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Functionalized carbon nanotube microfibers for chronic neural implants

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ABSTRACT

Background: Much progress has been made at the interface between neural tissue and electrodes for neurophysiology. However, there continues to be a need for novel materials that integrate well with the nervous system and facilitate neural recordings with longer-term sustainability and stability. Such materials have the potential to improve clinical approaches and provide important tools for basic neuroscience research.
New method: In this paper, we explore the use of dry-spun untreated or functionalized carbon nanotube fibers as implantable electrodes for neural recordings from insects over extended time periods.
Results: Measurements of fly eyes responding to light flashes illustrate the suitability of these materials for recording both the low- and high-frequency components of neural signals. Repeated recordings show good sustainability, especially with functionalized carbon nanotube fibers. In particular, recordings from the optic lobes of Madagascar hissing cockroaches last for at least 8 weeks.
Comparison with existing method(s): Electrophysiological research continues to rely heavily on metal electrodes that are good for short-lived preparations but less suitable for longer-term recordings, as scar tissue formation and cytotoxicity tend to cause a gradual reduction in signals.
Conclusions: Functionalized carbon nanotubes are a promising novel material that can be used to obtain long-term or repeated stable recordings, which are necessary for longitudinal studies, or to maintain other neural tissue interfaces such as those in insect–machine hybrid robots. The introduced insect preparation can also be used for the relatively rapid and cost-efficient testing of novel electrode materials.

1. Introduction

There is much need for novel technologies that allow the integration of biological tissues with electronic circuitry. In particular, tight interactions are required between stimulation or recording devices and the nervous system. Some clinically important approaches have been developed, such as deep brain stimulation for Parkinson's disease and other neurological conditions (Pena et al., 2007). Other approaches are geared toward basic research, including a variety of projects that involve insects. For example, controlling the movement of insects using wearable electronics has previously been proposed as a viable strategy to assist in search and rescue missions. Toward that end, substantial efforts have been made to optimize stimulation parameters that, for example, allow humans to direct the movements of cockroaches (Erickson et al., 2015). The benefit of using insect–machine hybrid robots as important research tools has also been recently recognized (Ando and Kanzaki, 2020). Thus far, such technology has been implemented in a variety of insects during walking and even during flight (Li and Sato, 2018; Sato and Maharbiz, 2010).

One limitation that has impeded progress in this line of research is the availability of biocompatible electrodes that allow for chronic implantation and longer-term experiments. A variety of materials, often in the form of small probes, have been used as electrodes that are inserted into an insect shortly prior to the experiment, with stimulation and or recording typically maintained for up to a few hours. Several studies geared towards remotely controlling insect flight, have reported successful interfaces for 30 min intervals (reviewed by Sato and Maharbiz, 2010). Using fiber tetrodes to record from freely moving cockroaches allowed for a successful machine–neuron interface for 2–4 h (Guo et al., 2014). Regarding sustainability, perhaps the most impressive study involved the use of flexible cuff electrodes to record from the neck of a tropical bush cricket, which allowed responses to be picked up 3 days post implantation (Hartbauer et al., 2012).

The majority of electrodes are made from relatively stiff materials, which likely has a negative effect on durability. From chronically implanted electrodes in vertebrates, it has become clear that electrode

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rigidity can lead to scar tissue formation, presumably due in part to physical abrasions caused by constant micromotions (Gilletti and Muthuswamy, 2006). Such scar tissue can gradually attenuate the contact between the recording or stimulating probe and the neural tissue. Another possible reason for scar tissue formation is the toxicity exerted by some metals. Recent advances in neuroscience research have shown carbon nanotubes (CNTs) to be a particularly promising material for interfaces between metal/alloy-based electrodes and neurons (Scaini and Ballerini, 2018; Shi et al., 2016; Vitale et al., 2015). CNTs are tubular molecular structures synthesized using carbon, with a diameter of less than 100 nanometers. They are typically synthesized in a chemical vapor deposition reactor on a silicon substrate. Due to van der Waals forces acting among them a vertical array of CNTs can be transformed into a soft fiber-like material by dry spinning (Alvarez et al., 2015, 2019). This involves the manual pulling together of arrays of CNT molecules, and physically twisting them into a bundle. This method is particularly beneficial, as it directly utilizes pristine CNT that are free of catalysts and hence minimally contaminated with potential toxins. These arrays need no further chemical processing; instead the physical action of pulling and twisting allows them to self-assemble in air, into a continuous ribbon that can be spun into a fiber of the desired diameter (from ten to hundreds of micrometers). The resulting fiber is soft and flexible, similar to a thread of yarn, while also being conductive and, due to its microstructure, having a large surface area. The suitability of this material for neural recordings and stimulation has already been demonstrated for surface recordings on fly eyes and the internal activation of the antennal nerve in Madagascar hissing cockroaches (Alvarez et al., 2020). Material characterization showed that in addition to being electrically conductive, CNT fibers have a high rate of electron transfer and low impedance when compared to gold and silver wires of comparable diameters (Alvarez et al., 2020). It is important to note that in this forerunner from our group, the focus had been on exploring the principal suitability of these CNT fibers for neurophysiology. Based on our encouraging results, we here take our studies a step further into the exploration of another variant of CNT fiber as well as the suitability of the material for recordings over extended time periods, exploring the interaction of the fiber with the surrounding neural tissue. Encouraging other data exists, such as that the enhanced electron transfer ability of CNT materials has also previously been used for implants in *Manduca* moths (Tsang et al., 2012). In this case, the integration of CNT–gold nanocomposites into the probes helped reduce the interfacial electrical impedance in a setup for recording from and stimulating freely flying moths.

Dry-spun CNT fibers can be further modified, for example, by coating (Alvarez et al., 2014, 2013), and cut to desired lengths or otherwise configured for integration into microelectrodes. In addition, such fibers can be chemically altered, including by acetone treatment, which results in densification (Alvarez et al., 2014; Liu et al., 2010), and by plasma functionalization, which introduces carboxyl and hydroxyl groups, thus rendering the fibers hydrophilic (Adusei et al., 2019). We therefore refer to them here as HCNT (hydrophilic CNT) fibers. Due to the wet nature of animal tissue, the latter method has been shown to improve the fiber–tissue interface (Vardharajula et al., 2012) that could potentially result in enhanced sustainability for physiological recordings.

In this study, we examined the suitability of dry-spun CNT fibers for extended recording from insects into which they have been implanted and left for extended periods of time. To contrast the efficiency and durability of pristine and functionalized CNT fibers with the performance of other materials, we also implanted silver and gold wire electrodes. To test functionality, we obtained electroretinograms (ERGs), which show the electrical signal of photoreceptors in response to light flashes and compared the quality and amplitude of the recordings obtained 1 and 4 days post electrode implantation in flies (*Sarcophaga bullata*). In addition, we obtained tissue samples to evaluate the fiber–tissue interface 4 days post implantation using histological and transmission electron microscopy imaging. Finally, we evaluated the durability of our functionalized fibers by implanting them into the eyes of long-lived Madagascar hissing cockroaches (*Gromphadorhina portentosa*). Neural signals were successfully obtained for 8 weeks or longer, which to the best of our knowledge sets a new longevity record for implanted recording electrodes in insects. Taken together, our studies on insects illustrate new approaches for obtaining stable long-term recordings. Moreover, the introduced preparation allows for the relatively quick and efficient evaluation of the performance of new materials for chronic neural recordings, thus identifying materials that will also likely be beneficial for neurophysiological studies in vertebrates. We found that functionalized, dry-spun CNT fibers are a particularly promising material for future applications involving insects with wearable electronics and other brain tissue electronic interfaces.

2. Methods

2.1. Animals

S. bullata flies were obtained as pupae from VWR (Radnor, PA) and raised in incubators at 25 °C with access to sugar and water. They were kept under a light/dark cycle of 14 h light and 10 h dark. Madagascar hissing cockroaches (*G. portentosa*) were the offspring of individuals kindly provided by the laboratory of Dr. Josh Benoit (University of Cincinnati). The cockroaches were raised on a combination diet of cat and dog food, supplemented by apples, carrots, and other vegetables, while kept in a laboratory at approximately 23 °C with access to natural daylight through a nearby window. Juveniles were separated as late nymphs and selected for experiments within the first 2 months of maturation.

Surgical implantation of electrodes and electrophysiology: To evaluate the suitability of different electrode materials for neurophysiological recordings, we implanted various electrodes into the eyes of flies (*S. bullata*). To do so flies were first anesthetized on ice and then kept on top of an ice block for the duration of the surgery. As electrode materials are too soft to penetrate the cuticle, a small hole needed to be made on the eye surface which then could be used as entrance point for the electrode. To do so a sharp insect pin was used, and gently pressed onto the eye surface while moved along the eye surface, till it was able to penetrate the layer of cuticular lenses. Immediately after the hole was present, a short piece of electrode was inserted through that hole, through the underlying ommatidia and into the optic lobes. The latter two types of tissue were sufficiently soft for all the materials to be successfully penetrated. Particular care was taken to insert electrodes of all materials approximately equally deep into the tissue. Once the electrode was positioned, the hole was sealed with dental wax. A second electrode, which served as a reference electrode, was inserted and secured in a similar fashion near the wing base. To minimize bias through the order of implantation, flies were implanted in small batches, with several materials being implanted in each session. At a later time (3–6 h for the first recording and 69–80 h for the second recording), the flies were mounted with wax onto a stick and placed into the physiology rig. For recordings, electrically conductive paint (#474-COM-11521, Mouser Electronics, Mansfield, TX) was used to make contact between the portion of the implanted electrode extending outside the head or thorax and the silver wire connected to the physiology rig. For the cockroaches, electrodes were implanted in a similar way, except conductive glue was used to permanently attach the electrodes to a small plug. In addition, small steel plates were attached to the thorax and abdomen, which allowed the cockroaches to be held in place using magnets. This setup enabled repeated recordings to be taken after each cockroach was plugged into the physiology and secured via magnetic attachment (Fig. 5B). The first recording was typically performed 5–8 days post implantation with subsequent recordings obtained on a weekly basis. All recordings were performed in a Faraday cage using standard electrophysiological equipment, including an A-M Systems 3000 AC/DC differential amplifier with a differential head stage (A-M Systems, Inc.,

Sequim, WA), a Tektronix 5111 A oscilloscope (Tektronix, Inc., Beaverton, OR), and an iWorx data acquisition system (HAI 118, iWorx Systems, Inc., Dover, NH). Data were acquired at a sampling rate of 10,000 Hz, stored on a PC using iWorx LabScribe software (iWorx Systems, Inc., Dover, NH), and analyzed using customized programs (available upon request) in MATLAB (The MathWorks, Inc., Natick, MA) that specifically detected the magnitude of the photoreceptor response to light stimulation. Light stimulation was provided by an LED (LED490–06, Roithner Lasertechnik GmbH, Vienna, Austria) with an attached optical fiber (FB143–500-ND, Digi-Key, Thief River Falls, MN), the end of which was placed near the surface of the eye. To ensure that the receptors were not saturated for our evaluation, we performed recordings at 8 different intensity levels (4.28×10^{10}, 2.78×10^{11}, 7.20×10^{11}, 1.75×10^{12}, 5.42×10^{12}, 1.04×10^{13}, 5.53×10^{13}, and 1.07×10^{14} photons/cm^2/s). Based on these pilot experiments, a light intensity of 5.53×10^{13} photons/cm^2/s was chosen, as the signal was well below saturation.

Electrode materials: The following electrode materials were evaluated: (1) silver wire (size 0.002, 99.99%, California Fine Wire Company, Grover Beach, CA); (2) gold wire (size 0.002, 99.99%, California Fine Wire Company, Grover Beach, CA); (3) CNT fiber fabricated as described in Alvarez at al. (2020), which is referred to as "CNT fiber or CNT electrode" with 50 μm diameter; and (4) functionalized CNT fiber fabricated using oxygen plasma treatment, as previously reported (Adusei et al., 2019), which increased the hydrophilicity of the CNT fiber for an improved fiber–tissue interface and hence is referred to as "HCNT fiber or HCNT electrode". These electrodes were 50 μm for the fly and 90 μm for the cockroach experiments. Although the CNT and HCNT fibers that were used in these experiments are currently not commercially available, they can potentially be obtained by request, and similar fibers are also available through Huntsman International LLC (The Woodlands, TX). All electrode materials were simply cut at the insertion site with small scissors, leading to a slightly compressed end-configuration with no specific sharpening.

Histological assessment: To obtain histological images of neural tissue (optic lobes) and electrode interfaces, fly heads were fixed at 4 °C in a solution of 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in Sorensen's phosphate buffer (pH 7.4) for up to 24 h. Subsequently, the tissue was rinsed and fixed in a 1% osmium tetroxide (OsO_4) solution for 1 h on ice and then 1 h at room temperature. Next, the tissue was rinsed with water before being dehydrated in a series of increasingly concentrated ethanol solutions and finally acetone. The tissue was then embedded in an ultralow viscosity embedding medium (Polysciences, Inc., Warrington, PA), sectioned at intervals of 8–10 μm, and mounted on slides. Images of representative sections were taken using a Retiga 2000R digital camera. For each preparation, care was taken to focus on sections that showed minimal electrode insertion-related injury in the immediate vicinity, which however resulted in some variation of exactly which part of the fly eye was imaged. Between 5 and 9 successful preparations were evaluated for each electrode type.

Transmission Electron microscopy: Tissue for electron microscopy analyses was processed, with a few modifications, as described in Wolff (2011). In brief, flies which had electrodes inserted for about 3 days were anesthetized on ice and then fixed with an EM fixative composed of 4% paraformaldehyde, 3.5% glutaraldehyde, and 1% tannic acid in Sorensen's phosphate buffer (all from Electron Microscopy Sciences, Hatfield, PA). The heads were separated from the body and bisected sagittally with careful attention paid to the eyes and inserted electrodes to avoid damaging any relevant tissue. After fixation, the tissue was washed with Sorensen's phosphate buffer (pH 7.2) and placed in 2% OsO_4 (Electron Microscopy Sciences, Hatfield, PA) on ice for 1 h and then at room temperature for 1 h. Subsequently, the tissue was washed, placed in 2% uranyl acetate for 16–20 h, washed again, and dehydrated in a series of increasingly concentrated ethanol solutions and then acetone. After embedding the tissue in an ultralow viscosity embedding medium (Polysciences, Warrington, PA) following the manufacturer's recommendations, the polymer blocks were sectioned with a rotary microtome at 8 μm intervals until the area of interest was close to the surface. Semithin and ultrathin sections of the resin-embedded samples were then obtained using a Reichert Ultracut E ultramicrotome. Resin-embedded samples sectioned at ~80 nm were visualized using a JEOL JEM 1230 transmission electron microscope with an AMT Advantage Plus 2k × 2k digital camera.

3. Results

3.1. Evaluation of electrode materials using photoreceptor responses of fly eyes

As a first step toward evaluating the suitability of different materials for experiments that require the longer-term implantation of electrodes, we implanted gold, silver, CNT, and HCNT electrodes into the eyes of *S. bullata* flies. These implants were then used to extracellularly record the electric response (ERG) of the photoreceptors and first-order interneurons on the first and fourth days post implantation. ERGs differ slightly depending on the organism. In flies (Belušič, 2011), the ERG is complex (Fig. 1A), with a transient positive response (indicative of the synaptic activation of first-order interneurons), a sustained negative receptor potential, and a transient negative response (also indicative of the activation of first-order interneurons). Gold electrodes were implanted in 12 flies, which gave an average response of 2.4 mV (s = 1.0) on day 1. Six of these flies also gave viable recordings on day 4, with an average response of 2.6 mV (s = 1.5). A comparison of the individuals with successful recordings on both days (Fig. 1B) revealed that five out of six showed a slightly lower signal 4 days post implantation as compared to 1 day post implantation. Nine flies were successfully implanted with silver wire electrodes, resulting in an average receptor potential of 3.0 mV (s = 1.1), and six flies still had viable signals on day 4, with an average response of 2.3 mV (s = 1.2). A comparison of the individuals with successful recordings on both days (Fig. 1C) showed a decrease in the response for five of the flies, whereas an increased response was observed for one fly. The fourteen flies with successfully implanted CNT fiber electrodes gave an average response of 1.7 mV (s = 0.7). Eight flies still had viable signals on day 4, with an average response of 1.7 mV (s = 1.1). A comparison of the individuals with successful recordings on both days showed some improvement in the signal for four of the eight flies (Fig. 1D). Fourteen flies were also implanted with HCNT fiber electrodes, showing an average response of 1.8 mV on day 1 (s = 0.9). Twelve flies also gave successful recordings on day 4, with an average response of 2.4 mV (s = 0.8). A comparison of the individuals with successful recordings on both days showed an improvement in the signal on day 4 for 10 of the 12 flies (Fig. 1E). Based on a two-way ANOVA there was a significant difference between electrode types (with $p < 0.5$), but not for day 1 versus day 4, and not for the interaction term. Based on a post hoc Tukey test, the only significance was between silver and CNT electrodes (with silver electrodes resulting in stronger signals than the CNT electrodes). To further evaluate the sustainability of these electrodes over 4 days, we calculated the % change for each group (Fig. 1F). The only electrode type that showed a positive change was the HCNT electrodes, with an average improvement of 49%. Based on a single-factor ANOVA, there was a significant difference among these groups ($p = 0.0095$). Based on a Tukey–Kramer post-hoc test, there was a significant difference specifically between HCNT and silver electrodes.

A critical property of an electrode for neural recordings is its ability to accurately capture the higher-frequency components of a signal. To evaluate the electrode materials in that capacity, we examined the on- and off-transients of the ERG signal (Fig. 2A), both of which are indicative of the activation of first-order interneurons in the fly visual system (Belušič, 2011). A single-factor ANOVA of all the day 1 and day 4 datasets revealed no significant differences between any of the groups. The on-transients noticeably improved between day 1 and day 4 for all

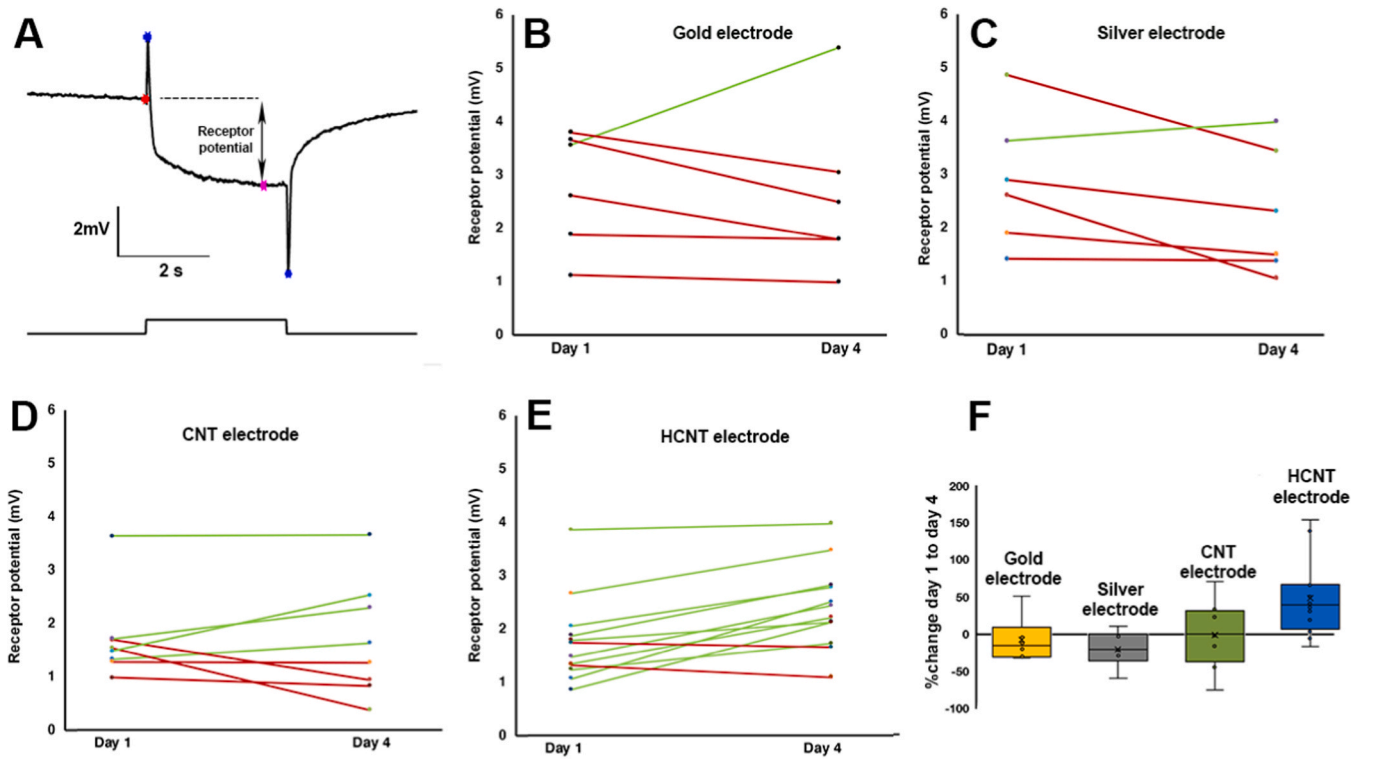

Fig. 1. Sustainability of implanted electrodes based on the receptor potential of flies. A. ERG recording with an HCNT electrode 4 days after implantation. The ERG response in flies has several components, including a sustained receptor potential that appears as an electronegative response (top trace) for the duration of light stimulation (bottom trace). B-E. Comparisons of the magnitudes of the photoreceptor responses between day 1 and day 4 recordings, with green lines indicating increases, and red lines indicating decreases. B. With gold electrodes the detected signal decreased for all but one of the implanted flies. C. With silver electrodes the detected signal decreased for all but one of the implanted flies. D. With CNT electrodes the detected signal decreased in half of the flies. E. With HCNT electrodes the detected signal increased in all but two of the implanted flies. F. Comparison of the % change in signal amplitude for each material from day 1 to day 4. An increase in signal strength was only observed for HCNT electrodes.

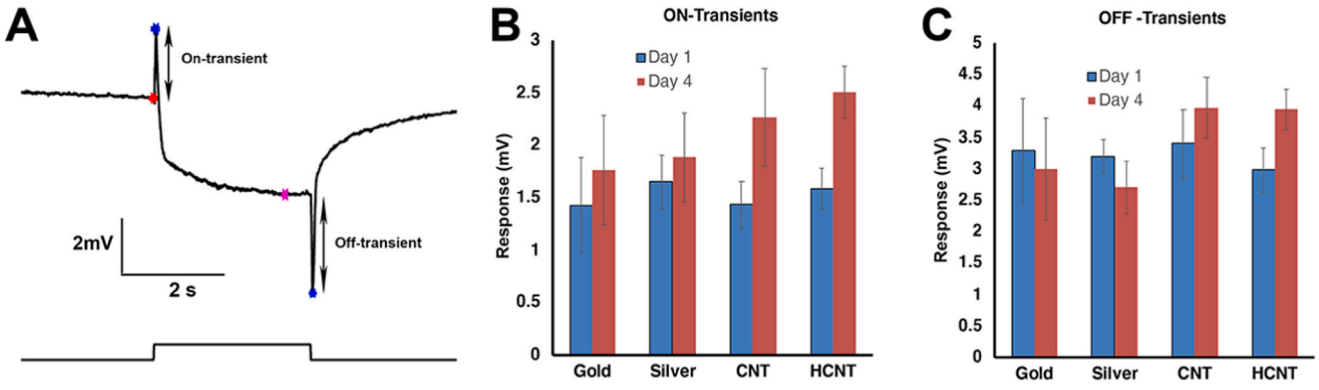

Fig. 2. Sustainability of implanted electrodes based on ERG transients in flies. A. ERG recording with an HCNT electrode 4 days after implantation. The ERG response in flies has several components, including on- and off-transients in response to the onset (top trace) and termination of light stimulation (bottom trace). B. On-transient detected using different electrodes. All electrode types clearly resolved the on-transients. A comparison of the on-transients between day 1 and day 4 recorded with different electrode materials revealed a general trend of increased signals over the investigated time period, with a significant increase observed for HCNT electrodes. C. Off-transient detected using different electrodes. All electrode types also resolved the off-transients.

four electrode materials (Fig. 2B). A Student's *t*-test revealed a significant difference (with $p = 0.08$ in a two-tailed test) between the two sets of HCNT electrode measurements. However, there were no significant differences between the two measurements with any of the other electrode materials. Relatively few changes were observed between the day 1 and day 4 measurements of the off-transients (Fig. 2C). Apart from a one tailed Student's *t*-tests test between day 1 and day 4 for the HCNT electrode (p value of 0.028) none of the comparisons between the day 1 and day 4 recordings for any of the electrode materials revealed a significant difference.

3.2. Histological and ultrastructural assessment of electrode materials

To gain insights into how the different electrode materials interact with neural tissue, we performed a basic histological assessment. Ethyl gallate staining was used to visualize the interface between each electrode type and the surrounding neural tissue. After completion of the electrophysiological experiments, we examined some of the flies in

which electrodes had been implanted 4 days earlier (Fig. 3). The obtained histological cross-sections illustrate that each electrode type was indeed implanted in neural tissue, albeit there was some variation in exactly how each electrode was positioned, and from where within the neuropil sections were taken. As shown in Fig. 3A, with the gold electrode, slight distortions occur in the surrounding tissue. In particular, small gaps between the electrode and surrounding tissue were visible with most gold and silver electrodes (Fig. 3A and B) but not with the CNT and HCNT fibers (Fig. 3C and D). In some preparations, the nearby tissue showed signs of stress and degeneration, including the formation of vacuoles. Although these effects were very difficult to quantify, and in part may have related to implantation related injury, it is noteworthy that they appeared to be particularly common in preparations with silver electrodes. Both the CNT and HCNT electrodes appeared to integrate particularly well with the surrounding tissue, with a generally tight interface.

To further investigate the interactions between the CNT and HCNT fibers and neural tissue, we examined the preparations using transmission electron microscopy (Fig. 4). The low-magnification cross-sectional image of a CNT fiber electrode embedded in resin reveals a dense meshwork of fibers (Fig. 4A), with relatively loose and corrugated borders. In the high-magnification image (Fig. 4B), individual CNTs are visible, many of which are relatively loose at the border between the CNT fiber and the surrounding embedding medium. Fig. 4C shows a cross-sectional image of a CNT fiber that was embedded in the fly optic neuropil for 4 days prior to fixing the tissue. Here, a relatively sharp border exists at the interface between loose CNT bundles and neural tissue. In contrast, densification is observed around the perimeter of the HCNT fiber and its interface with the surrounding tissue is particularly tight (Fig. 4D).

3.3. *Long-term chronic implantation of HCNT electrodes in Madagascar hissing cockroaches*

To evaluate the suitability of HCNT electrodes for measurements over extended time periods, we implanted HCNT fibers into both eyes of long-lived Madagascar hissing cockroaches, which were attached with conductive glue to a small plug that was secured to the cockroach using super glue. Small steel plates were also attached, allowing an ERG recording from each cockroach to be obtained on a weekly basis by simply attaching them to a magnetic holder and plugging them into the physiology rig (Fig. 5A and B). The ERG signal shape in insects can be variable, and on- and off- transients have not always been observed, including in cockroaches. For example, they have been observed in the cockroach *Leucophaea maderae* (Colwell and Page, 1989) but not in *Blattella germanica* (Chang and Lee, 2001), and variable shapes and amplitudes even were found between different recording positions on the eye surface (French et al., 2015). While it is not completely clear what causes these differences, it is know that some of the ERG properties in compound eyes are influenced by a resistance barrier within their eyes (Heisenberg, 1971; Shaw, 1975). Our recordings did not have on- and off-transients but were characterized by a sustained electronegative response to a light flash. Fig. 5C illustrates the response from an implanted cockroach, with an average amplitude of 1.9 mV for the first recording. After 8 weeks, a comparable recording was achieved from the same individual (Fig. 5D), with an average amplitude (across individuals) of 1.6 mV. Eight cockroaches had successful implants in the left eye; however, two were excluded from the dataset because they only allowed for recordings during the first week. For the remaining cockroaches, data from two implants were obtained for 6 weeks, whereas the other four implants lasted at least 8 weeks, with one lasting 10 weeks and one 12 weeks (not illustrated). Of the four successful implants into the right eye, one lasted at least 6 weeks, one 8 weeks, one 10 weeks, and one 12 weeks. Recordings also were attempted with implanted gold wires, but these were unsuccessful as the material was too soft to persist. Fig. 5E illustrates the average photoreceptor response over 8 weeks. Substantial variation arose, with the signals from some individuals being more discernable than those from others. To better illustrate how well the signal was maintained over the 8 week period, we obtained the relative photoreceptor response by normalizing the signal to the maximum value for each cockroach eye (Fig. 5F).

4. Discussion

Within the last decade, there has been a remarkable increase in technologies and materials that can potentially be used to interface with biological tissues (Rastogi et al., 2018; Scaini and Ballerini, 2018). Among them, novel electrode approaches based on dry-spun CNT fibers are particularly promising (Alvarez et al., 2020; McCallum et al., 2017). Although CNTs are hydrophobic and potentially toxic (Ali-Boucetta et al., 2011), a variety of approaches have emerged to improve their interactions with tissue, including functionalization approaches that

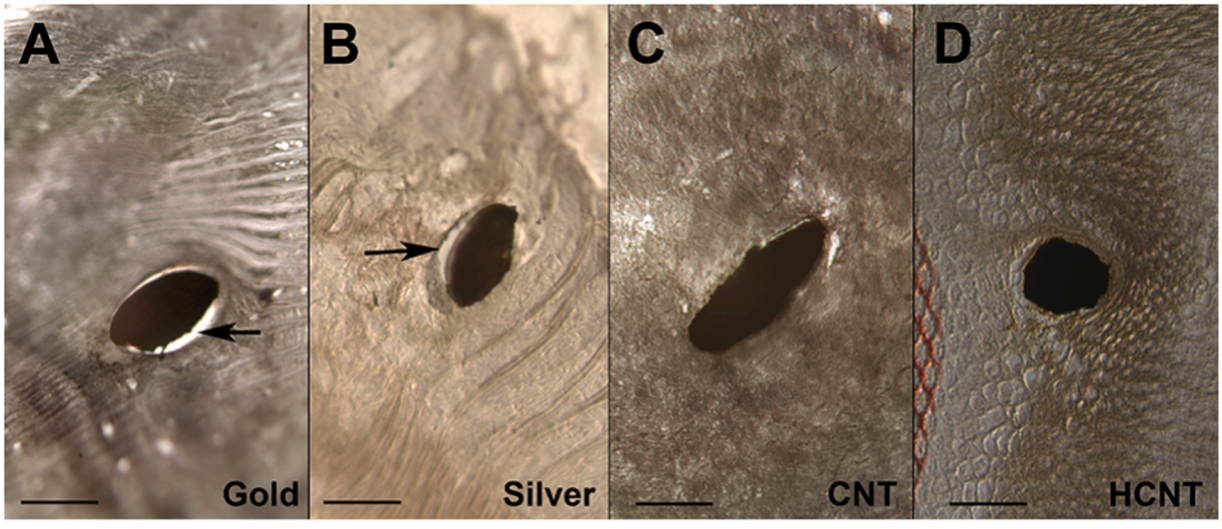

Fig. 3. Histological examination of electrode–tissue interfaces. A. Cross-section of a gold electrode that was implanted into the optic lobe of a fly 4 days prior to fixing the tissue. A small gap exists between the metal electrode and the tissue (arrow). B. Cross-section of a silver electrode, which shows a similar gap at the metal–tissue interface (arrow). C. Cross-section of a CNT electrode, which shows a relatively tight interface with the neural tissue. D. Cross-section of an HCNT electrode, which shows a seamless interface with the fly neural tissue, with very little to no disturbance of the surrounding tissue. Scale bars represent 50 μm.

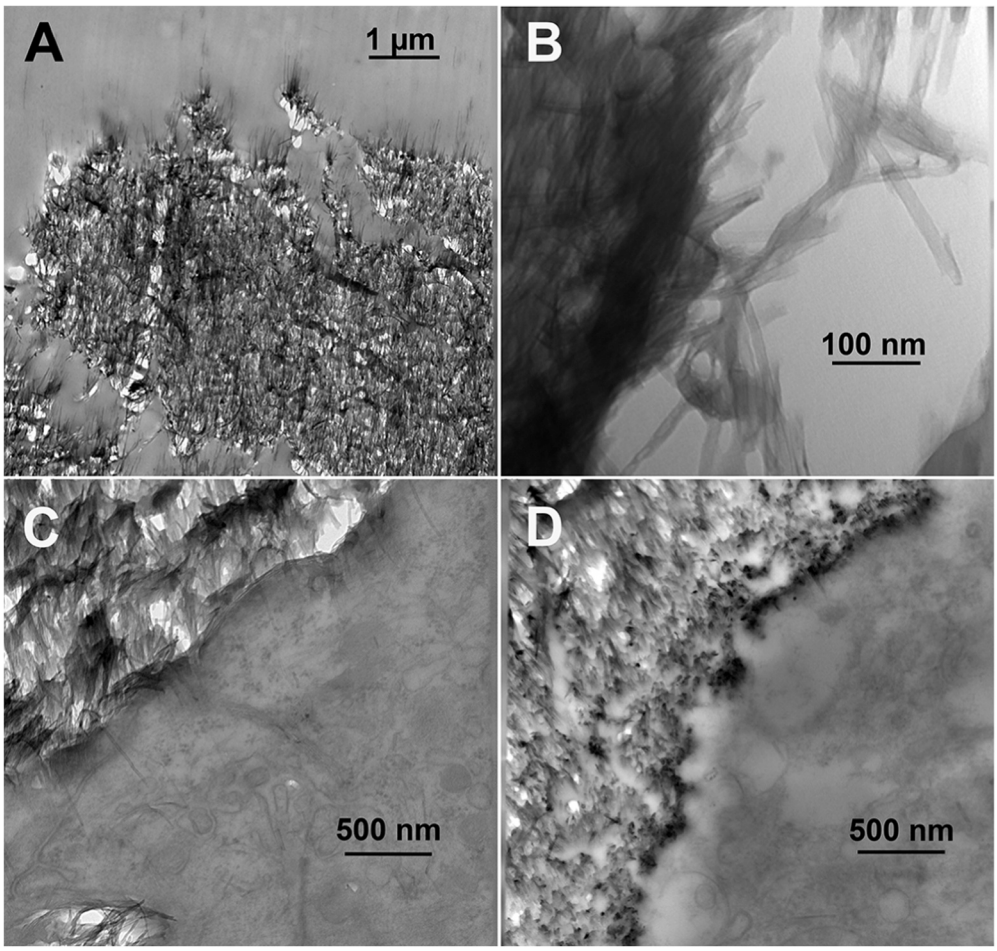

Fig. 4. Ultrastructural investigation of CNT and HCNT fiber electrodes. A. Low-magnification cross-sectional image of a CNT fiber embedded directly in resin showing that the internal organization of these electrodes is based on a dense array of CNT fibers. B. Corresponding high-magnification image showing individual CNTs and bundles, some of which are loosely arranged near the periphery of the electrode. C. Cross-sectional image of a CNT fiber electrode implanted into a fly optic neuropil 4 days prior to fixation. A relatively sharp border exists between the neural tissue and the hydrophobic electrode. D. Cross-sectional image of an HCNT fiber implanted into a fly optic neuropil 4 days prior to fixation. The HCNT is visibly denser at its periphery, which appears to allow for very close contact with the neural tissue, possibly due to its hydrophilic nature.

alter the outer CNT surface (Vardharajula et al., 2012). However, the practical performance of many possible configurations remains unclear. In addition, the diversity of new materials and the availability of techniques to modify existing materials highlight a need for relatively quick and cheap methods to test different electrode designs. Currently, bioelectronic approaches are typically explored in rodents (Li et al., 2020), and there is a need for paradigm organisms that are easier to work with. In this study, we demonstrated how the chronic implantation of electrodes into the insect visual system, which is part of their central nervous system, can be used in this context.

A successful electrode needs to make good connections, have a high electron transfer ability and good conductance, and remain stable over long periods of times. In addition, it is important to capture the higher frequency components of a signal. A comparison of the abilities of silver, gold, untreated CNT, and functionalized CNT (HCNT) electrodes to capture neural activation patterns from fly eyes suggested that HCNT fibers are particularly promising. In this experiment, different electrodes were implanted invasively into the optic neuropils of flies, which are part of their central nervous system, in order to assess the biological compatibility of electrodes to tissue and evaluate sustainability of recordings over a period of time. The flies were then exposed to short flashes of light and the neural response was recorded as a typical ERG. The ERG recording consisted of a sustained component that directly represented the response of the photoreceptor cells (Belušič, 2011), which in flies are neurons. For all four investigated materials, the sustainability of some preparations was sufficient to obtain successful recordings on both the day of electrode implantation and 4 days later. However, sustained recordability was only achieved for 50% of the flies implanted with gold electrodes, 66% implanted with silver electrodes, 57% implanted with untreated CNT electrodes, and 85% implanted with HCNT electrodes. While these differences could be attributable to a variety of factors (such as minor differences during the electrode placement surgery), the relatively high rate of successful day 4 recordings for HCNT electrodes is suggestive of a particularly compatible electrode configuration. A similar trend was observed for the fraction of experiments in which the day 4 recording showed a higher signal than the day 1 recording (Fig. 1). Specifically, only 1 out of 6 recordings showed an improvement in the signal for silver or gold electrodes, half showed improvement for untreated CNT electrodes, and 10 out of 14 showed improvement for HCNT electrodes. Our experiments show that during the relatively short time frame that we addressed in these experiments, all materials were adequate to get recordings on both days, albeit the CNT electrodes performed significantly worse than the silver electrodes. At the same time, we observed consistent improvement primarily in HCNT electrodes. Looking at the percentage change in the overall signal strength, only the HCNT electrodes showed improvement (Fig. 1F), with a significant difference when compared to the silver electrodes. Although additional experiments will be necessary to determine possible differences between these materials at longer time scales, and if carbon nanotube-based electrodes indeed can sustain recordings beyond what could be done with metal electrodes, these findings are encouraging in terms of the HCNT configuration forming a particularly sustainable electrode interface. All the electrode materials performed well in regard to the higher-frequency components of the ERG, which are indicative of the synaptic activation of the interneurons of the visual pathway (Heisenberg, 1971). A single-factor ANOVA of all the day 1 and day 4 datasets revealed no significant differences between any of the groups in either scenario, indicating that all the materials

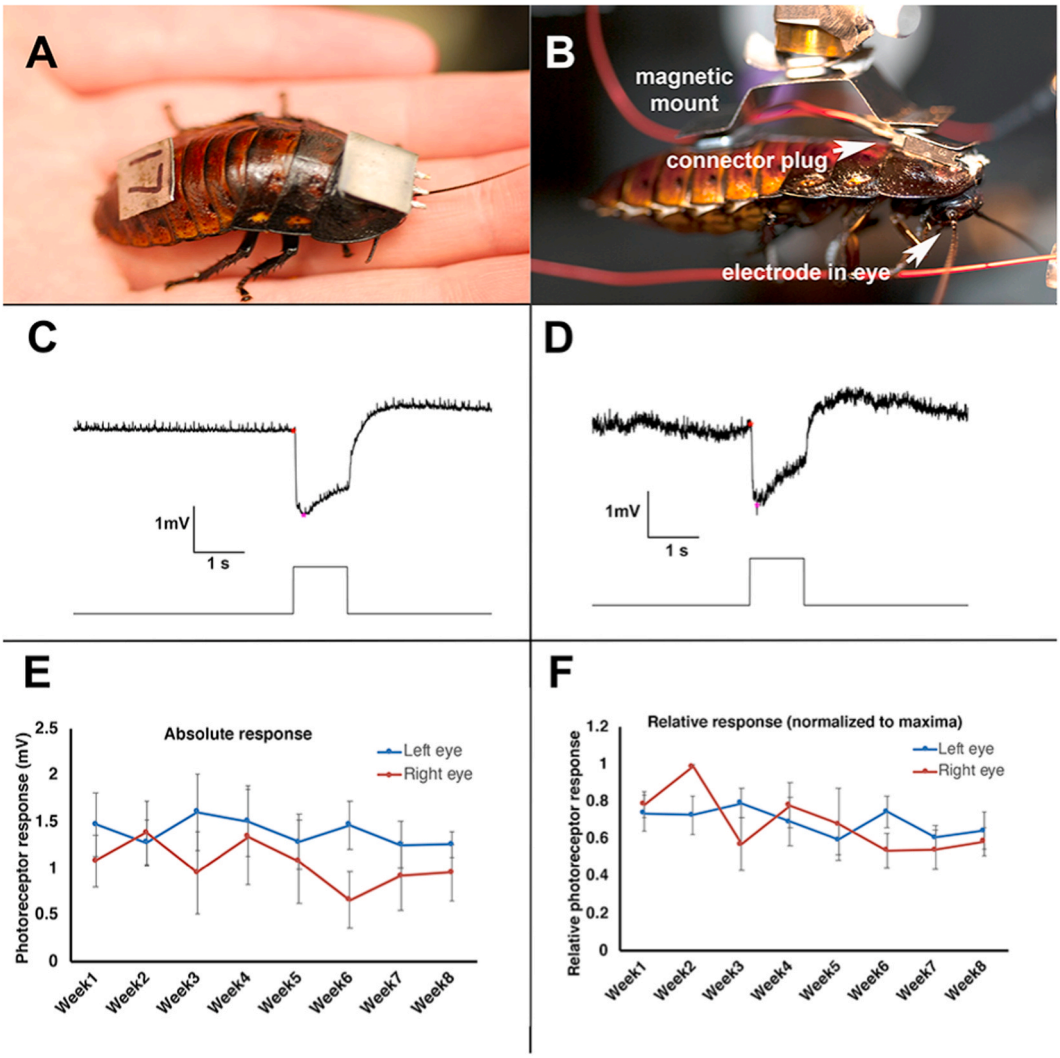

Fig. 5. Chronic implantation of HCNT electrodes into cockroaches for repeated recordings over many weeks. A. To facilitate long-term recordings, HCNT fibers were implanted into the eye and attached to a connector, and small steel plates were attached to the thorax and abdomen. B. For weekly recordings, cockroaches were attached to a magnetic mount and the physiology rig was directly plugged into the permanently mounted connector. C. ERG recording from an eye with an HCNT electrode 1 week after implantation. The ERG response (top trace) to a light flash, the timing of which is shown by the bottom trace. D. ERG recording from the same eye 8 weeks after implantation. E. Average photoreceptor responses over an 8 week period (N = 4–6 for the left eye and 3–4 for the right eye. F. Relative photoreceptor responses over an 8 week period, as obtained by normalizing the signal to the maximum response for each eye.

adequately captured these high-frequency signals. Silver is a commonly used and well-proven electrode material. Thus, our two configurations with novel electrode materials (CNT and HCNT) do not lose relevant higher-frequency components through unwanted capacitive currents (as is sometimes observed with other materials such as glass electrodes), making them suitable for applications that involve such components. Exactly how high frequencies can be to be successfully recorded remains to be explored. It is important to note that the signal that we recorded is relatively large (in the range of mV) when for example compared to vertebrate brain recordings, in which the signal is more typically around 100 μV (Cogan, 2008). Our recordings also were characterized by considerable noise levels (around 0.15 mV) the magnitude of which however was consistent across electrode types. Since this level of noise is not unusual for insect recordings with our setup, and it is substantially higher than when electrodes are measured in saline solution, it most likely represented neural noise, the combined signal of unrelated nearby neural processes. Further exploration will be necessary to evaluate electrodes for the types of recordings that are typically executed in vertebrate brains.

Consistent with the physiological measurements, a histological examination showed particularly tight integration between the tissue and the two CNT materials (Fig. 3). Conversely, both the gold and silver electrode preparations typically showed some level of separation between the electrode and the tissue. While these gaps may represent a sectioning artifact rather than a lack of proximity, it is also noticeable that the neural tissue surrounding the metal electrodes is sharply delineated and, in some cases, appears to have formed a border, possibly indicative of scar tissue. In comparison, both the CNT and HCNT electrodes appeared better integrated.

To further investigate the electrode–tissue interface, we examined the CNT and HCNT electrodes using electron microscopy (Fig. 4). Images of the fiber alone revealed a corrugated outer surface that contributes to a large interface, which is further enhanced by CNTs loosely connected to the CNT fiber that protrude into the surrounding medium. These observations are consistent with previous reports of large surface areas for this type of material (Peigney et al., 2001). For the CNT electrode implanted in fly neural tissue, a relatively sharp border between the fiber and neural tissue remained 4 days after implantation. However, a relatively tight interface was observed with the HCNT fiber, which may be due to the relatively dense nature of the fiber perimeter as well as the

hydrophilic nature of this functionalized fiber. It is conceivable that it is this tight and dense interface that allowed the HCNT fiber to perform particularly well in our physiological comparisons.

As our initial assessment on flies revealed HCNT fibers to be a particularly promising material, we evaluated the longer-term stability in Madagascar hissing cockroaches (*G. portentosa*), which are relatively long lived. Here, we again used ERG recordings to compare signals over extended time periods. A particular challenge for these experiments was that free movement of the cockroach head must be maintained to allow the animals to feed. Consequently, the HCNT fibers were somewhat exposed as they ran from the middle of the head to the thorax, where they were attached to the connector. Indeed, the majority of preparations that were lost over time exhibited visually identifiable rips in this region, presumably due to grooming. Nevertheless, we succeeded in recording from a representative set over a minimum of 8 weeks (Fig. 5), with one individual yielding a clear signal for 12 weeks. The cockroach ERG contained only a sustained component (without the transients specific to flies), the strength of which remained remarkably stable in many cases (see Fig. 5C and D for a comparison of the recordings from week 1 and week 8). However, in a few cases, the strength of the signal declined somewhat over this extended time period, and in some recordings the noise level increased. The latter was not due to the addition of any specific noise frequency, but rather a general increase in baseline noise, consistent with a slight increase in electrode resistance. These observations suggest that the fiber configuration could be further improved. Nevertheless, these results are indicative of remarkable durability and to the best of our knowledge represent the longest time periods successfully recorded from an electrode in any insect.

In addition to providing a method for the relatively fast and cost-efficient testing of different electrode materials, our approach illustrates a technology for recording neural signals from insects over long time periods, which is a powerful tool for the types of recording and stimulation approaches mentioned in the introduction. In addition, the ability to record repeatedly from an electrode placed in exactly the same position could make longitudinal studies possible. For example, the ERG recording allows the sensitivity of a given eye to be assessed directly, and sustained recordings over longer time periods could be used to assess how such sensitivity is modulated at different times of the day, over longer time periods, depending on the developmental state, or even by nutrition or other experimental parameters. While we used light pulses as a relatively easy stimulation method, as the fly visual system is part of the central nervous system, it is expected that similar recordings could be obtained from other parts of the central and peripheral nervous systems. Hopefully, our findings will help advance such studies and promote the use of insects as a rapid and cost-effective approach for testing the many new electrode materials that have recently been reported.

CRediT authorship contribution statement

Elke K. Buschbeck: Conceptualization, Methodology, Project administration, Writing – original draft, Funding acquisition. **Anh Duc Le**: Investigation, Formal analysis. **Carly Kelley**: Investigation, Formal analysis. **Md Abdul Hoque**: Investigation. **Noe T. Alvarez**: Conceptualization, Resources, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors have no conflicts of interests to disclose.

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