12]. Thus, if it exists, the interneuronal anterograde and retrograde exchange of exosomal lipids, proteins and RNAs would represent an ideal way for mediating the synaptic changes necessary for plasticity; direct information transfer allowed by exosomes would be far more efficient than that allowed by direct cell–cell contacts or secreted soluble factors.

Several criteria must be fulfilled for exosomes to play a role in the plasticity of the nervous system: (i) MVBs must...
Neuronal endosomes underlie several aspects of synaptic plasticity

In neurons, endosomes are present in both pre- and postsynaptic compartments. Electron microscopy observations of the adult hippocampus revealed the presence of MVBs appearing as large vacuoles delimited by a single membrane and containing a varying number of 50–80 nm membrane vesicles. MVBs are mainly detected in dendritic shafts and inside a limited number of spines, which are small protrusions containing postsynaptic parts of glutamatergic synapses [13]. Several in vivo paradigms revealed that movements of MVBs to synapses of hippocampal neurons are tightly linked to synaptic plasticity [14]. In cultured neurons, LTP (long-term potentiation), a form of synaptic plasticity now widely accepted as a model of learning and memory processes, correlates with the recruitment of recycling endosomes into or near spines, where they fuse with the plasma membrane [15,16]. Fusion of the endosomal membrane to the surface increases the spine volume and thereby synaptic efficiency. Furthermore, glutamate receptors present in the limiting membrane of endosomes become inserted at the neuronal surface, diffuse laterally to synaptic sites and accumulate at postsynaptic densities. Since the new inserted cargoes are inserted at synapses undergoing plastic changes. The loss of AMPA glutamate receptors is a potent activator of MVB fusion to the plasma membrane and thereby of exosome secretion. Thus fusion of endosomes with the plasma membrane of dendrites is tightly regulated by synaptic activity and is necessary for the early steps of synaptic plasticity.

Neurons secrete exosomes in a tightly regulated way

We demonstrated previously that cultured cortical neurons release exosomes [19]. As in the case of other cells, exosomes isolated from neuronal culture media floated on sucrose gradients at a density of 1.1–1.2 g/ml and contained both Alix [ALG-2 (apoptosis-linked gene 2)-interacting protein X] and Tsg101 (tumour susceptibility gene 101). Alix is a cytoplasmic protein which interacts with Tsg101 of the ESCRT (endosomal sorting complex required for transport) machinery necessary for the formation of ILVs (intraluminal vesicles) accumulating inside MVBs [20–22]. Endophilin A, which also interacts with Alix [23], was not detected in exosomes, showing that entry of cargoes into ILVs is regulated. Incorporation of membrane proteins in ILVs is also selective as exosomes contained AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid), but not NMDA (N-methyl-D-aspartate) glutamate synaptic receptors.

Three other studies reported constitutive secretion of exosomes by neurons [24–26]. These studies, as well as our initial one, used embryonic neurons cultured for only 3–8 days. In short-term cultures, neurons make only a few synapses and neurite outgrowth is still ongoing. Thus exosomal release could reflect in part the fusion of late endosomes/lysosomes at growth cones known to occur during neurite elongation [27]. To test whether fully mature neurons can also release exosomes, we have studied exosomal release from cortical neurons cultured for 15 days [28]. Even if the basic level of exosomal release by these cultures was very low, it was drastically increased after raising cytosolic calcium with a calcium ionophore. Electron microscopic examination of cultures treated for 1 min with ionomycin revealed clusters of exosomes at the surface of dendrites, thus visualizing the fusion of MVBs with the plasma membrane. These cultures, which contain both glutamatergic and GABAergic neurons making functional networks, were used to test the influence of synaptic activity on exosomal release. For this, we incubated the cultures with GABA (γ-aminobutyric acid) receptor antagonists, such as picrotoxin or bicucullin, which alleviate inhibitory activities within the networks and thereby increase synaptic glutamatergic activity. Such treatments rapidly (10–15 min) and massively augmented the secretion of exosomes in a way that is dependent on glutamate ionotropic receptors [28]. Taken together, our data suggest that calcium entry through synaptic glutamate receptors is a potent activator of MVB fusion to the plasma membrane and thereby of exosome secretion. The enhanced secretion of AMPA-receptor-containing exosomes following glutamatergic synaptic activation, underlines exosomal release as a way of local elimination of receptors at synapses undergoing plastic changes. The loss of AMPA receptors upon extensive synaptic activation could be a mechanism of homeostatic synaptic scaling, necessary for adjusting the strength of all of a neuron’s excitatory synapses to stabilize firing [29]. In this scenario, regulation of the pool of surface synaptic receptors by exosome secretion would be a local event, avoiding retrograde transport of MVBs necessary to hydrolyse the receptors in lysosomes, which are only present in proximal dendrites and soma.

The fate of exosomes released by neurons

In the immune system, exosomes were first shown to be internalized by [30], and more recently to fuse directly with [12], the plasma membrane of dendritic cells. Exosomes released in the CNS (central nervous system) parenchyma could be phagocytosed by macrophage-like cells, such as oligodendrocyte-derived exosomes which are internalized by microglial cells [31]. Astrocyte endfeet enwrapping a number of glutamatergic synapses can also endocytose/phagocytose cellular debris [32] and could thus capture exosomes released...
at synapses. Transfer of exosomes might also occur between spines of the same neuron or across synapses to end up in afferent neurons. Indeed, the diameter of neuronal exosomes (\(\sim 60\) nm) is compatible with endocytosis by clathrin-coated pits at presynaptic boutons, spines or dendritic shafts [33]. In line with this, we recently found that a fragment of tetanus toxin, known to cross synapses in \(\textit{vivo}\), is endocytosed by neurons to end up inside MVBs and be released by way of exosomes [28]. Neuronal exosomes containing the toxin incubated on cultured neurons bound to and entered synapses presynaptically (M. Chivet, C. Javalez and R. Sadoul, unpublished work), thus suggesting that the toxin crosses synapses in association with exosomes. Theoretically, membrane of exosomes could fuse with the plasma membrane or, if they are endocytosed, with endosomal membranes. Backfusion of ILVs has been demonstrated to occur inside MVBs [34] and could thus concern exosomes, which have the same origin. Fusion of exosomes with cell-surface, or endosomal, membranes, would add new transmembrane proteins acting on the synaptic physiology such as AMPA receptors. It would also allow the release into the cytosol of the exosome content, including signal transduction molecules capable of modifying receiving neurons. We have recently observed that exosomes secreted by neurons contain miRNAs (M. Chivet, C. Javalez and R. Sadoul, unpublished work). Given that single miRNAs have multiple targets, the impact of exosome-mediated local transfer of miRNA on the pattern of translated mRNAs in receiving neurons may be quite extensive. Accordingly, Xin et al. [35] reported recently that exosomes secreted by mesenchymal cells influence neurite outgrowth apparently through \(\text{miR-133b}\) contained inside exosomes.

### Relevance of neuronal exosomes for neurodegenerative diseases

Exosomes carry pathogenic proteins such as \(\alpha\)-synuclein, \(\text{PrP}^{\text{Sc}}\) (abnormal disease-specific conformation of prion protein), APP (amyloid precursor protein) C-terminal fragments and phosphorylated tau, which are involved in Parkinson’s, prion and Alzheimer’s diseases respectively. The scarp form of the prion protein (i.e. \(\text{PrP}^{\text{Sc}}\)) can be secreted via exosomes and remains infectious under this form [36]. Thus, similarly to tetanus toxin, prions could propagate from the periphery to the CNS by way of trans-synaptic exchange of exosomes. \(\alpha\)-Synuclein secreted together with exosomes released by neuroblastoma cells causes cell death of recipient neuronal cells suggesting that \(\alpha\)-synuclein secretion via exosomes serves to amplify and propagate Parkinson’s disease-related pathology [37]. The catabolism of APP giving rise to the amyloidogenic C-terminal APP fragment occurs in endosomes and this fragment, as well as \(\text{A}\beta\) (amyloid \(\beta\)-peptide), is released by way of exosomes [25,38–40]. Furthermore, whereas endogenous tau was not secreted inside neuronal exosomes [19], it was detectable in exosomes from cells overexpressing the protein, suggesting that exosomes may contribute to eliminate the excess of intracellular tau [41,42]. The tau pathology could spread \(\textit{in vivo}\) through trans-synaptic transmission, and it is tempting to speculate that exosomes contribute to the spreading of the Alzheimer’s disease pathology throughout interconnected cortical areas [43]. These puzzling hypotheses require \(\textit{in vivo}\) work (i) to show that exosomal release from MVBs occurs \(\textit{in situ}\), (ii) to find out the privileged site of this release, and (iii) to demonstrate trans-synaptic exchange of exosomes. Furthermore, even though the activity-dependent release of exosomes suggests a genuine function of exosomes in synaptic plasticity, molecular tools to specifically block MVB fusion with the plasma membrane must be developed to test this hypothesis. Nevertheless, studies on exosomes in the CNS are bound to shed new light on intercellular exchanges within the brain and to open new avenues towards understanding how neurodegenerative diseases spread over time throughout the nervous system.

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