ACS Chemical Neuroscience

Review

pubs.acs.org/chemneuro

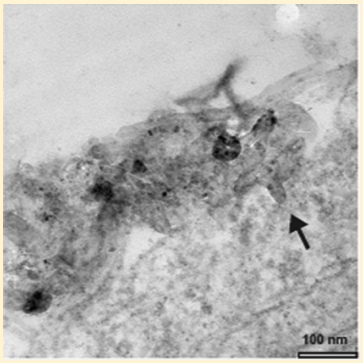
Carbon Nanotubes: Artificial Nanomaterials to Engineer Single Neurons and Neuronal Networks

Alessandra Fabbro,[†,‡] Susanna Bosi,[†] Laura Ballerini,*[,‡] and Maurizio Prato[†]

[†]Department of Chemical and Pharmaceutical Sciences, University of Trieste, Trieste, Italy

[‡]Life Science Department, University of Trieste, Trieste, Italy

ABSTRACT: In the past decade, nanotechnology applications to the nervous system have often involved the study and the use of novel nanomaterials to improve the diagnosis and therapy of neurological diseases. In the field of nanomedicine, carbon nanotubes are evaluated as promising materials for diverse therapeutic and diagnostic applications. Besides, carbon nanotubes are increasingly employed in basic neuroscience approaches, and they have been used in the design of neuronal interfaces or in that of scaffolds promoting neuronal growth *in vitro*. Ultimately, carbon nanotubes are thought to hold the potential for the development of innovative neurological implants. In this framework, it is particularly relevant to document the impact of interfacing such materials with nerve cells. Carbon nanotubes were shown, when modified with biologically active compounds or functionalized in order to alter their charge, to affect neurite outgrowth and branching. Notably, purified carbon nanotubes used as scaffolds can promote the formation of nanotube−neuron hybrid networks, able per se to affect neuron integrative abilities, network connectivity, and synaptic plasticity. We focus this review on our work over several years directed to investigate the ability of carbon nanotube platforms in providing a new tool for nongenetic manipulations of neuronal performance and network signaling.



KEYWORDS: *Carbon nanotubes, nanotechnology, cultured neuronal network, synapse, short-term plasticity, patch clamp recordings*

The discovery and manipulation of innovative nanomaterials, such as carbon nanotubes (CNTs), are becoming increasingly helpful in biomedical applications in general[1,2] and in neuroscience research approaches and developments in particular, thus providing new tools able to specifically interact with the nervous system and with neurons at the nanoscale.[3] CNTs have been alternatively proposed as growth substrates promoting neuronal development, scaffolds for nerve tissue engineering, electrode coating, or neuronal interfaces for long-term implants.[4−10] In their soluble form, CNTs are also promising nanovectors for drug delivery and molecular sensing applications.[11−13]

Since their discovery in 1991 by Ijima,[14] CNTs have shown outstanding mechanical, thermal, and conductive properties: these unique nanoobjects made of one or more rolled-up graphene sheets possess high surface area, high mechanical strength but ultralight weight, rich electronic properties, and excellent chemical and thermal stability.[15] These properties make CNTs very promising in different technological fields; in particular, CNTs were used as conductive composites, energy storage and energy conversion devices, sensors, field emission displays and radiation sources, hydrogen storage media and nanometer-sized semiconductor devices, probes, and interconnects (for a review, see ref 16). Their poor solubility and their apparently high toxicity have been faced in the past decade via functionalization of the CNT surface by means of many different approaches (Figure 1a) aimed at increasing their solubility and lowering their toxic effects to promote biomedical applications.[17] CNTs have been proposed as biosensors (see ref 18 for a review), ion channel blockers,[19] biocatalysts,[20] photothermal probes in cancer therapy,[21] and nanovectors.[22]

In the past decade, CNTs have been largely investigated as substrates for the development of neuronal circuits; in such a use, the development of a hybrid neuronal−nanomaterial network served also as a platform to examine neuronal detection of and reactions to, environmental physical and chemical features. In particular, the ability of CNTs to modulate neuronal behavior at either the structural (synaptogenesis and neurite elongation)[23−27] and functional (synaptic efficacy)[10,27−30] level was shown. In this respect, interfacing neurons with purified CNTs emerged as an effective tool for manipulating neuronal activity in single cells, in a network of synapses, and, more recently, in multilayered tissue explants.[10,27−30]

The leading scope of this review is to highlight experiments addressing the ability of synthetic CNTs to directly and specifically interact with cultured brain networks and their role in re-engineering neuronal and synaptic performance.

Special Issue: Biological Engineering in Neuroscience

Received: April 30, 2012
Accepted: May 22, 2012
Published: May 22, 2012

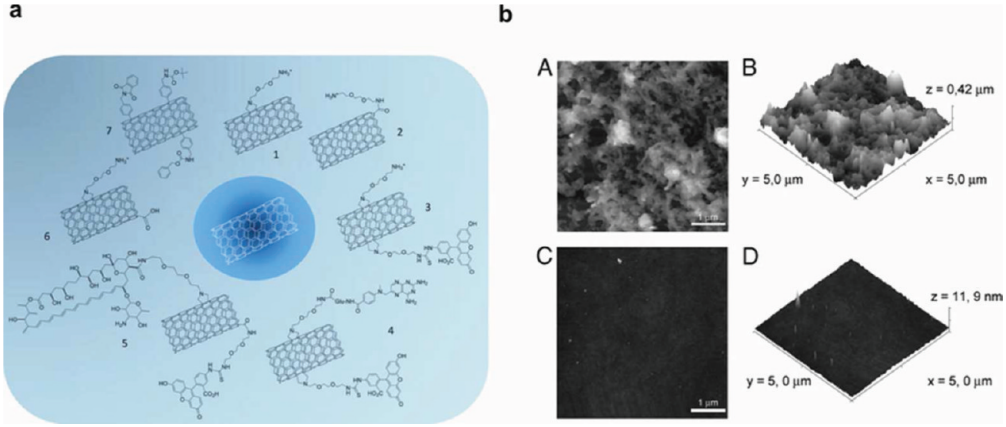


Figure 1. Carbon nanotubes as scaffolds for neuronal growth. (a) Examples of chemical modifications on carbon nanotubes. Reprinted with permission from ref 17. Copyright 2011 The Royal Society of Chemistry. (b) MWCNT and glass growth supports used as the control condition in most studies are characterized by different roughness. AFM images (A,C) and three-dimensional plot profiles (B,D) of MWCNT and glass substrates, respectively. Reprinted with permission from ref 27. Copyright 2011 The Society for Neuroscience.

SCAFFOLDS FOR NERVE TISSUE ENGINEERING AND CARBON NANOTUBES

Scaffolds made of different kinds of biomaterials are being investigated as substrates able to favor neuronal growth and axonal regeneration. In order to provide stromal support in the case of lesions at the level of the central nervous system (CNS) and to overcome the inhibitory environment (for example, in the case of spinal lesions),[31−33] scaffolds selected for CNS tissue engineering have to be biocompatible with the host tissue without inducing inflammatory and immune reactions, reduce the astrocytic reaction and glial scar formation, allow neuronal adhesion and axonal extension within a three-dimensional architecture, offer proper physical support to cells and axons, provide physical properties similar to the native environment, and have a tunable rate of degradation without inflammation induced by degradation products.[34] The architecture of the scaffold structure is crucial for a successful impact on neuronal regeneration: for example, the introduction of longitudinal tubular constructs provides physical guidance for axonal regrowth and cell migration, and thus may enhance nerve regeneration.[35] Moreover, also the micro- and nanotopography at the cellular level are fundamental for successful nerve regeneration,[34] and several studies have shown that cells behave differently on aligned or randomly oriented fibers.[36] The main characteristic of these materials is a longitudinal organization mimicking the natural structure of the axonal pathways within the brain and the spinal cord.[34]

More recently, the design of 3D scaffolds incorporating conducting nanostructures was proposed in order to develop smart biomaterials for the engineering of electrically propagating tissues.[2] For example, carbon nanotubes were used to implement a composite sponge[37] with high electrical conductivity, ideally placed to support the cultivation of neurons.

In this vision, CNTs can be exploited by virtue of their tubular shape together with their conductivity features to produce the best substrate for neural engineering. CNTs and their composites have been used as, or incorporated into, scaffolds to investigate their influence on the growth of neural processes. Variously patterned CNTs offered a two-dimensional or three-dimensional guidance for the elongation and growth of neurites.[38,39]

CNTs have been functionalized with molecules like polyethylene imine[24] and neurotrophines,[40] incorporated in agarose,[41] deposited by a layer-by-layer technique together with polyelectrolytes in supported assemblies or as freestanding films:[42,43] regardless of the functionalization used, altogether these studies hint at the development of CNTs-based platforms for neuro-regeneration purposes.

The application of carbon nanotubes in neuroscience research has been oriented toward the use of two different types of material: (i) single-walled CNTs (SWCNTs), composed of a single graphene sheet rolled-up and closed at each end by a hemispherical fullerene cap, and (ii) multiwalled CNTs (MWCNTs), composed of numerous concentric graphene cylinders. Several features of CNT structure make them intriguing candidates for nervous system applications, e.g., their shape and size, reminiscent of the morphology of the smallest neuronal arborizations,[4] or their high electrical conductivity and surface area which may increase charge injection capacity of CNT-based microelectrodes[6] (Figure 1b). In addition, nonfunctionalized CNTs may affect neuronal signaling by means of CNT/cell membrane "direct" interactions. The following sections will expand this issue, discussing evidence of the CNT-mediated modulation of a single neuron, synaptic, and network activities.

The focus of this review is on the use of purified CNTs, while the ability of functionalization and covalent and noncovalent modifications of CNTs to tune their ability to support neuronal growth are reviewed in detail elsewhere.[9,44,45]

IMPACT OF CARBON NANOTUBES ON MONOLAYERED SYNAPTIC NETWORKS: SPONTANEOUS ACTIVITY AND SINGLE CELL SIGNALING

Several studies support the biocompatibility of CNTs scaffolds (SWCNTs or MWCNTs, as-prepared, functionalized, or codeposited with polymers) when used *in vitro* to sustain neuronal growth and axonal elongation and branching.[23−29,42,46−48] These first studies reporting CNTs' ability to sustain neuronal growth mostly investigated the impact of such substrates on neuronal morphology, and the issue of the signaling changes displayed by neurons grown on CNT-based structures was neglected; in particular pure, nonfunctionalized CNTs were rarely tested. In fact, while the first report on the

dx.doi.org/10.1021/cn300048q | *ACS Chem. Neurosci.* 2012, 3, 611−618

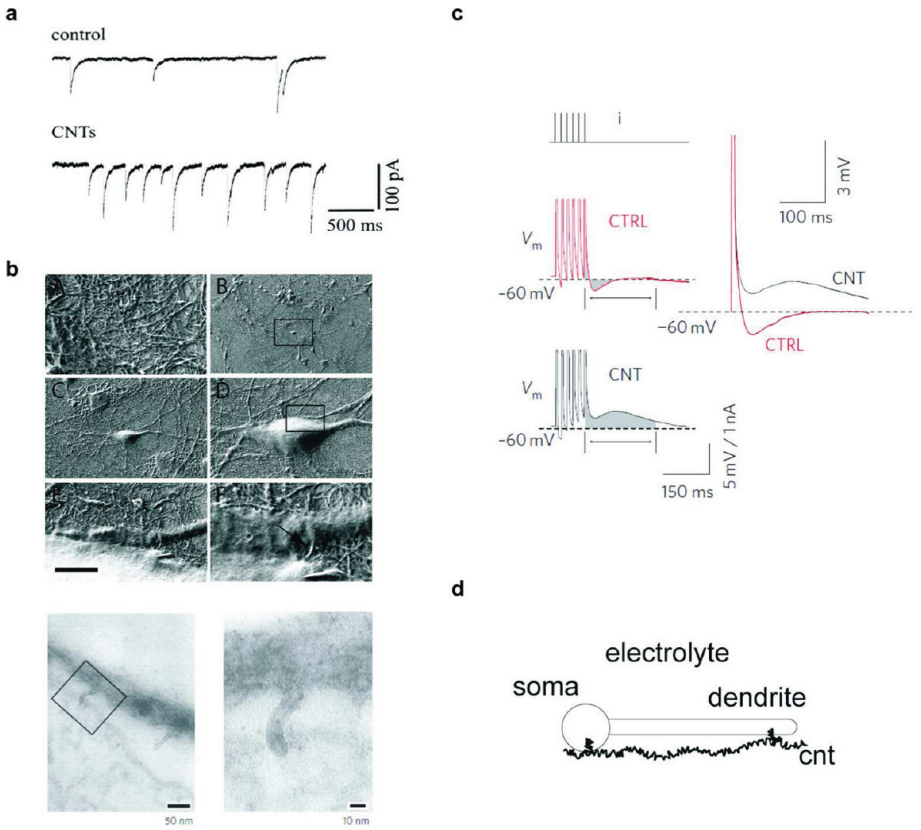


Figure 2. Neurons in close contact with CNT scaffolds show improved network activity and single-cell integrative abilities. (a) Exemplificative voltage-clamp recordings of postsynaptic currents (PSCs) recorded from hippocampal neurons cultured on glass or on CNT substrates; PSC frequency is significantly increased in CNT cultures. Reprinted from ref 28. Copyright 2005 American Chemical Society. (b) Top, scanning electron microscopy images showing SWCNTs substrate features (A) and the close and intimate contacts between CNTs and the neuronal membrane of cultured hippocampal neurons (subsequent micrographs in B–F). Reprinted with permission from ref 29. Copyright 2007 The Society for Neuroscience. Scale bar (in E): A, 1 μm; B, 200 μm; C, 25 μm; D, 10 μm; E, 2 μm; F, 450 nm. Bottom, transmission electron microscopy micrographs from planar sections of hippocampal cultures grown on CNTs, showing a single nanotube (highlighted in the box) "pinching" the neuronal membrane (bottom part of the image). Reprinted with permission from ref 30. Copyright 2009 Nature Publishing group. (c) Hippocampal neurons cultured on control glass (CTRL) or on CNT substrate (CNT) were forced to fire a train of six action potentials (by injection of current steps, i; top) in order to assess the presence of an additional afterhyperpolarization or afterdepolarization at the end of the train (gray shadow). CNT scaffolds significantly increased the fraction of neurons showing an afterdepolarization. Reprinted with permission from ref 30. Copyright 2009 Nature Publishing group. (d) The "electrotonic" hypothesis has been formulated in order to explain the increased ability to generate an afterdepolarization in neurons cultured on a CNTs layer. The hypothesis assumes that intracellular compartments are electrically exposed to CNTs, which could act as electrical shortcuts between distal cellular compartments. Reprinted with permission from ref 30. Copyright 2009 Nature Publishing group.

ability of CNT layers to sustain neuronal growth dates back to 2000,[23] the first one investigating neuronal electrical activity when cells are grown on CNTs appeared later, in 2005.[28] Using single cell recording, the patch-clamp technique, we measured synaptic and firing activity from neurons which were reconstructing functional networks interfaced to nonfunctionalized MWCNTs. We showed that dissociated hippocampal neurons, after 8 days of culturing interfaced to MWCNT supports, always displayed a strong potentiation in their spontaneous activity (mainly detected as an increase in the frequency of spontaneous, network-driven postsynaptic currents-PSC; Figure 2a), in comparison with that of controls grown on pure glass supports. Later, we further demonstrated that, in a similar fashion, SWCNTs interfaced to neurons are equally able to induce a potentiation of spontaneous synaptic activity in cultured hippocampal networks.[29] This increase in spontaneous activity of cultured brain circuits was not due to the building up of networks of different sizes with respect to control growth supports, as the neuronal density (quantified by immunocytochemistry experiments) was similar in the two culturing conditions, as well as the cell body size and the number of neurites emerging from the soma.[28,29] The control and neurons grown on CNTs also showed similar membrane passive properties (input resistance, capacitance, and resting potential), again indicating similar cellular dimensions and healthy state in both culturing conditions.[27−30]

The extraordinary intimate and tight contacts between neurons and CNTs were documented by means of scanning and transmission electron microscopies (SEM and TEM), which show the presence of direct and close juxtapositions between CNTs and neuronal membranes[10,29,30] (Figure 2b). Process entanglement has indeed been demonstrated to play a key role in neuronal anchoring to rough surfaces, such as CNTs.[49] The presence of nanocontacts between neuronal membranes and CNTs suggested the possibility of the occurrence of their direct electrical coupling, an issue explored in detail only in 2009.[30] By means of single cell electrophysiological techniques, this study showed that CNTs are able

dx.doi.org/10.1021/cn300048q | ACS Chem. Neurosci. 2012, 3, 611−618

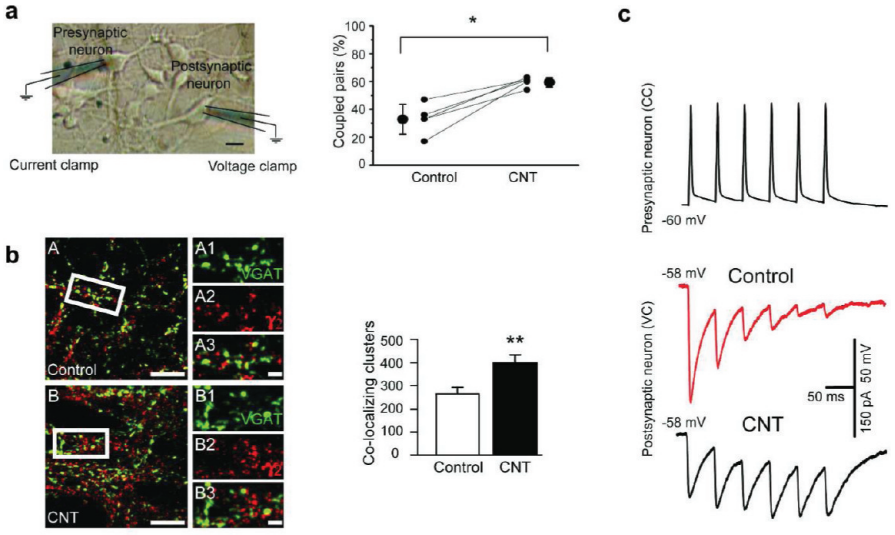


Figure 3. (a) Left, bright-field image of the experimental setting employed for paired patch-clamp recordings from hippocampal neurons cultured on glass or on CNTs scaffolds. The presynaptic neuron is forced to fire one or more action potentials, and the evoked postsynaptic currents (PSCs) are recorded from the postsynaptic neuron. Scale bar is 15 μm. Right, cultures interfaced to CNTs show a strong increase in the probability of finding connected cell pairs. (b) Left, confocal images from immunocytochemistry experiments highlighting the presynaptic component (vescicular GABA transporter,VGAT, clusters, in green) and the postsynaptic component (GABA receptor γ_2 subunit clusters, in red) in control (A) and CNT cultures (B), whose colocalization is the morphological evidence of the presence of synapses. A1−3 and B1−3 show the VGAT and GABA receptor γ_2 subunit signals separately (A1, A2, and B1, B2) or merged (A3, B3) in control and CNT cultures, respectively. Scale bars: A and B, 10 μm; A1−3 and B1−3, 2 μm. Cultures on CNTs show a marked increase in the number of colocalized clusters, i.e., synapses (right). (c) In paired recordings on control neurons, a train of action potentials elicited in the presynaptic neuron usually evokes depressing PSCs in the postsynaptic neuron, while in cells cultured on CNT scaffolds PSCs are usually not depressing. Reprinted with permission from ref [27]. Copyright 2011 The Society for Neuroscience.

to mold the integrative abilities of cultured hippocampal neurons and that this ability is potentially due to a direct electrical coupling between CNTs and neuronal membranes. In this work, single neurons were forced to fire brief trains of action potentials at different frequencies to maximize the integration of regenerative properties in the different (proximal and distal) neuronal compartments.[50] Neurons grown on CNT scaffolds were significantly more prone to generate and summate back-propagating action potentials, showing an additional somatic depolarization after high frequency trains of action potentials (Figure 2c). Back-propagating action potentials are a regenerative property of certain classes of neurons which play important roles, for example, in the fine-tuning of synaptic activity, in the expression of spike-timing-dependent plasticity, or in governing the release of modulatory messengers; altogether, the ability to back-propagate action potentials can regulate synaptic plasticity in both the short- and long-terms.[51−55] The improved generation of back-propagating action potentials brought about by CNTs implies that these materials modulate single cell excitability. This may even account for the activity boost detected at the network level (i.e., increased PSCs), a hypothesis supported by theoretical modeling.[30] Intriguingly, theoretical simulations also put forward the hypothesis that the CNT carpet could impact on back-propagating action potential generation by a direct electrical shortcut between nearby dendritic compartments (the so-called "electrotonic hypothesis";[30] Figure 2d): this shortcut would account for the dendritic extra depolarization observed after the action potential train and might be mediated by the direct contact between CNTs and neuronal membranes.[30,56,57] However, the observation that the presence of direct electrical connections between neurons in the network was extremely low[27] ruled out the possibility that additional presence of CNT-mediated shortcuts at the network level could be responsible for the CNT ability to improve functional connectivity of neuronal networks.[27] Besides the suggestive hypothesis that electrical signals traveling along neuronal membranes be shortcut and directly delivered to distant compartments, CNT/neuron interactions may be used to specifically instruct neurons toward a more excitable phenotype.

The impact of CNT scaffolds on single cell excitability highlighted the exceptional nature of this nanomaterial and its potentials even in the field of neurobiology. CNT meshworks used as supports for neuronal growth are essentially characterized by their roughness and conductivity[27,30] (Figure 1b). Substrates for neuronal growth different from CNTs, with either conductivity or nanoroughness comparable to that of CNT, are not able to improve dendritic regenerative ability (i.e., the back-propagating action potentials), thus indicating that both properties are necessary for CNT substrates to manipulate neuronal performance.[30,45] The summation of CNT nanotopography and physical and chemical properties provides neurons with a great deal of information, thus suggesting that neuronal contacts to CNT scaffolds might activate multiple and complex adhesion-mediated, intracellular signaling cascades.[1,2]

CARBON NANOTUBES IMPACT ON MONOLAYERED SYNAPTIC NETWORKS: TUNING OF SYNAPTIC CONNECTIVITY AND PLASTICITY

To thoroughly understand CNT effects on neuronal transmission, regardless of the modulation of single-cell excitability, it is crucial to explore if and how CNTs can modulate neuronal synaptic properties. A recent work directly faced this issue by means of simultaneous recording of pairs of interconnected neurons in dissociated hippocampal cultures grown on CNT

dx.doi.org/10.1021/cn300048q | ACS Chem. Neurosci. 2012, 3, 611−618

growth supports or on control glass[27] (Figure 3a). Action potentials were evoked in the first (presynaptic) neuron by injecting a depolarizing current pulse, while the evoked unitary postsynaptic currents (PSCs, usually $GABA_A$ receptor-mediated in these experimental conditions)[27] were recorded in the second (postsynaptic) cell. In this study, we showed that culturing neurons on CNT scaffolds almost doubled the probability of finding monosynaptically connected neurons, when compared to controls, i.e., CNTs guide the build-up of more synapses than in control conditions. In the same work, we further demonstrated that the strong rise in the coupling probability was due to a massive increase in synaptic density: immunofluorescence colocalization experiments reported the morphological evidence of an increase in the number of GABAergic synaptic contacts in networks grown on CNTs layers compared to that of controls[27] (Figure 3b). This strong boosting in neuronal network connectivity is proposed to be the major mechanism by which CNTs increase the frequency of spontaneous PSCs of cultured networks reported in the previous studies[27−29] and fosters CNTs as a powerful artificial growth support able to promote *de novo* formation of synapses.

A surprising ability of CNTs in modulating neuronal physiology emerged in the same work,[27] i.e., CNTs also affect the short-term dynamic of synaptic connections. A striking feature of neuronal transmission is the short-term dynamics of synaptic connections that govern their processing abilities; that is, the synapses may display a transient alteration in strength when activated repetitively.[58−60] By means of paired patch-clamp experiments, the authors elicited high frequency trains of action potentials in the presynaptic cell and recorded the GABAergic PSCs in the postsynaptic one. In control synapses, the PSCs' amplitude became progressively smaller during the train, indicating a synaptic short-term depression[61,62] (Figure 3c). Conversely, PSCs' amplitude remained almost stable, or even potentiated, in neurons cultured on CNT supports (Figure 3c), indicating that synapses formed under these growing conditions display an improved efficacy. The CNT-mediated effect on synaptic plasticity was due to a modulation of the probability of GABA release at synapses by CNTs (i.e., a release-dependent mechanism). Indeed, increasing the release probability in CNT cultures by raising the extracellular $[Ca^{2+}]$ abolished the short-term potentiation (thus making CNT synapses more similar to control ones), while decreasing the extracellular $[Ca^{2+}]$ in control cultures reduced synaptic depression (making controls more similar to CNT synapses).[27]

A very interesting, although unexpected, feature of CNT impact on synaptic networks, is that CNT modulations are governed by independent mechanisms.[27] Synaptogenesis (i.e., the increased probability of finding connected couples in paired recordings) due to CNT supports has been shown to be independent of neuronal activity, as it persisted even when activity was suppressed by chronically blocking action potential generation by tetrodotoxin (TTX). Conversely, the chronic block of activity by TTX in cultures grown on CNT scaffolds converted their observed short-term potentiation into short-term depression. In other words, the short-term synaptic plasticity of activity-deprived CNT cultures remains similar to that of controls, even if CNT scaffolds are still able to promote a strong increase in global neuronal connectivity.[27] The mechanisms underlying this dichotomy are still not clear. We hypothesized that the adhesion of neurons to the substrate is an important factor able per se to instruct neuronal behavior[7,30,63−65] through the activation of complex and diverse intracellular signaling cascades.[1,2,66,67] Thus, the reported CNTs effects are mediated, at least in part, by the adhesive interactions between CNTs and neuronal membranes, regardless of the action potential generation. In agreement with this hypothesis, Jin and colleagues[68] recently demonstrated that PC12 cells differentiated toward the neuronal lineage, when grown on a MWCNT-covered nanofibrous structure, show a very strong increase in the expression of the adhesion molecule FAK (a cytoplasmic tyrosine kinase playing a key role in the signaling pathway of integrins, the membrane receptors mediating cell adhesion to the substrate),[66,67,69] compared to the control, non-CNT-covered nanofibrous structures.[68] The activation of specific intracellular cascades might favor the synaptogenesis observed in cultures grown on CNT scaffolds. Alternatively, CNT substrates could influence the deposition of extracellular matrix molecules, and the specific composition of the so-called "perineuronal net" could in turn affect synaptogenesis.[70] All of the suggested mechanisms are far from being proved by experimental evidence, and further work is needed to definitely shed light on the molecular mechanisms linking CNTs to neuronal network functional dynamics.

CARBON NANOTUBES INTERFACED TO CNS EXPLANTS: TRANSLATING NETWORK MODULATION FROM LOCAL TO REMOTE IN A MULTILAYERED TISSUE

Most studies investigating CNT platforms and neuronal properties required the use of relatively simplified experimental models such as the monolayer of dissociated brain cells.[23−30,46] Although networks of dissociated neurons remain a convenient model, even showing a certain complexity,[71] only recently, explant cultures were used to interface CNT scaffolds to multilayered neuronal networks. We characterized the long-term impact on neuronal performance of interfacing spinal cord explants with scaffolds of purified MWCNTs.[10] In our experimental model, we cocultured spinal cord and dorsal root ganglia (DRG) slices on a film of purified, non-functionalized CNTs or on control glass, and we explored how explants interfaced with the CNT substrates in the long term (up to three weeks) developed; in particular, the growing ability of neurites and the functional properties of spinal neurons were assessed. In agreement with our previous reports (see above), CNTs efficiently sustained spinal neurons survival and growth *in vitro*. Interestingly, spinal explants grown on CNT scaffolds showed a significant increase in the number and length of neuronal fibers outgrowing the spinal tissue. This behavior was accompanied by a substantial increase in the number of growth cones at the tips of the extending neurites. We further focused on the fibers elongating on CNTs or on control glass and found, by means of atomic force microscopy (AFM), that fibers grown in contact with the CNT layer adapted differently to the substrate, basically increasing their adhesion area. This was confirmed by force spectroscopy curves acquired from AFM experiments that showed how neuronal fibers grown on CNTs displayed different intrinsic elastic properties, being less stiff than those grown on control glass. The last finding indicated the ability of neuronal fiber to adapt to CNT substrate, suggesting an improved neuronal adhesion, leading to fibers flattening.[10] In addition, in analogy to what was previously reported,[29,30] also in cultured explants TEM and SEM highlighted extensive and very tight contacts between CNTs and neurite membranes (Figure 4a).

dx.doi.org/10.1021/cn300048q | ACS Chem. Neurosci. 2012, 3, 611−618

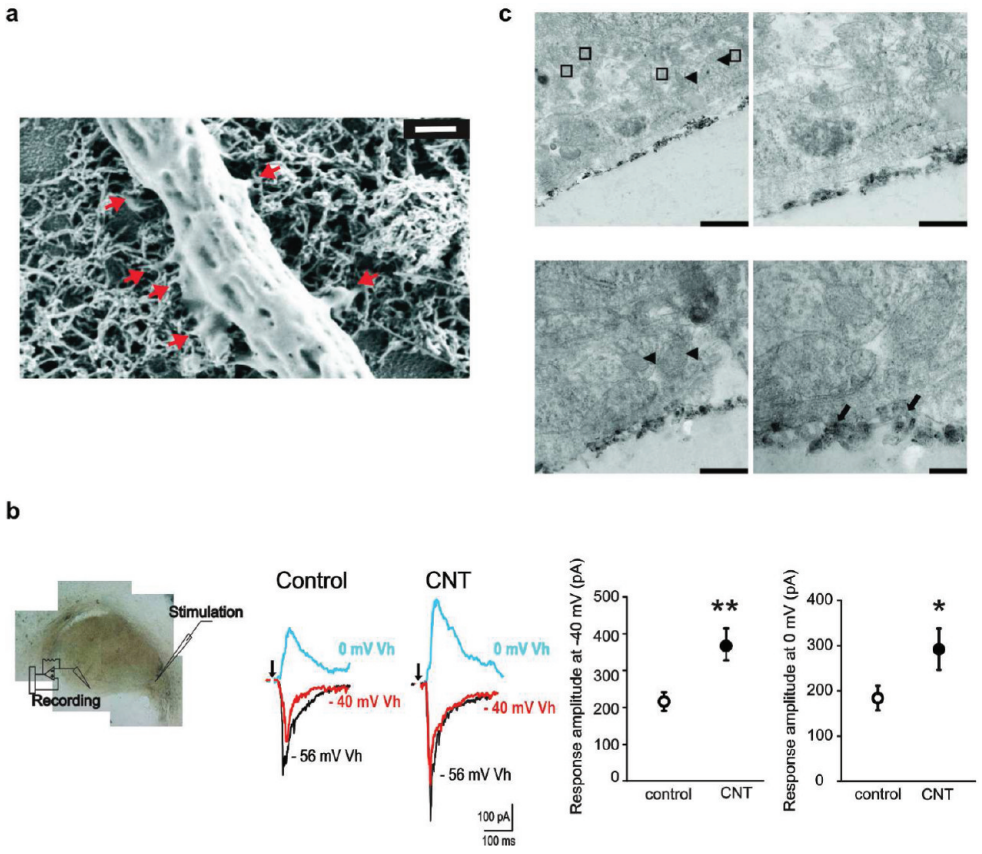


Figure 4. CNT scaffolds support the growth of spinal explants and remotely boost synaptic connectivity. (a) Scanning electron microscopy micrograph of a peripheral neuronal fiber of a spinal explant grown on a CNT layer, with numerous and very tight contacts between CNTs and the neuronal membrane (red arrows). Scale bar is 500 nm. (b) Left, schematic representation of the experimental setting: the dorsal root ganglion was electrically stimulated, while the evoked postsynaptic currents (ePSCs) were recorded from homolateral ventral interneurons. Middle, superimposed ePSCs recorded from interneurons from explants cultured on glass or on CNTs at the resting membrane potential (−56 mV), at the reversal potential for inhibitory currents (−40 mV, red; the excitatory component of the evoked response), and at the reversal potential for excitatory currents (0 mV, blue; the inhibitory component of the evoked response). The amplitude of both the excitatory and the inhibitory components was strongly increased in explants interfaced to CNT scaffolds (right). (c) Transmission electron microscopy micrographs from sagittal sections of spinal explants grown on CNTs, showing healthy tissue (mitochondria are highlighted by arrowheads and neuronal microtubule sections by boxes) and close contacts between CNTs and the bottom part of the explant (arrows). Scale bars: top left, 1 μm; top right, 500 nm; bottom left, 500 nm; bottom right, 200 nm. Reprinted from ref 10. Copyright 2012 American Chemical Society.

Yet, CNT driven changes in neuronal fiber elongation, morphology, and elastomechanical properties involved direct adhesion of neuronal membranes on CNTs. A step beyond these considerations emerged in the same work[10] where we reported, for the first time, the impact of CNT interfaces to cell layers distant from the CNTs themselves. More explicitly, CNT scaffolds are able to modulate the functional performance of neurons spatially far from the scaffold. We investigated synaptic activity by performing patch-clamp recordings from neurons located in the superficial layer of the ventral, premotor area of the explants, i.e., 4−5 cell layers far from the CNTs substrate. We took advantage of the good preservation of the physiological neuronal connections in cultured explants. In particular, in this model in-growing afferent DRG fibers establish functional connections toward the spinal ventral areas; we therefore analyzed the postsynaptic currents evoked in ventral interneurons by the focal electrical stimulation of the homolateral DRG. The evoked PSCs (ePSCs) comprise both an excitatory and an inhibitory component.[72] These two synaptic components were dissected out by exploiting their different reverse potential values. Both inward and outward currents contributing to the polysynaptic ePSCs were strongly increased in amplitude when recorded from explants interfaced to CNT scaffolds (Figure 4b). In addition, a significant increase in the amplitude of the spontaneous (ongoing) synaptic currents was also detected by recording ventral interneurons.[10] The potentiating effect was not due to an increased DRG fibers innervation, thus suggesting the presence of a more complex modulation of synaptic activity.

It is important to point out that the neurons recorded in these experiments were not in direct contact with the CNTs substrate. It is therefore suggested that CNTs affect neuronal firing activity and synaptic connectivity in the neuronal layer directly facing CNT scaffold (in agreement with previous findings on dissociated neurons[27,28,30]), where CNTs intimately and extensively interact with membranes (Figure 4c),[10] and that this alteration is remotely translated into an amplification of synaptic signals at the level of ventral interneurons relatively far from the CNT interface.

While we ruled out the possibility that the described effects could be, at least in part, accounted for by a CNT-mediated increase in the number of presynaptic release sites or by a local increase in firing activity,[10] the real mechanisms underlying this effect are still obscure and further studies are necessary to

dx.doi.org/10.1021/cn300048q | ACS Chem. Neurosci. 2012, 3, 611−618

clarify these issues. Besides, these results put forward the intriguing possibility to exploit CNT technology to remotely direct neuronal behavior in complex systems and might contribute to the progress in the development of innovative, CNT-based neuronal interfaces and implantable devices.

CONCLUSIONS

In summary, CNTs represent a unique material to shape neuronal signaling, as CNT scaffolds used as substrates for neuronal growth *in vitro* are able to affect single cell integrative abilities, to promote neuronal network connectivity and synaptic plasticity and to remotely increase the efficacy of synaptic responses. What the real nature of the interaction is between CNTs and neuronal membranes and what the fine mechanisms are mediating CNTs impact on neuronal performance still remain a fascinating mystery, and further studies are needed to definitely shed light on this issue. Nevertheless, the results here described pave the way to the CNTs' application to more complex systems; the challenge is now to transfer CNT technology to *in vivo* applications, to develop next-generation devices able to efficiently impact neuronal performance in a controlled fashion.

AUTHOR INFORMATION

Corresponding Author

*Laura Ballerini, Life Science Department, Center for Neuroscience B.R.A.I.N. University of Trieste, via Giorgieri 1, I-34127, Trieste, Italy. Tel: +39 040 558 2411. Fax: +39 040 5582011. E-mail: lballerini@units.it.

Funding

Financial support from NEURONANO-NMP4-CT-2006-031847 and CARBONANOBRIDGE ERC-2008-227135 to L.B. and M.P. is gratefully acknowledged.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Place, E. S., Evans, N. D., and Stevens, M. M. (2008) Complexity in biomaterials for tissue engineering. *Nat. Mater. 8*, 457−470.

(2) Dvir, T., Timko, B. P., Kohane, D. S., and Langer, R. (2011) Nanotechnological strategies for engineering complex tissues. *Nat. Nanotechnol. 6*, 13−22.

(3) Silva, G. A. (2006) Neuroscience nanotechnology: progress, opportunities and challenges. *Nat. Rev. Neurosci. 7*, 65−74.

(4) Gilmore, J. L., Yi, X., Quan, L., and Kabanov, A. V. (2008) Novel nanomaterials for clinical neuroscience. *J. Neuroimmune Pharm. 3*, 83−94.

(5) Keefer, E. W., Botterman, B. R., Romero, M. I., Rossi, A. F., and Gross, G. W. (2008) Carbon nanotube coating improves neuronal recordings. *Nat. Nanotechnol. 3*, 434−439.

(6) Kotov, N. A., Winter, J. O., Clements, I. P., Jan, E., Timko, B. P., Campidelli, S., Pathak, S., Mazzatenta, A., Lieber, C. M., Prato, M., Bellamkonda, R. V., Silva, G. A., Wong Shi Kam, N., Patolsky, F., and Ballerini, L. (2009) Nanomaterials for neural interfaces. *Adv. Mater. 21*, 3970−4004.

(7) Lee, W., and Parpura, V. (2009) Wiring neurons with carbon nanotubes. *Front. Neuroeng. 2*, 8.

(8) Shein, M., Greenbaum, A., Gabay, T., Sorkin, R., David-Pur, M., Ben-Jacob, E., and Hanein, Y. (2009) Engineered neuronal circuits shaped and interfaced with carbon nanotube microelectrode arrays. *Biomed. Microdevices 11*, 495−501.

(9) Malarkey, E. B., and Parpura, V. (2010) Carbon nanotubes in neuroscience. *Acta Neurochir. Suppl. 106*, 337−341.

(10) Fabbro, A., Villari, A., Laishram, J., Scaini, D., Toma, F. M., Turco, A., Prato, M., and Ballerini, L. (2012) Spinal cord explants use carbon nanotube interfaces to enhance neurite outgrowth and to fortify synaptic inputs. *ACS Nano 6*, 2041−2055.

(11) Pantarotto, D., Singh, R., McCarthy, D., Erhardt, M., Briand, J. P., Prato, M., Kostarelos, K., and Bianco, A. (2004) Functionalized carbon nanotubes for plasmid DNA gene delivery. *Angew. Chem., Int. Ed. 43*, 5242−5246.

(12) Al-Jamal, K. T., Gherardini, L., Bardi, G., Nunes, A., Guo, C., Bussy, C., Herrero, M. A., Bianco, A., Prato, M., Kostarelos, K., and Pizzorusso, T. (2011) Functional motor recovery from brain ischemic insult by carbon nanotube-mediated siRNA silencing. *Proc. Natl. Acad. Sci. U.S.A. 108*, 10952−10957.

(13) Ghoshmitra, S., Diercks, D. R., Mills, N. C., Hynds, D. L., and Ghosh, S. (2012) Role of engineered nanocarriers for axon regeneration and guidance: Current status and future trends. *Adv. Drug Delivery Rev. 64*, 110−125.

(14) Ijima, S. (1991) Helical microtubules of graphitic carbon. *Nature 354*, 56−58.

(15) Ajayan, P. M. (1999) Nanotubes from carbon. *Chem. Rev. 99*, 1787−1799.

(16) Prithu, S., and Prerit, A. (2008) Recent advances in carbon nanotube-based electronics. *Mater. Res. Bull. 43*, 2517−2526.

(17) Bianco, A., Kostarelos, K., and Prato, M. (2011) Making carbon nanotubes biocompatible and biodegradable. *Chem. Commun. (Cambridge, U.K.). 47*, 10182−10188.

(18) Yang, W., Ratinac, K. R., Ringer, S. P., Thordarson, P., Gooding, J. J., and Braet, F. (2010) Carbon nanomaterials in biosensors: should you use nanotubes or graphene? *Angew. Chem., Int. Ed. 49*, 2114−2138.

(19) Park, K. H., Chhowalla, M., Iqbal, Z., and Sesti, F. (2003) Single-walled carbon nanotubes are a new class of ion channel blockers. *J. Biol. Chem. 278*, 50212−50216.

(20) Feng, W., and Ji, P. (2011) Enzymes immobilized on carbon nanotubes. *Biotechnol. Adv. 29*, 889−895.

(21) Moon, H. K., Lee, S. H., and Choi, H. C. (2009) In vivo near-infrared mediated tumor destruction by photothermal effect of carbon nanotubes. *ACS Nano 3*, 3707−3713.

(22) Klumpp, C., Kostarelos, K., Prato, M., and Bianco, A. (2006) Functionalized carbon nanotubes as emerging nanovectors for the delivery of therapeutics. *Biochim. Biophys. Acta 1758*, 404−412.

(23) Mattson, M. P., Haddon, R. C., and Rao, A. M. (2000) Molecular functionalization of carbon nanotubes and use as substrates for neuronal growth. *J. Mol. Neurosci. 14*, 175−182.

(24) Hu, H., Ni, Y., Montana, V., Haddon, R. C., and Parpura, V. (2004) Chemically functionalized carbon nanotubes as substrates for neuronal growth. *Nano Lett. 4*, 507−511.

(25) Galvan-Garcia, P., Keefer, E. W., Yang, F., Zhang, M., Fang, S., Zakhidov, A. A., Baughman, R. H., and Romero, M. I. (2007) Robust cell migration and neuronal growth on pristine carbon nanotube sheets and yarns. *J. Biomat. Sci.-Polym. E 18*, 1245−1261.

(26) Malarkey, E. B., Fisher, K. A., Bekyarova, E., Liu, W., Haddon, R. C., and Parpura, V. (2009) Conductive single-walled carbon nanotube substrates modulate neuronal growth. *Nano Lett. 9*, 264−268.

(27) Cellot, G., Toma, F. M., Varley, Z. K., Laishram, J., Villari, A., Quintana, M., Cipollone, S., Prato, M., and Ballerini, L. (2011) Carbon nanotube scaffolds tune synaptic strength in cultured neural circuits: novel frontiers in nanomaterial-tissue interactions. *J. Neurosci. 31*, 12945−12953.

(28) Lovat, V., Pantarotto, D., Lagostena, L., Cacciari, B., Grandolfo, M., Righi, M., Spalluto, G., Prato, M., and Ballerini, L. (2005) Carbon nanotube substrates boost neuronal electrical signaling. *Nano Lett. 5*, 1107−1110.

(29) Mazzatenta, A., Giugliano, M., Campidelli, S., Gambazzi, L., Businaro, L., Markram, H., Prato, M., and Ballerini, L. (2007) Interfacing neurons with carbon nanotubes: electrical signal transfer and synaptic stimulation in cultured brain circuits. *J. Neurosci. 27*, 6931−6936.

(30) Cellot, G., Cilia, E., Cipollone, S., Rancic, V., Sucapane, A., Giordani, S., Gambazzi, L., Markram, H., Grandolfo, M., Scaini, D., Gelain, F, Casalis, L., Prato, M., Giugliano, G., and Ballerini, L. (2009) Carbon nanotubes might improve neuronal performance by favouring electrical shortcuts. *Nat. Nanotechnol. 4*, 126−133.
(31) Busch, S. A., and Silver, J. (2007) The role of extracellular matrix in CNS regeneration. *Curr. Opin. Neurobiol. 17*, 120−127.
(32) Fitch, M. T., and Silver, J. (2008) CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. *Exp. Neurol. 209*, 294−301.
(33) Rolls, A., Shechter, R., and Schwartz, M. (2009) The bright side of the glial scar in CNS repair. *Nat. Rev. Neurosci. 10*, 235−241.
(34) He, J., Wang, X. M., Spector, M., and Cui, F. Z. (2012) Scaffolds for central nervous system tissue engineering. *Front. Mater. Sci. 6*, 1−25.
(35) Spivey, E. C., Khaing, Z. Z., Shear, J. B., and Schmidt, C. E. (2012) The fundamental role of subcellular topography in peripheral nerve repair therapies. *Biomaterials 33*, 4264−4276.
(36) Corey, J. M., Lin, D. Y., Mycek, K. B., Chen, Q., Samuel, S., Feldman, E. L., and Martin, D. C. (2007) Aligned electrospun nanofibers specify the direction of dorsal root ganglia neurite growth. *J. Biomed. Mater. Res. A 83*, 636−645.
(37) Gui, X., Cao, A., Wei, J., Li, H., Jia, Y, Li, Z., Fan, L, Wang, K., Zhu, H., and Wu, D. (2010) Soft, highly conductive nanotube sponges and composites with controlled compressibility. *ACS Nano 4*, 2320−2326.
(38) Zhang, X., Prasad, S., Niyogi, S., Morgan, A., Ozkan, M., and Ozkan, C. S. (2005) Guided neurite growth on patterned carbon nanotubes. *Sensor Actuator B 106*, 843−850.
(39) Nguyen-Vu, T. D. B., Chen, H., Cassell, A. M., Andrews, R. J., and Meyyappan, M. (2007) Vertically aligned carbon nanofiber architecture as a multifunctional 3-d neural electrical interface. *IEEE Trans. Biomed. Eng. 54*, 1121−1128.
(40) Matsumoto, K., Sato, C., Naka, Y., Kitazawa, A., Whitby, R. L. D., and Shimizu, N. (2007) Neurite outgrowths of neurons with neurotrophin-coated carbon nanotubes. *J. Biosci. Bioeng. 103*, 216−220.
(41) Lewitus, D. Y., Landers, J., Branch, J. R., Smith, K. L., Callegari, G., Kohn, J., and Neimark, A V. (2011) Biohybrid carbon nanotube/agarose fibers for neural tissue engineering. *Adv. Funct. Mater. 21*, 2624−2632.
(42) Gheith, M. K., Sinani, V. A., Wicksted, J. P., Matts, R. L., and Kotov, N. A. (2005) Single-walled carbon nanotube polyelectrolyte multilayers and freestanding films as a biocompatible platform for neuroprosthetic implants. *Adv. Mater. 17*, 2663−2667.
(43) Wu, Z. R., Ma, J., Liu, B. F., Xu, Q. Y., and Cui, F. Z. (2007) Layer-by-layer assembly of polyelectrolyte films improving cytocompatibility to neural cells. *J. Biomed. Mater. Res. A. 81*, 355−362.
(44) Fabbro, A, Cellot, G, Prato, M, and Ballerini, L. (2011) Interfacing neurons with carbon nanotubes: (re)engineering neuronal signaling. *Prog. Brain Res. 194*, 241−252.
(45) Fabbro, A., Toma, F. M., Cellot, G., Prato, M., and Ballerini, L. (2012) Carbon Nanotubes and Neuronal Performance in *Nanomedicine and the Nervous System* (Martin, C. R., Preedy, V. R., and Hunter, R. J., Eds.), pp 183−206, Science Publishers, Enfield, NH.
(46) Ni, Y., Hu, H., Malarkey, E. B., Zhao, B., Montana, V., Haddon, R. C., and Parpura, V. (2005) Chemically functionalized water soluble single-walled carbon nanotubes modulate neurite outgrowth. *J. Nanosci. Nanotechnol. 5*, 1707−1712.
(47) Lu, Y., Li, T., Zhao, X., Li, M., Cao, Y., Yang, H., and Duan, Y. Y. (2010) Electrodeposited polypyrrole/carbon nanotubes composite films electrodes for neural interfaces. *Biomaterials 31*, 5169−5181.
(48) Voge, C. M., and Stegemann, J. P. (2011) Carbon nanotubes in neural interfacing applications. *J. Neural Eng. 8*, 011001.
(49) Sorkin, R., Greenbaum, A., David-Pur, M., Anava, S., Ayali, A., Ben-Jacob, E., and Hanein, Y. (2009) Process entanglement as a neuronal anchorage mechanism to rough surfaces. *Nanotechnology 20*, 015101.
(50) Larkum, M. E., Kaiser, K. M., and Sakmann, B. (1999) Calcium electrogenesis in distal apical dendrites of layer 5 pyramidal cells at a critical frequency of back-propagating action potentials. *Proc. Natl. Acad. Sci. U.S.A. 96*, 14600−14604.
(51) Markram, H., Lübke, J., Frotscher, M., and Sakmann, B. (1997) Regulation of synaptic efficacy by coincidence of postsynaptic APs and EPSPs. *Science 275*, 213−215.
(52) Dan, Y., and Poo, M. M. (2004) Spike timing-dependent plasticity of neural circuits. *Neuron 44*, 23−30.
(53) Waters, J., Schaefer, A., and Sakmann, B. (2005) Back-propagating action potentials in neurones: measurement, mechanisms and potential functions. *Prog. Biophys. Mol. Biol. 87*, 145−170.
(54) Zilberter, Y., Harkany, T., and Holmgren, C. D. (2005) Dendritic release of retrograde messengers controls synaptic transmission in local neocortical networks. *Neuroscientist 11*, 334−344.
(55) Kuczewski, N., Porcher, C., Ferrand, N., Fiorentino, H., Pellegrino, C., Kolarow, R., Lessmann, V., Medina, I., and Gaiarsa, J. L. (2008) Backpropagating action potentials trigger dendritic release of BDNF during spontaneous network activity. *J. Neurosci. 28*, 7013−7023.
(56) Massobrio, G., Massobrio, P., and Martinoia, S. (2008) Modeling the neuron-carbon nanotube-isfet junction to investigate the electrophysiological neuronal activity. *Nano Lett. 8*, 4433−4440.
(57) Pogodin, S., and Baulin, V. A. (2010) Can a carbon nanotube pierce through a phospholipid bilayer? *ACS Nano 4*, 5293−5300.
(58) Zucker, R. S., and Regehr, W. G. (2002) Short-term synaptic plasticity. *Annu. Rev. Physiol. 64*, 355−405.
(59) Catterall, W. A., and Few, A. P. (2008) Calcium channel regulation and presynaptic plasticity. *Neuron 59*, 882−901.
(60) Fioravante, D., and Regehr, W. G. (2011) Short-term forms of presynaptic plasticity. *Curr. Opin. Neurobiol. 21*, 269−274.
(61) Abbott, L. F., Varela, J. A., Sen, K., and Nelson, S. B. (1997) Synaptic depression and cortical gain control. *Science 275*, 220−224.
(62) Schneggenburger, R., Sakaba, T., and Neher, E. (2002) Vesicle pools and short-term synaptic depression: lessons from a large synapse. *Trends Neurosci. 25*, 206−212.
(63) Corey, J. M., and Feldman, E. L. (2003) Substrate patterning: an emerging technology for the study of neuronal behavior. *Exp. Neurol. 184*, S89−96.
(64) Chen, S. X., Tari, P. K., She, K, and Haas, K. (2010) Neurexin-neuroligin cell adhesion complexes contribute to synaptotropic dendritogenesis via growth stabilization mechanisms in vivo. *Neuron 67*, 967−983.
(65) Ferrari, A., Cecchini, M., Serresi, M., Faraci, P., Pisignano, D., and Beltrami, F. (2010) Neuronal polarity selection by topography-induced focal adhesion control. *Biomaterials 31*, 4682−4694.
(66) Mitra, S. K., Hanson, D. A., and Schlaepfer, D. D. (2005) Focal adhesion kinase: in command and control of cell motility. *Nat. Rev. Mol. Cell. Biol. 6*, 56−68.
(67) Hoffman, B. D., Grashoff, C., and Schwartz, M. A. (2011) Dynamic molecular processes mediate cellular mechanotransduction. *Nature 475*, 316−323.
(68) Jin, G. Z., Kim, M., Shin, U. S., and Kim, H. W. (2011) Neurite outgrowth of dorsal root ganglia neurons is enhanced on aligned nanofibrous biopolymer scaffold with carbon nanotube coating. *Neurosci. Lett. 501*, 10−14.
(69) Myers, J. P., and Gomez, T. M. (2011) Focal adhesion kinase promotes integrin adhesion dynamics necessary for chemotropic turning of nerve growth cones. *J. Neurosci. 31*, 13585−13595.
(70) Berardi, N., Pizzorusso, T., and Maffei, L. (2004) Extracellular matrix and visual cortical plasticity: freeing the synapse. *Neuron 44*, 905−908.
(71) Shein-Idelson, M., Ben-Jacob, E., and Hanein, Y. (2011) Engineered neuronal circuits: a new platform for studying the role of modular topology. *Front. Neuroeng. 4*, 10.
(72) Galante, M., Nistri, A., and Ballerini, L. (2000) Opposite changes in synaptic activity of organotypic rat spinal cord cultures after chronic block of AMPA/kainate or glycine and $GABA_A$ receptors. *J. Physiol. 523*, 639−651.