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A Novel Anti-inflammatory Surface for Neural Electrodes**

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Brain implants, once limited to the realm of science fiction, have gradually become scientific reality,[1,2] aided by advances in microelectrode technology. The clinical benefits of brain implants are far-reaching and include improvements in quality of life for patients suffering from neurological impairments and diseases, such as Parkinson's disease, epilepsy, blindness, and paralysis. However, a number of major bottlenecks currently hinder the realization of the therapeutic potential of brain implants, one of which being the ability of the implant to function reliably over the remaining lifetime of the patient. It is generally believed that many complications in long-term implant functionality are due to an adverse brain tissue response elicited by the implant. This inflammatory response, featured by the formation of the astroglial scar, can result in isolation of the implant from target neurons both electrically and mechanically, and is arguably the biggest stumbling block in realizing chronic recordings from neural electrodes. A common strategy to dampen the tissue response is local delivery of anti-inflammatory drugs directly at the implant-tissue interface.[3–5] Although such approach has been shown to successfully moderate host response during the acute phase,[6] alone it may be inadequate to have long-term functional consequences, due to limitations in the duration of drug release. Since the sustained chronic tissue response to the implant is considered to be a result of both foreign body reaction[7] and mechanical mismatch induced micromotion,[8,9] we propose to modulate the sustained tissue response by endowing the implant with an intrinsic anti-inflammatory surface. Here, we demonstrate through both in vitro cell culture studies and in vivo rodent studies, that immobilized alpha-MSH creates an inherently anti-inflammatory neural electrode surface such that it significantly attenuates glial response for at least 4 weeks post-implantation.

Alpha-MSH is an endogenous tridecapeptide that is secreted by pituitary cells, astrocytes, monocytes, keratinocytes, etc., and is found in the skin, brain, and other tissues.[10,11] Among its broad, potent anti-inflammatory functions, studies have shown alpha-MSH inhibits pro-inflammatory cytokines and neurotoxic nitric oxide (NO) production by microglia stimulated with beta-amyloid and interferon-gamma, which simulated inflammation associated with Alzheimer's disease.[12] Since microglia, the resident macrophages in the brain, are the frontline defense cells against invasive implants, the neuroimmunomodulatory peptide alpha-MSH was selected in our study to directly mitigate microglial response to foreign brain implants.

Coupling of the active molecule to the neural implant surface was accomplished through silane chemistry and the use of a hetero-bifunctional crosslinker with both thiol- and amino-reactive moieties,[13] as demonstrated in Figure 1. The surface coverage of alpha-MSH peptide was 0.0212 nmol cm^{-2}, quantified by application of sulfo-SDTB that reacts with primary amine, specifically in this case, those presented from the lysine residue and the N-terminus of the alpha-MSH peptide.

In order to first investigate whether the alpha-MSH peptide remains biologically active when covalently immobilized to the Si surface, we cultured primary rat microglial cells on the peptide modified surface and subjected the culture to lipopolysaccharide (LPS) bacterial endotoxin, which elicits an inflammatory response in vitro. As shown in Figure 2A, the production of nitric oxide (NO), an indicator of inflammation, was reduced to nearly 50% of that observed in the control culture, suggesting that the anti-inflammatory property of alpha-MSH was preserved during the tethering process. Moreover, we examined the gene expression of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-alpha) and interleukin 1 (IL-1) using real time RT-PCR. Both of these cytokines play a key role in mediating the inflammation process in vivo, and microglia are the known major cellular source for production of these cytokines. Modulating the level of TNF-alpha and IL-1 is critical as excessive production of these factors could not only amplify the inflammation process by stimulating glial cell proliferation and activation, but also cause neuronal death. LPS is commonly used to induce microglial activation and cause production of neurotoxic pro-inflammatory cytokines. This is consistent with our finding where the mRNA expression level of TNF-alpha had an over three-fold increase in the control Si group after subjecting to LPS stimulation (Fig. 2B). In contrast, TNF-alpha expression

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Figure 1. Reaction scheme for immobilizing anti-inflammatory neuropeptide alpha-MSH on silicon neural implant surface.

Figure 2. Effect of immobilized alpha-MSH on LPS-stimulated microglia in vitro. (A) NO production by microglial cells cultured on the control silicon surface and alpha-MSH immobilized silicon surface. Real time RT-PCR analysis revealed fold change in gene expression of pro-inflammatory cytokines TNF-alpha (B) and IL1 (C) from microglial culture with and without LPS stimulation.

from microglia cultured on the alpha-MSH tethered Si surface showed ~ 40% decrease upon LPS stimulation. Alpha-MSH reduces expression of TNF-alpha due to its ability to block proinflammatory signaling such as activation of nuclear factor κB (NF κB).[14,15] The change in the gene expression of the other cytokine, IL-1, was more marked, with an increase of over three orders of magnitude observed in the control group (Fig. 2C). For the peptide-presenting surface, although there was a slight increase in IL-1 expression after LPS stimulation, the increase was much milder compared to the control, i.e. less than 7% of the change that was detected in the control case. Altogether, these results suggest that the neuropeptide alpha-MSH immobilized on the Si surface remains biologically active and exerts anti-inflammatory effects via direct interaction with microglial cells.

After confirming the anti-inflammatory capability of the peptide-immobilized Si surface in vitro, the in vivo bioactivity of the alpha-MSH surface was investigated. Michigan-type, planar, single-shank Si microelectrodes were functionalized with the alpha-MSH peptide and implanted into the motor cortex area of rat brain. This area was chosen based on the usefulness of motor commands to be used as control signals for a neuroprosthetic device, as recently demonstrated in humans.[1] Extensive primate research has also focused on the motor cortex region.[16,17] To minimize variation between animals, an untreated electrode was implanted into the corresponding contralateral location of the same rat to serve as a control. Tissue response at two different time points was examined, with one representing the acute phase (one week), and the other representing the chronic phase (four weeks). It is known that microglia and astrocytes are among the active contributors to the scar formation. Using specific cellular markers and immunohistochemical staining, the response of microglia and astrocytes was evaluated. One week after electrode implantation an increase in the number of ED1-positive cells (indication of activated microglia/macrophages) within ~100 μm radius of the interface was observed with the unmodified Si electrode (Fig. 3A). The majority of these ED1-positive cells were packed right at the electrode-tissue interface, where they assumed morphology typical of activated macrophages. Further away from the interface, the ED1-positive cells were less compact, and appeared to be smaller and bearing processes. In contrast to this control condition, microglial response to the alpha-MSH peptide functionalized electrode at the same time point was much milder. As shown in Figure 3B, ED1-positive staining surrounding the electrode was significantly diminished. Most of the cells were bearing processes with relatively few amoeboid in shape. Quantitatively, ED1 immunoreactivity around the implant was significantly lower in the case where the electrode was modified with alpha-MSH peptide, ~ 67% less than that of the untreated control ($p < 0.05$). It suggests that the tethered peptide remained functionally viable in vivo and was able to suppress the microglial activation around the electrode. Four weeks

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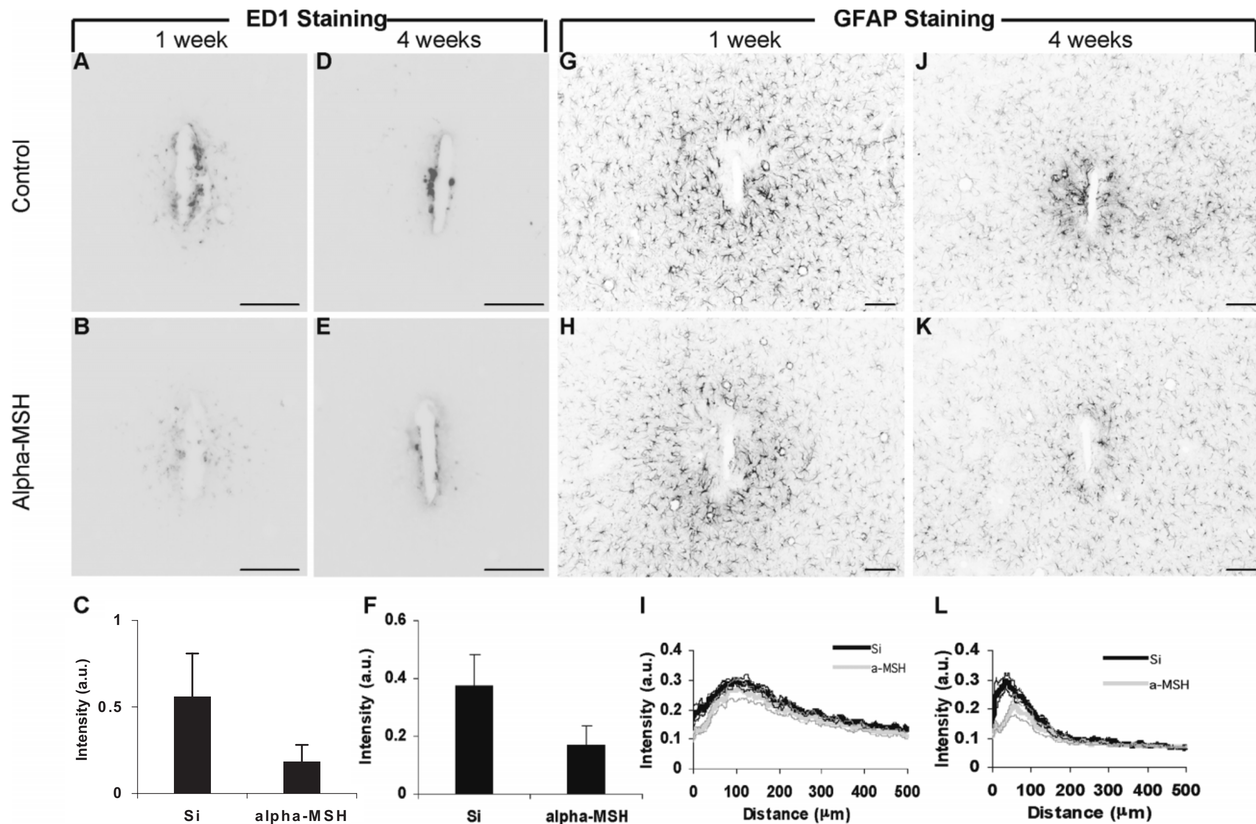

Figure 3. ED1 staining for reactive microglia/macrophages on tissue sections from brains implanted with control electrode (A & D) and alpha-MSH immobilized electrode (B & E) at 1 week and 4 weeks post implantation, as well as the corresponding quantification results (C & F). Astrocyte response was also evaluated using GFAP staining for the control electrode (G & J) and alpha-MSH immobilized electrode (H & K) at 1 week and 4 weeks post implantation. Quantitative comparison of GFAP immunoreactivity between the control and alpha-MSH immobilized electrode was made via GFAP intensity profiles as a function of distance from the insertion site (I & L), shown as the average intensity (thick line) ± SEM (thin line). Scale bar = 100 μm.

post implantation the activated microglia evolved into a thin compact layer of amoeboid-like cells isolating the control electrode from the brain parenchyma (Fig. 3D). It is hypothesized that the persistent microglial activation is due to stimuli originating from the indwelling electrode as well as associated implant micromotion-generated mechanical strain/stress.[7,18] A thin layer of faint ED1 staining was observed for the peptide modified electrode (Fig. 3E), and the intensity was ~ 50% less than that of the control ($p < 0.05$). Importantly, this indicates the tethered neuropeptide was able to exert anti-inflammatory effect even after four weeks in vivo, likely due to its direct contact with the target microglial cells. It is unclear, however, whether the improved microglial response is a result of less "frustrated" phagocytosis, or a reduction in micromotion induced inflammation, or a combination of both. While no difference was observed in terms of glial fibrillary acidic protein (GFAP) immunoreactivity between the control electrode and peptide modified electrode after one week of implantation shown by the average intensity line profile (Fig. 3I), a statistically significant difference in reactive astrocytic response was noted after four weeks in vivo. Similar to microglial response, the GFAP positive zone progressed into a tighter sheath encapsulating the control electrode insertion site (Fig. 3J) in contrast to the broader and more dispersed appearance seen after one week (Fig. 3G). Within a radius of about 50 μm from the insertion site, GFAP intensity was significantly lower around the peptide modified electrode than the control (Fig. 3L). Even though the tethered alpha-MSH peptide was not in direct contact with astrocytes, it is possible the observed reduction in GFAP immunoreactivity is related to the less intense microglia activation. Reduced microglial modulation of astrocyte response through a reduction in pro-inflammatory molecular mediators, such as cytokines and reactive oxygen species, is one possible explanation for the observed reduction in long-term GFAP expression. We further examined whether the immobilized alpha-MSH peptide could directly influence pro-inflammatory cytokine expression in vivo, as we have demonstrated occurs in vitro. Using in situ hybridization we were able to visualize the spatial profile of TNF-alpha mRNA expression at the interface. As shown in Figure 4A, the majority of TNF-alpha expression was distributed at the implant-tissue interface, which corresponds to the location of activated microglia/macrophages. It further suggests that microglia express this pro-inflammatory

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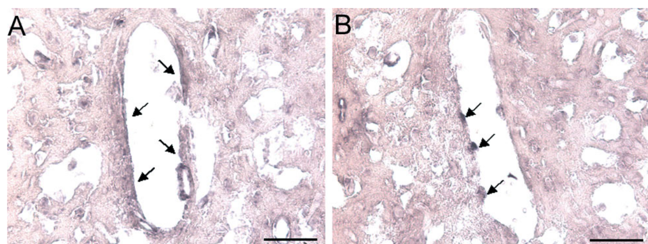

Figure 4. In situ hybridization of tissue sections from brains implanted with control electrode (A) and alpha-MSH immobilized electrode (B) at 1 week post implantation, using probes specific for TNF-alpha mRNA. Arrows indicate examples of TNF-alpha expressing cells. Scale bar = 50 μm.

cytokine around implanted electrodes.[19] In contrast, TNF-alpha expression was lower and scattered around the alpha-MSH peptide modified electrode, which is consistent with the lower ED1 immunoreactivity observed (Fig. 3B). Results from in situ hybridization along with immunostaining results strongly support that the anti-inflammatory effect of the immobilized alpha-MSH peptide on the surface of the neural implant is maintained in vivo into chronic time points.

In summary, we have demonstrated that surface immobilization of an anti-inflammatory peptide can significantly decrease inflammation around neural electrodes implanted for at least 4 weeks in adult rodent brain. The neuroimmunomodulatory peptide alpha-MSH retained its anti-inflammatory property while immobilized to the implant surface, and was able to directly modulate microglial response by modulating the expression of inflammatory cytokines. This novel strategy therefore can potentially improve the reliability of chronic neural implants in vivo.

Experimental

Chemicals and Materials: Polished silicon wafers with a 10000 Å thick oxide layer were ordered from University Wafer. (3-Aminopropyl) triethoxysilane (APTES), *N*-succinimidyl-6-maleimidylhexanoate (EMCS), anhydrous toluene, and acetonitrile were purchased from Sigma-Aldrich and used without any further purification. The alpha-MSH analogue, HS-CH_2CH_2CO-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, purified by high-performance liquid chromatography, was obtained from Biopeptide. Sulfosuccinimidyl-4-O-(4,4′-dimethoxytrityl)butyrate (sulfo-SDTB) was bought from Prochem Inc. The lipopolysaccharide (LPS) from *E. Coli* was from Sigma.

Peptide Immobilization: Neuropeptide alpha-MSH immobilization to silicon surface was achieved by using an aminosilane linker group following procedure used by Xiao and Textor [13]. Briefly, 1 × 1 cm^2 silicon samples were first cleaned with a piranha solution (3:1 concentrated H_2SO_4/30% H_2O_2, v/v) for 30 min. Caution: piranha solution reacts violently with organic solvents and should be handled with extreme care! Silanization of the surface was achieved by immersing the samples into a solution containing 30 mL dry toluene and 0.5 mL APTES for 3 h at 120 °C. After incubation period, samples were ultrasonically washed with chloroform five times, acetone twice, and methanol five times, and were extensively rinsed with water. The heterobifunctional crosslinker EMCS was then coupled to the amine terminated surface by allowing the substrates to incubate in a 5 mM solution of EMCS in acetonitrile at room temperature for 1 h. Substrates were then rinsed extensively with acetonitrile and acetone. After drying with nitrogen, these maleimide-grafted substrates were ready for peptide immobilization. This was achieved by subjecting the substrates to a 2.0 mM peptide solution (pH 6.5) at room temperature for 2 h. Following this treatment, the peptide-grafted silicon samples were washed thoroughly with water, dried with nitrogen, and stored in argon.

Estimation of Surface Density of Immobilized Peptide: A highly sensitive colorimetric assay using sulfo-SDTB was applied to quantify the surface peptide concentration [20–22]. A 0.1 mM sulfo-SDTB solution was prepared by dissolving 3 mg sulfo-SDTB in 1.0 mL of DMF and further diluting to 50 mL with 50 mM sodium bicarbonate buffer (pH 8.5). One millimeter of this solution was then added to wells of 24-well plates containing Si samples and incubated for 1 h at room temperature. Subsequently, the samples were rinsed three times with the sodium bicarbonate buffer, and 400 μL of 35% perchloric acid was added to each sample to release the dimethoxytrityl cation that led to color development. The absorbance of the solution in each well was then measured at 498 nm. From the known extinction coefficient of the dimethoxytrityl cation (ε = 70000 M^{-1} cm^{-1}), surface concentration of the immobilized peptide was calculated.

Cell Culture: Microglial cells were obtained using a modification of the procedure by Giulian and Baker [23]. Briefly, mixed glial cells dissociated from neonatal Sprague–Dawley rat cerebral hemispheres were plated in 75 cm^2 poly-L-lysine coated tissue culture flasks (Fisher) at a density of one brain per flask in culture medium consisting of DMEM-F12 media (Gibco) supplemented with 20% fetal bovine serum and 1% penicillin/streptomycin. After 7–10 days, flasks were lightly shaken to release microglial cells into the media supernatant, and these floating microglia were subsequently centrifuged into a pellet, and resuspended in DMEM-F12 medium supplemented with 10% fetal bovine serum.

LPS Stimulation Assay: LPS stimulation assays were conducted by seeding 100,000 microglial cells per cm^2 on alpha-MSH immobilized as well as untreated control 1 × 1 cm^2 Si wafers, which were placed in individual wells of a 24-well culture plate and cultured for 24 h in 5% CO_2 atmosphere at 37 °C. The culture was then subjected to 1 ng mL^{-1} LPS stimulation. Accumulation of NO_2^-, a stable end product extensively used as an indication of nitric oxide (NO) production by the microglial cultures was assayed with Griess reagent (Promega). In addition, total RNA was extracted from the cell culture using the materials and protocol provided in the RNeasy Mini Kit (Qiagen). Subsequently, real-time RT-PCR reactions were performed to evaluate gene expression of the pro-inflmmatory cytokines TNF-alpha and IL-1 [24].

Electrode Implantation: Single shank neural electrodes were obtained from NeuroNexus Technologies. Shank dimensions were 5 mm in length, 33–200 μm wide and 15 μm thick. Peptide immobilization to the electrode was carried out using the same procedure described above. Animal procedures adhered to the National Institute of Health (NIH) guideline and followed an approved protocol by the Institutional Animal Care and Use Committee (IACUC) at Georgia Institute of Technology. Adult male Sprague-Dawley rats were used. Surgical procedures reported in previous work were applied [24]. Briefly, animals were anesthetized under isoflurane, and in each animal, two 3.2 mm burr holes were created at locations +0.2 mm anterior and ±3.0 mm lateral to the bregma. Control electrode and alpha-MSH immobilized electrode were then implanted into the brain through these two burr holes via hand insertion. Afterwards, the burr holes were covered with 1% SeaKem Agarose (Cambrex) gel, and the craniotomy was furthered sealed using dental acrylic. The skin was sutured shut with 4-0 monofilament nylon and the animals were monitored carefully until full recovery.

Immunohistology: Animals were prepared for immunohistology one week and four weeks after surgery (n = 4 animals per time point). Procedures described in previous study were followed [24]. To examine brain tissue response in the cortical region, 30 μm thick cortical sections taken from all brains from each group were stained simulta-

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neously for the antibody of interest. Specifically, ED-1 was used as marker for reactive microglia and macrophages, and GFAP for astroctyes. Sections were then mounted and fluorescent images were acquired using a Microfire digital camera and a Zeiss Axioskop2 Plus upright microscope. Quantitative analysis of immunohistological data was performed using custom software developed in MATLAB (Mathworks) [24]. All statistical inferences were made between alpha-MSH immobilized electrode and untreated control electrode using Student's *t*-test analysis (two-tailed) between like parameters.

In Situ Hybridization: GreenStar* Digoxin-Hyperlabeled Oligonucleotide Probe specific against TNF-alpha was custom designed and synthesized by GeneDetect. Frozen brain sections that were 7 μm thick were hybridized with the probe following the protocol provided by the manufacturer [25]. Indirect signal detection was accomplished using an anti-DIG antibody conjugated to alkaline phosphatase.

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