Photosensitivity of Neurons Enabled by Cell-Targeted Gold Nanoparticles

Highlights

- AuNPs can be targeted to neurons for optical excitation using biological ligands
- These targeted AuNPs are highly resistant to convective washout
- This technique can excite activity in mouse hippocampal slices
- The mechanism depends on the rate of change of temperature, not on temperature itself

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In Brief

Carvalho-de-Souza et al. show that ligand-conjugated gold nanoparticles robustly attach to neurons and trigger action potentials in response to pulses of light. The mechanism of stimulus involves fast increases in membrane temperature, which change membrane capacitance and induce depolarization.
Photosensitivity of Neurons Enabled by Cell-Targeted Gold Nanoparticles

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SUMMARY

Unmodified neurons can be directly stimulated with light to produce action potentials, but such techniques have lacked localization of the delivered light energy. Here we show that gold nanoparticles can be conjugated to high-avidity ligands for a variety of cellular targets. Once bound to a neuron, these particles transduce millisecond pulses of light into heat, which changes membrane capacitance, depolarizing the cell and eliciting action potentials. Compared to non-functionalized nanoparticles, ligand-conjugated nanoparticles highly resist convective washout and enable photothermal stimulation with lower delivered energy and resulting temperature increase. Ligands targeting three different membrane proteins were tested; all showed similar activity and washout resistance. This suggests that many types of ligands can be bound to nanoparticles, preserving ligand and nanoparticle function, and that many different cell phenotypes can be targeted by appropriate choice of ligand. The findings have applications as an alternative to optogenetics and potentially for therapies involving neuronal photostimulation.

INTRODUCTION

Selective optical stimulation of specific classes of excitable cells is a major goal in neurobiology. Optical stimulation avoids many of the problems inherent to electrodes, such as invasiveness and lack of specificity, and can thus allow for novel experimental designs. A common method to achieve this optical stimulation is through optogenetics, wherein molecules such as channelrhodopsin are selectively expressed in different cell types; exposure to light opens these channels and causes cell depolarization (Packer et al., 2013; Zhang et al., 2006). While this technique is undoubtedly powerful, it requires gene transfection to achieve cellular expression of the light-sensitive proteins. As such, it is not currently suitable for use in a variety of systems including human subjects, where gene therapy remains highly experimental (Ginn et al., 2013).

One potential alternative to optogenetic techniques is the direct optical stimulation of unmodified neurons (Allégre et al., 1994; Hirase et al., 2002; Lugo et al., 2012). Direct optical stimulation approaches have included the use of infrared (IR) (Wells et al., 2005) wavelengths, although the mechanism of this effect was not initially understood. Recently, Shapiro and collaborators (Shapiro et al., 2012) showed that for IR wavelengths, laser-induced fast changes in the temperature of the local aqueous medium can heat the cell membrane and produce capacitive currents. Further experimentation and modeling demonstrated that changes in the cells’ membrane capacitances were sufficient to produce the observed depolarizing currents and action potentials under physiological conditions. However, while direct heating of bulk solution with IR light is effective, it is an imprecise way to stimulate neurons and may cause off-target effects or cellular damage.

Gold nanorods (AuNRs) have been investigated as a more targeted alternative due to their ability to absorb near-infrared (NIR) light and efficiently convert this energy to heat. This heating effect has a short range, and since NIR light is not strongly absorbed by water, the bulk aqueous media is unaffected. AuNR heating has been used to excite cultured neurons and exposed nerves by depositing the rods in proximity to cell membranes (Eom et al., 2014; Yong et al., 2014). Spherical gold nanoparticles (AuNPs) with a 20 nm diameter are similar to AuNRs in that they can absorb light and convert this energy to heat (Roper et al., 2007), but their plasmon absorption band exhibits a peak near 523 nm rather than in the NIR range. The relatively strong absorbance of the 20 nm spherical AuNPs at 523 nm, i.e., at wavelengths typically prominent in indoor as well as daylight ambient illumination, suggests that photothermal neuronal excitation mediated by these AuNPs might have utility for applications such as the restoration of light-induced signaling within the retina of patients with photoreceptor degenerative disease. However, while the short-range heating effect of AuNPs and AuNRs allows for selective heating of a localized environment, it also has a significant drawback: the particles must be extremely close to the cells of interest in order to produce any effect.
Here we report an approach of potentially wide applicability for highly localized AuNP-mediated photostimulation of neurons by conjugating the AuNPs to functional groups that specifically bind to external motifs of neuronal membrane proteins. Similar strategies have previously been used to target nanoparticles to cancer cells to facilitate imaging and photothermal destruction of malignancies (Huang et al., 2006; Sokolov et al., 2003). We conjugated AuNPs to high-avidity ligands of three different membrane proteins of dorsal root ganglion (DRG) neurons and tested these conjugates for their ability to confer light-responsiveness to these cells. The investigated ligands included a synthetic molecule based on Ts1 neurotoxin from the venom of the Brazilian scorpion Tityus serrulatus, which binds voltage-gated sodium channels (Barhanin et al., 1982; Dang et al., 2014; Possani et al., 1977); antibodies targeting the TRPV1 ion channel; and antibodies targeting the P2X3 receptor ion channel. The protein targets of all three of these ligands are known to be expressed in the membrane of DRG neurons (Hayes et al., 2000; Kostyuk et al., 1981; Xiang et al., 1998). We found that each of these AuNP conjugates binds to cultured DRG neurons and enables optical triggering of action potentials with remarkable robustness at low AuNP concentrations while displaying significant resistance to washout. By contrast, unconjugated AuNPs required higher concentration to allow for optical stimulation and washed away immediately upon solution exchange. The fact that this technique works well with all tested ligands suggests that this strategy is quite general. By appropriate selection of nanoparticle geometry, as well as choice of attached ligand, this conjugation technique should allow for selective stimulation of many excitable cell types with wavelengths ranging from the visible to NIR. With further development, nanoparticle-based direct photostimulation could provide an alternative to optogenetic techniques in situations where genetic manipulation is impractical.

**RESULTS**

**Non-Functionalized AuNPs Enable Photoexcitation but Wash Out Quickly**

Our first experiment was to determine whether DRG neurons can be excited with visible light in the presence of 20 nm diameter AuNPs. DRG neurons were patch clamped in the whole-cell configuration and transiently exposed to a 532 nm laser pulse (Figure 1A). AuNPs were added via perfusion through one side of a theta capillary, and they were washed away by perfusion of fresh buffer through the other side of the capillary (Figure 1B). Cells were stimulated with 300 pA of depolarizing current to produce a control action potential and verify cellular excitability; 200 ms subsequent to the electrically stimulated action potential, a 174 mW laser pulse at 532 nm was delivered to the neuron for 1 ms. The laser was focused on the cell under study to produce an average irradiance of \( \approx 31 \) kW/cm\(^2\) (Figure S1). In the absence of AuNPs, no response to the laser flash was observed, either in the form of depolarization or membrane damage (Figure 1C). However, perfusion of 50 nM AuNPs onto the neurons rendered about 80% of tested cells (23 out of 29) sensitive to the laser pulse and an optically induced action potential was produced. This effect depended strongly on the AuNP concentration near the cell, and active perfusion of fresh buffer rapidly washed the particles out, abolishing their effect. A second addition of 50 nM AuNPs restored light sensitivity. The time course of the effect emphasizes that the non-functionalized AuNPs are removed from the cell surface within seconds of a perfusion wash (Figure 1D). Even without active washing, diffusion of AuNPs away from a cell is sufficient to abolish optical sensitivity within about 2 min (Figure S1).

**Ts1-Conjugated AuNPs Are Highly Resistant to Washing**

We next attempted to induce AuNP localization to neuronal membranes by conjugating synthetic Ts1 to nanoparticles. Ts1, a neurotoxin that binds voltage-gated sodium channels without blocking them (Campos et al., 2007), was biotinylated

**Figure 1. Action Potentials Can Be Stimulated by 532 nm Light Using AuNPs**

(A) Diagram of the experimental setup. The cell is patch clamped in a whole-cell configuration (pipette on the left), and both AuNP addition and perfusion washing are accomplished via the theta capillary on the right. Abbreviations are as follows: AOM, acousto-optic modulator; ND, neutral density filters; DIC, dichroic mirror; OBJ, microscope objective; AMP, amplifier; LPF, low-pass filter; ADC, analog-to-digital converter. Many parts of the diagram are not drawn to scale.

(B) AuNPs were perfused over a patch-clamped DRG neuron through one side of a theta capillary. After a sufficient optical response is observed, fresh buffer is perfused over the cell from the other side of the capillary, washing away the AuNPs.

(C) Representative traces of current-clamped DRG cells firing action potentials in response to two different stimuli: a 300 pA, 1 ms current injection (left side, blue bars) and a 174 mW, 1 ms 532 nm laser pulse (right side, green bars). Initially, cells were responsive only to the electrical stimulus, but a bolus of AuNPs sensitized the cells to light. Washing removed enough AuNPs from the cell that the laser effect became insufficient to trigger an action potential. Optical excitability returned when a second bolus was added to the bath.

(D) An active washing system demonstrates how the laser effect is dependent on AuNP concentration near the cell. Upon washing, the laser effect rapidly disappears within seconds. “Peak response” is the maximum voltage reached following a laser pulse. See also Figure S1.
and conjugated to streptavidin-coated AuNPs (Figure S2 and Supplemental Experimental Procedures). Upon application of this AuNP-Ts1 conjugate at 20 nM, a fraction of the cells tested (9 out of 21) became optically excitable with 1 ms laser pulses using powers as low as 126 mW, although 174 mW was a more reliable stimulus (Figures 2A and 2B). Significantly, many of these DRG neurons stayed sensitive to the laser pulse and fired action potentials for approximately 30 min under continuous perfusion wash (Figure 2C). The fact that the AuNP-Ts1 conjugate did not show equivalent activity in all tested cells and that it worked in a significantly lower fraction of cells as compared to non-functionalized AuNPs (p = 0.016, Fisher’s exact test) is expected given the variable expression profiles of sodium channel types among different DRG neurons (see Discussion). Additionally, during these experiments, the neurons could be stimulated many times by the same 1 ms duration pulses of light without any indication of cell membrane damage or failure of the nanoparticle-cell linkage. These data suggest that the Ts1 promoted a close association of the AuNP with the neuronal membrane for the duration of the experiment. Importantly, under current clamp, the laser-induced membrane depolarization magnitude was proportional to laser power until the threshold potential was reached, at which point an action potential fired (Figure 2D). Furthermore, under voltage clamp, the membrane currents induced by the laser pulse were linearly modulated by laser power at a constant voltage (Figure S3). Similarly, at a constant laser power, DRG neurons treated with AuNP-Ts1 showed optically induced currents with a linear dependence on membrane potential and a reversal potential near 0 mV (Figure S3). Together, these data show that the neurons are efficiently bound by the AuNP-Ts1 conjugate and that this link is well behaved and stable over long periods.

One practical consideration with thermal stimulation methods is the risk of off-target or damaging effects from the temperature increase. Accordingly, minimizing temperature changes in a neuron’s surrounding environment is probably advantageous as long as the neuron itself remains optically excitable. To investigate this, we measured the bath temperature ∼2 μm away from the cell during laser stimulation. In the absence of AuNP-Ts1 (top two traces), no temperature changes were observed 2 μm away from a DRG cell during either electrical (blue bar) or optical (green bar) stimulation. After adding AuNP-Ts1 and washing away excess nanoparticles (bottom two traces), an increase in temperature was measured during optical stimulation. Temperature traces are single recordings with noise subtraction and are filtered at 1 kHz.

A procedure identical to that used with non-functionalized AuNPs (Figure 1B) is unable to wash AuNP-Ts1 from the cell. As with non-functionalized AuNPs, DRG neurons initially respond only to electrical stimuli (blue bars: 500 pA, 1 ms), but addition of AuNP-Ts1 sensitizes them to optical stimuli (green bars: 174 mW, 532 nm, 1 ms). Here, however, washing does not quickly eliminate optical excitability. Even after 20 min of continuous washing, neurons labeled with AuNP-Ts1 remain optically excitable. Increasing laser power causes increasing cell depolarizations, triggering action potentials once the cell’s threshold voltage is reached. 174 mW is necessary to reliably stimulate this particular neuron. Inset: sample traces for each laser power.

In the absence of AuNP-Ts1 (top two traces), no temperature changes were observed 2 μm away from a DRG cell during either electrical (blue bar) or optical (green bar) stimulation. After adding AuNP-Ts1 and washing away excess nanoparticles (bottom two traces), an increase in temperature was measured during optical stimulation. Temperature traces are single recordings with noise subtraction and are filtered at 1 kHz. See also Figures S2 and S3.

Figure 2. Optical Stimulation of DRG Neurons with AuNP-Ts1
(A) A procedure identical to that used with non-functionalized AuNPs (Figure 1B) is unable to wash AuNP-Ts1 from the cell.
(B) As with non-functionalized AuNPs, DRG neurons initially respond only to electrical stimuli (blue bars: 500 pA, 1 ms), but addition of AuNP-Ts1 sensitizes them to optical stimuli (green bars: 174 mW, 532 nm, 1 ms). Here, however, washing does not quickly eliminate optical excitability.
(C) Even after 20 min of continuous washing, neurons labeled with AuNP-Ts1 remain optically excitable.
(D) Increasing laser power causes increasing cell depolarizations, triggering action potentials once the cell’s threshold voltage is reached. 174 mW is necessary to reliably stimulate this particular neuron. Inset: sample traces for each laser power.
(E) In the absence of AuNP-Ts1 (top two traces), no temperature changes were observed 2 μm away from a DRG cell during either electrical (blue bar) or optical (green bar) stimulation. After adding AuNP-Ts1 and washing away excess nanoparticles (bottom two traces), an increase in temperature was measured during optical stimulation. Temperature traces are single recordings with noise subtraction and are filtered at 1 kHz.
(F) With nanoparticles present in the bulk solution (before washing), a large change in temperature is observed. Active perfusion washing leaves only the nanoparticles tightly bound to the surface. The cell remains optically excitable, but the temperature change measured 2 μm from the cell decreases dramatically. Green bar shows the optical stimulus. Traces are averages of 20 recordings and are filtered at 1 kHz. See also Figures S2 and S3.
Functionalized AuNPs Allow for Repetitive Stimulation without Evidence of Cellular Damage

A question of central interest is whether there is an intrinsic limitation on the frequency of stimulation when using AuNPs, perhaps due to temperature buildup or some other effect. This issue is complicated by the fact that DRG neurons are a heterogeneous population with widely varying maximum firing frequencies; DRG somas have maximum firing frequencies from as low as 3 Hz to above 150 Hz depending on the DRG cell type (Waddell and Lawson, 1990). To explore this, we treated DRG neurons with AuNP-Ts1 and stimulated them with trains of laser pulses. In our studies, the maximum firing rate we were able to observe was 40 Hz. In this case, a series of 1 ms laser pulses delivered at 40 Hz for a total of 3 s stimulated an action potential with every stimulus, whereas a similar train at 50 Hz did not (Figure 3A). In other cells, we observed maximum firing frequencies as low as 15 Hz. An important distinction, then, is whether this maximum rate was limited by some property of the optical stimulus or whether we were simply observing the intrinsic maximum firing rate of the neuron. To address this, we compared a cell’s maximum firing rate when stimulated optically versus when stimulated with an electrical current injection (Figure 3B). Optical and electrical stimuli of similar magnitudes (as measured by the rate of change of membrane potential during the respective stimuli) and equivalent 1 ms durations produced similar responses from the cell, as both trains stimulated action potential responses to about one-third of the stimuli. We therefore conclude that in this case, the observed firing frequency was limited by the intrinsic properties of the neuron and was not a characteristic of stimulation with AuNPs. To corroborate this point, we tested the effect of the laser stimulus under voltage clamp at $-100$ mV (Figure 3C). At this membrane potential, neuronal channel activity is virtually eliminated and the laser-induced capacitive currents can be observed without confounding ion channel activity. We observed that the induced currents produced by laser pulses at 200 Hz are of similar magnitude to those produced by 40 Hz laser pulses and have no apparent abnormalities. This provides further evidence that the observed maximum action potential firing rate of 40 Hz is unrelated to the optical stimulation and indicates that optical excitation with AuNPs may be possible at much higher frequencies in other neuron types.

An important concern with this technique is whether stimulation mediated by AuNPs is harmful to a cell. While a single stimulus can kill a cell if excessive laser power is used (likely due to overheating), this does not seem to be a concern at lower power levels suitable for producing excitation. During this study of repetitive stimulation, some cells were optically stimulated to produce more than three thousand action potentials with no evidence of toxicity or cellular damage induced by the particles or the optical stimulation. Furthermore, over the course of these thousands of stimuli, there was no detected decrease in optical excitability. Cells that initially showed good responses to optical stimuli continued to fire action potentials in response to laser
represents 100 laser pulse (far left frame), while the others begin 100 ms, 180 ms, and 260 ms (far right frame) following the optical stimulus. The baseline image to subtract are shown, each showing the average activity over four consecutive 80 ms windows following the stimulus. The first window begins 20 ms after the start of the laser pulse (far left frame), while the others begin 100 ms, 180 ms, and 260 ms (far right frame) following the optical stimulus. The baseline image to subtract was created by averaging 180 ms of data from before the stimulus. The data in these images are from the same acquisition as in (B). The scale bar in the first image represents 100 μm. Gaussian image filtering has been applied to these images (see Supplemental Experimental Procedures for details). See also Figure S4.

pulses until the patch seal failed. While these observations provide evidence against acute forms of toxicity at typical power levels, they do not rule out a chronic effect occurring over hours, days, months, or longer. Further investigations would need to be performed before AuNP stimulation is used for long-term studies.

AuNP-Ts1 Allows Optical Stimulation of Hippocampal Brain Slices and Electrode-free Investigation of Activity

The above results, which demonstrate the utility of AuNPs for stimulating isolated DRG neurons in culture, led to the question whether similar AuNP-based optical stimulation is feasible in a complex neurological tissue. For this further test, we employed an all-optical method that avoided the need for electrodes of any kind. The experiments utilized acute slices of mouse hippocampus, a commonly used ex vivo brain tissue preparation (Biscoe and Duchen, 1985). To provide optical stimulation, we injected a small bolus of AuNP-Ts1 into the CA1 region of the hippocampal slice (Figure 4A). Afterward, the entire slice was perfused with the voltage-sensitive infrared dye indocyanine green (ICG) (Treger et al., 2014). We chose this dye because both its absorption and emission occur in the NIR spectrum and its fluorescence thus has excellent tissue penetration. For the experiment, ICG fluorescence was continuously monitored in the infrared while a short pulse of 532 nm light was delivered to the nanoparticles. A clear, transient reduction in ICG fluorescence can be seen in response to a 225 mW, 10 ms optical stimulus (Figure 4B). As ICG shows a decreasing fluorescence with increasing membrane potential, this signal implies cellular depolarization, as expected. The brain slices required a significantly stronger optical stimulus compared to cultured DRG neurons. This is likely due to the fact that the spot size of the 532 nm laser was much larger in the brain slice setup (due to a lower numerical aperture objective and scattering in the tissue), thus reducing the power density of the spot. In addition to simple depolarization, paired-pulse facilitation was observed in response to closely spaced stimuli (Figure S4). Finally, this technique allows for spatial maps of neural activity to be constructed. Figure 4C shows a visual time course of neural activity. Immediately following the stimulus, significant depolarization predominates in the region of observation. Over time, this evolves toward a mixture of small, localized regions of depolarization and hyperpolarization that average out to the baseline level of activity. These data show that combining AuNPs with a voltage-sensitive dye such as ICG can allow for neurological studies in complex tissues without the physical restrictions associated with electrodes.

Antibodies Can Be Used to Bind AuNPs to Target Cells

Although Ts1 proved capable of binding AuNPs to DRG neurons, we next pursued a more general strategy for linking AuNPs to a target of interest by using antibodies as the AuNP-anchoring molecules. For the case of DRG neurons, we chose TRPV1 and the P2X3 receptor as antibody targets since these proteins are more highly expressed in neurons than in glia. Antibodies to these two targets (TRPV1ab and P2X3ab, respectively) were chemically biotinylated (see Supplemental Experimental Procedures) and incubated with DRG cells overnight. After multiple buffer exchanges the next day, recording began. As expected, the neurons were insensitive to light before streptavidin-functionalized AuNPs (AuNP-SAs) were present, whereas exposure to AuNP-SAs rapidly sensitized both TRPV1ab- and P2X3ab-labeled cells to 126 mW, 1 ms optical stimuli (Figures 5A and 5B). As with AuNP-Ts1, not all neurons treated with AuNP-SAs became sensitive to optical stimuli (11 out of 19), likely due to variable expression of the target membrane proteins (see Discussion). Antibody-labeled neurons that became sensitized to laser pulses retained this sensitivity for more than 20 min under continuous convective washing (Figures 5C and 5D). Thus, the AuNPs were tightly linked to DRG cells by the antibodies.
Despite the workability of the biotinylated antibodies as AuNP-anchoring molecules, they require either chemical biotinylation of primary antibodies or the purchase of biotinylated primary antibodies, which must often be custom-ordered at higher cost. Since it is often more convenient to use unmodified primary antibodies, we investigated the alternative AuNP binding strategy of using AuNPs functionalized with secondary antibodies (AuNP-2ab). We first incubated DRG neurons overnight with unmodified TRPV1ab or P2X3ab antibodies. As before, we washed the cells extensively the next day prior to recording. Once again, no optical excitability was detected before application of the nanoparticles (Figures 6A and 6B). Addition of AuNP-2abs to the cells quickly conferred sensitivity to the 126 mW, 1 ms laser pulses to a fraction of the tested cells (9 out of 15), and this photosensitivity remained resistant to washout (Figures 6C and 6D). AuNP-2ab bound to unmodified primary antibodies performed similarly to AuNP-SAs bound to biotinylated primary antibodies in terms of ability to sensitize neurons to light. In future applications, the choice of which to use would likely depend on convenience for, or specific requirements of, the intended application.

A third labeling strategy attempted with the antibodies was to pre-mix biotinylated TRPV1ab or biotinylated P2X3ab with AuNP-SA to produce AuNP-antibody conjugates (AuNP-TRPV1ab and AuNP-P2X3ab); we then applied these pre-formed conjugates to the neurons. Both AuNP-TRPV1ab and AuNP-P2X3ab at concentrations of 20 nM rendered neurons light sensitive (Figures S5 and S6). Furthermore, both AuNP-TRPV1ab and AuNP-P2X3ab were highly efficient; only 48 mW of light delivered to the neuron for 1 ms was sufficient to reliably produce action potentials with these pre-formed conjugates, whereas this power was never sufficient to trigger action potentials using the other AuNP preparations. However, presumably as a consequence of this high efficiency of light energy collection, the AuNP-antibody (AuNP-ab) conjugates mediated thermal damage of the neurons, likely because they formed large clusters (see Discussion). Following a laser pulse, an abnormal and persistent membrane depolarization indicating cell damage was frequently observed following the action potential, along with loss of excitability. In most cases, the resting potential spontaneously recovered and excitability returned after several seconds (Figures S5 and S6). Although the improved optical sensitivity of the neurons might be advantageous for some applications, the increased propensity for cellular damage may well be limiting in other circumstances.

**AuNP-Based Optical Excitability Is due to Heat-Induced Changes in Membrane Capacitance**

A mechanism has been described whereby a rapidly delivered pulse of heat directly depolarizes cell membranes by transiently changing their capacitances (Shapiro et al., 2012), which was the basis of our working hypothesis when using AuNPs. As noted
Above, AuNPs absorb 532 nm light and convert this energy into heat. Accordingly, we hypothesized that the light sensitivity in cells treated with AuNPs is intrinsically related to the membrane and does not involve temperature sensitivity of any membrane proteins. To investigate this mechanism, we tested the effects of AuNP treatment on light-induced electrophysiological properties of horizontal planar membranes composed of asolectin. Under voltage clamp, and following delivery of AuNPs to the upper surface of the bilayer, laser pulses as short as 0.1 ms induced currents that were similar to those produced in the neurons. The current amplitudes did not increase linearly with pulse duration; rather, they reached a saturating value with 0.5 ms and longer laser pulses (Figure 7A). These currents varied roughly linearly with laser power (Figure 7B). In addition, the currents were negative at negative voltages, changed polarity when the voltage was close to 0 mV, and were positive at positive voltages with a linear current-voltage relationship (Figure 7C). Temperatures measured near the membrane transiently rose in response to a laser pulse before slowly returning to baseline (Figure 7D). However, the measured temperatures continued to rise for several milliseconds following the end of a laser pulse, which was interesting and unexpected. Capacitances of the membranes were measured by applying a sinusoidal voltage to the system and calculating the resulting impedance (Supplemental Experimental Procedures). We found that the capacitance rapidly increased during a laser pulse and decreased much more slowly after the pulse was turned off (Figure 7E). The time constant of the decrease in capacitance following the end of a laser pulse was 10.2 ms with a 1 ms laser pulse (Figure 7F), considerably faster than was observed with direct IR heating (Shapiro et al., 2012), but still slow enough that it did not produce a significant $dC/dt$, which is necessary to induce a capacitive current. The dynamics of capacitance change largely reflected the observed temperature changes in the system, and both behaved somewhat nonlinearly with respect to pulse duration.

To help us understand the counterintuitive temperature observation, as well as interpret our broader results in the context of the hypothesized mechanism, we mathematically modeled a system of membrane-associated gold nanoparticles in response to incident laser pulses. The diffusion of heat from the nanoparticles to the membrane was solved for different nanoparticle geometries, and the effects of the resulting temperature changes above, AuNPs absorb 532 nm light and convert this energy into heat. Accordingly, we hypothesized that the light sensitivity in cells treated with AuNPs is intrinsically related to the membrane and does not involve temperature sensitivity of any membrane proteins. To investigate this mechanism, we tested the effects of AuNP treatment on light-induced electrophysiological properties of horizontal planar membranes composed of asolectin. Under voltage clamp, and following delivery of AuNPs to the upper surface of the bilayer, laser pulses as short as 0.1 ms induced currents that were similar to those produced in the neurons. The current amplitudes did not increase linearly with pulse duration; rather, they reached a saturating value with 0.5 ms and longer laser pulses (Figure 7A). These currents varied roughly linearly with laser power (Figure 7B). In addition, the currents were negative at negative voltages, changed polarity when the voltage was close to 0 mV, and were positive at positive voltages with a linear current-voltage relationship (Figure 7C). Temperatures measured near the membrane transiently rose in response to a laser pulse before slowly returning to baseline (Figure 7D). However, the measured temperatures continued to rise for several milliseconds following the end of a laser pulse, which was interesting and unexpected. Capacitances of the membranes were measured by applying a sinusoidal voltage to the system and calculating the resulting impedance (Supplemental Experimental Procedures). We found that the capacitance rapidly increased during a laser pulse and decreased much more slowly after the pulse was turned off (Figure 7E). The time constant of the decrease in capacitance following the end of a laser pulse was 10.2 ms with a 1 ms laser pulse (Figure 7F), considerably faster than was observed with direct IR heating (Shapiro et al., 2012), but still slow enough that it did not produce a significant $dC/dt$, which is necessary to induce a capacitive current. The dynamics of capacitance change largely reflected the observed temperature changes in the system, and both behaved somewhat nonlinearly with respect to pulse duration.

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were calculated (Figure S7, Supplemental Experimental Procedures). The results from the model were broadly in line with our experimental results and clarified some features of the system. For example, the above-noted continued rise in temperature after the end of the laser pulse appears to reflect the inability to place the temperature-measuring micropipette as close to the bilayer as is possible with DRG neurons, due to the geometry and optics of the bilayer system. This increased distance from the membrane caused a time lag in our observations as heat from the AuNPs required several milliseconds to diffuse to our measurement location (Figure S8). This lag also manifested as a slowed onset of temperature increase following a laser pulse; as expected, this delay was not present in the capacitance measurements, as those were recorded directly in the lipid membrane. An additional benefit of the model is that it allows a rough calculation of the amount of capacitive current generated by a single nanoparticle in response to a given laser pulse. Based on our calculations of the number of particles adhered to a DRG neuron combined with the total current produced by the particles, we find that a single particle under an irradiance of 18 kW/cm² produces approximately 0.75 pA of current. The depolarization produced by such a current will depend on the membrane capacitance of the cell under consideration.

**DISCUSSION**

In this work, we first showed that neurons can be stimulated with visible light in the presence of 20 nm spherical AuNPs. This was expected as neuronal stimulation has been previously shown with NIR wavelengths (Eom et al., 2014; Yong et al., 2014). While visible light has poorer tissue penetrance than NIR light (Bashkatov et al., 2005), it may be advantageous in applications such as vision restoration in diseased retina (see below). Importantly, the present experiments also demonstrate certain shortcomings of unmodified AuNPs for optical excitation. In particular, they must be present at relatively high concentrations to be effective, and they are easily washed away from the target neurons in a few seconds with convective solution exchange.

To overcome these limitations, we employed a strategy of conjugating the AuNPs to molecules that bind neuronal membrane proteins with high avidity as a strategy to place the AuNPs close to the neuronal membrane and avoid diffusion of the nanoparticles away from the neuron. These molecules included the scorpion toxin Ts1, which targets voltage-gated sodium channels, and antibodies that target TRPV1 and P2X3 ion channels expressed by DRG neurons. Remarkably, all of these ligands substantially facilitated binding of the AuNPs to the target neurons without impeding their excitatory capability. It is important to note that all three ligands tested showed activity in only a fraction of cells (29 out of 55, pooled together), whereas non-functionalized AuNPs worked in the majority of cells (23 out of 29). This difference is statistically significant at α = 0.05 (p = 0.0195, Fisher’s exact test) and is expected because DRG neurons are a highly diverse collection of cells representing the majority of somatosensory phenotypes in the body (Omri and Meiri, 1990). Cells of different sensory specialization can express very different profiles of membrane receptors, a fact that motivated this study and our approach to cell targeting. For example, TRPV1 is classically associated with thermosensitive neurons, while P2X3 receptors are usually associated with nociceptive neurons (Burnstock, 2000; Greffrath et al., 2003). Neurons of different phenotypes thus can exhibit markedly different levels of expression of these channels; in cells with low expression, AuNP binding would probably be insufficient to produce optical sensitivity with the laser powers used in this study. In contrast, non-functionalized nanoparticles were used at high concentration and without washing them out, allowing them to stimulate most excitable cells regardless of their expression profiles. Although ligand-functionalized nanoparticles did not readily attach to all neurons, when the nanoparticles did bind to a cell they were highly resistant to washout. Neurons treated with AuNP-Ts1 remained optically sensitized after more than 30 min of continuous convective solution exchange, and antibody-conjugated nanoparticles performed similarly. Importantly, even when AuNPs are bound to membrane ion channels such as TRPV1, their effect is independent of the activity of the channel. Specifically, they depolarize the membrane by directly changing membrane capacitance rather than by opening the ion channels to which they are ligated. This allows for much faster depolarization than in other strategies where nanoparticle heating leads to opening of temperature-sensitive ion channels (Stanley et al., 2012). The rapid depolarization enabled by the present technique involving membrane capacitance change is critical for temporally precise stimulation of neuronal activity. Finally, tight binding of nanoparticles to the target neuron allowed for minimization of off-target environmental heating once excess particles were removed. These properties are likely to be of significant benefit in vivo, where concentrations of exogenous molecules must be limited to avoid toxicity and where interstitial fluids are constantly being circulated. Additionally, given that all of the investigated molecules conjugated with AuNPs enabled robust photostimulation, it is likely that other AuNP-anchoring biological ligands can be used to preferentially target different classes of excitable cells. Although this work exclusively utilized 20 nm spherical gold nanoparticles with their associated absorption peak at 523 nm, this excitation regime may not be ideal for all purposes. For instance, stimulation with NIR light around 800 nm may be optimal for many in vivo applications due to its high tissue penetrance and much lower absorption by hemoglobin as compared to 532 nm light. Fortunately, a variety of different sizes and shapes of nanoparticles are commercially available, possessing plasmon band absorption peaks ranging from around 500 nm to more than 2 μm. The chemical functionalizations described here should be readily applicable to this array of nanoparticles, allowing optical stimulation with the wavelength best-suited to a specific application.

A particularly interesting phenomenon observed in these experiments is that the efficiency of photostimulation with AuNP-antibody conjugates exhibits a striking dependence on the labeling method. When the AuNP-streptavidin is mixed with biotinylated antibodies prior to incubation with neurons, the cells are much more strongly excited (often to the point of damage) than when the neurons are incubated with primary antibodies, washed with buffer, and then incubated with the functionalized AuNPs. The likely cause of both the improved excitation efficiency and the increased propensity for damage...
with the pre-mixed AuNP-antibodies is the formation of large AuNP clusters. In the case of AuNP-Ts1, a single toxin molecule can bind only one AuNP, so multiple nanoparticles cannot link together. In contrast, the biotinylation process probably attaches multiple biotin molecules at various sites to each antibody (Supplemental Experimental Procedures), while the nanoparticles have an average of nine streptavidin molecules per AuNP. This presumably allows for the formation of arbitrarily large branching complexes via multiple streptavidin-biotin reactions. Thus, a single reaction linking an antibody to its epitope on a neuron could potentially adhere many cross-linked nanoparticles to the neuron at a single location. Conversely, when the biotinylated antibodies and nanoparticles are applied sequentially, they cannot form clusters since they are not present in solution at the same time. The presence of large clusters is expected to greatly increase the number of AuNPs bound to a given neuron, thus increasing the heat generated in the cell’s immediate environment by a given incident optical power. While the increased tendency for cellular damage associated with these cross-linked AuNP structures makes their use questionable for neuronal stimulation, they may find utility in applications such as targeted cell ablation or cancer photothermal therapy where induction of cell death is the objective (Kennedy et al., 2011).

Our results obtained with planar lipid bilayers revealed that laser-induced heating of a lipid bilayer produces changes in bilayer capacitance. These changes drive currents that, in a cellular environment, would alter the transmembrane potential. This process occurred in the absence of any membrane proteins, indicating that ion channels and other membrane proteins are not necessary for the mechanism of neuronal stimulation via AuNPs. As expected when we conceived these experiments, this process is qualitatively similar to the method of direct heating with an IR laser (Shapiro et al., 2012). However, the cooling kinetics observed here after a laser pulse are faster by an order of magnitude or more. This disparity can be explained by the fact that the IR laser pulse is absorbed by a relatively large solution volume; the large excitation volume generates a large amount of heat, and dissipation of this heat requires a relatively long diffusion distance. AuNPs, by contrast, heat only their immediate environment, and the smaller total heat generation results in a shortened diffusion path length for cooling. Importantly, these experiments demonstrated that the size of the depolarizing current depends on the rate of change of temperature (i.e., the derivative), not on the temperature itself. This means that sizeable depolarizations can be obtained without raising the temperature to damaging levels by heating a cell quickly while keeping the duration of heating very short.

The present approach, employing a cell-targeting anchor to intimately localize the AuNP photosensor at the membrane for photothermal cell activation, represents a potentially widely applicable advance in the technology of neuronal optical stimulation. This work has potential applications in at least two distinct fields. First, it could be used as an alternative to optogenetics in fundamental research. Functionalized AuNPs allow for high-avidity binding and stimulation of neurons without damaging the cells. Furthermore, nanoparticles present potential advantages versus optogenetics. The use of functionalized AuNPs does not require transfection or genetic modification of the organisms or tissues of interest. This makes the AuNP technique particularly suitable for use in species in which genetic manipulation is less well-developed or in which the cells/tissue/organism under study cannot survive over an extended transfection period. As in the cases of pharmacological approaches for establishing light sensitivity of native, unmodified ion channels (Lester et al., 1980; Mourot et al., 2013; Yue et al., 2012), the period required for inducing light sensitivity with cell-targeted AuNP conjugates is essentially that needed for access of the administered conjugates to the cells (for in vitro preparations as in the present study, as little as several minutes). Despite these advantages, nanoparticles also have important limitations compared to optogenetics. In addition to stimulation, optogenetics can provide neuronal inhibition and optical readouts of neuronal activity via expression of molecules such as halorhodopsins and voltage-sensitive fluorescent proteins (Akemann et al., 2012; Zhang et al., 2007). Nanoparticles, to our knowledge, are strictly limited to stimulation as inhibition would require an optically induced cooling effect, which seems unlikely. Furthermore, unlike the essentially permanent expression of light-sensitive proteins induced by optogenetic modification of cells, the clearance and/or degradation of delivered AuNP conjugates probably limit the lifetime of cell photosensitivity induced by AuNP treatment; thus, for long-term study, AuNP-based methods would probably require repeat treatment. Another potential limitation of nanoparticles is their broad (and in the case of non-spherical particles, multiple) peaks of plasmon absorption. This may limit the ability to independently excite multiple cell types using different wavelengths, a technique that is possible with optogenetics (Prigge et al., 2012).

A second field of possible application of AuNP-enabled neuronal photostimulation is that of human therapeutics. One therapeutic goal that may be addressable via AuNP treatment is the enabling of direct photostimulation of retinal ganglion cells (RGCs) in patients who suffer from photoreceptor degenerative diseases, such as age-related macular degeneration or retinitis pigmentosa. RGCs are “retinal output” neurons that, in the healthy retina, transmit action potentials to the brain that encode visual signals initiated by the retina’s rod and cone photoreceptors. There is ample evidence that, in many instances of advanced-stage photoreceptor degenerative diseases, RGCs remain healthy despite the deterioration and loss of the native rods and cones (Margolis et al., 2008; Mazzoni et al., 2008; Medeiros and Curcio, 2001). In this situation, functionalized AuNPs similar to those used in the present study could be injected into the eye where they would bind to the RGCs, allowing light entering the eye to directly excite the RGCs and thus bypass the inoperative photoreceptors. The critical importance of achieving vision repair in photoreceptor degenerative diseases is motivating approaches that currently include opto-electronic, optogenetic, pharmacological, and other strategies to engineer light sensitivity of RGCs or other retinal neurons (Bharti et al., 2014; Bi et al., 2006; Caporale et al., 2011; Chader et al., 2009; Greenberg et al., 2011; Lagali et al., 2008; Polosukhina et al., 2012; Theogarajan, 2013). The present experimental findings with conjugated AuNPs open a new avenue that may benefit progress toward this important objective.
EXPERIMENTAL PROCEDURES

DRG cells were cultured from neonatal rats and used 1 to 7 days thereafter (see Supplemental Experimental Procedures for more details on this and all other procedures). For experiments where antibodies were used to link AuNPs to neurons, the primary antibodies (TRPV1ab and P2X3ab) were added to the neuron culture medium and kept overnight in the incubator. Experiments were performed the next day, and AuNP-SA or AuNP-2ab was added during the experiment as shown in the data. For DRG neurons under current clamp, current injections were always 1 ms in duration and varied in amplitude from 300 pA to 700 pA based on what level was necessary to produce robust stimulation in a given cell. Similarly, laser pulses in DRG neurons were also always 1 ms in duration and power was varied as described in our results. All DRG experiments were performed using a 40x microscope objective lens to focus the laser onto the cells. Hippocampal slices were harvested from adult mice and immediately stored in artificial cerebrospinal fluid (aCSF) bubbled with carbon dioxide. Immediately before use, a slice was injected with a mixture of 20 nM AuNP-Ts1 and a fluorescent dye (to allow the injection site to be visualized). The slices were then immersed in a perfusion chamber and stained with ICG dissolved in aCSF for 5 min. Finally, perfusion of carbon-bubbled aCSF was turned on to clear away excess ICG and keep the tissue oxygenated. ICG was imaged using 780 nm excitation through a 20x objective in epifluorescence configuration through the glass floor of the perfusion chamber. Optimal stimulation was performed through a 32x objective mounted above the prep. Planar lipid bilayers were formed from asolectin in a horizontal hole 300 μm in diameter. Due to the geometry of the bilayer chamber, we were unable to use the same microscope objective as with the DRG neurons. Instead, a 10x objective was used. This results in a lower degree of focusing of the laser, and thus laser powers are not directly comparable between the two systems. Temperatures were measured by monitoring changes in resistance of calibrated micropipettes (Yao et al., 2009). Capacitances of planar bilayers were monitored by applying sinusoidal command voltages and observing the resulting sinusoidal currents. The impedance of the system is largely determined by the capacitance of the bilayer for high-frequency inputs. The command signal used in this work had a frequency of 5 kHz. Data analysis, mathematical modeling, and chemical syntheses are described in detail in the Supplemental Experimental Procedures. All animal protocols used in this work were approved by the University of Chicago Animal Care and Use Committee.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and eight figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.02.033.

AUTHOR CONTRIBUTIONS

The project was conceived by D.R.P. and F.B. All authors contributed to design of the research. J.L.C.-d.-S. performed the patch clamp and bilayer experiments, with close assistance by J.S.T. J.S.T. and J.L.C.-d.-S. performed the brain slice imaging experiments. J.L.C.-d.-S., J.S.T., and F.B. analyzed the electrophysiological data. S.B.H.K and B.D. designed, and B.D. performed, the chemical syntheses. F.B. and J.S.T. developed and carried out the computational modeling. J.S.T. and J.L.C.-d.-S. wrote the manuscript, with assistance by D.R.P. and F.B.

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Photosensitivity of Neurons Enabled by Cell-Targeted Gold Nanoparticles

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Figure S1. (A) The power distribution of the laser when focused through a 40x microscope objective. The laser was focused onto a slide coated in tetramethylrhodamine, and the resulting fluorescence was imaged with a CMOS camera. The pixel values were summed and normalized to 200 mW, the total power of the laser as measured with a commercial laser power meter. The size imaged by each pixel was calibrated by taking an image of a ruler with the same objective lens. This distribution represents the profile of light seen by the patch-clamped neurons in the work when using the laser at full power. The average irradiance was calculated by choosing the half-maximum value as the boundary of the laser spot, roughly corresponding to a circle of 5 μm radius. Within this boundary, the average intensity was 36.2 kW/cm² when using full laser power. (B) Diffusion of non-functionalized AuNPs away from DRG neurons causes loss of optical sensitivity. Initially, DRG cells are unresponsive to light as the peak voltage response (the maximum membrane potential reached following a laser pulse) is simply equal to the resting potential. Addition of non-functionalized AuNPs quickly sensitized the cells to light, and they reliably fired action potentials on every pulse. Even without active perfusion washing, however, the non-functionalized AuNPs eventually began to diffuse away from the cells and they lost excitability, firing action potentials with a progressively lower probability over the course of about two minutes. This contrasts with active washing where the cells became completely unresponsive to light within seconds. As expected, addition of a new bolus of AuNPs to the cell restored sensitivity to light. Black arrows show additions of 50 nM AuNP boluses.
**Figure S2.** Chemical synthesis of biotinylated Ts1. (A) Synthetic strategy for the preparation of Ts1-PEG3-Biotin. (B) Purified Ts1-PEG3-Biotin. Inset ESMS Calc. 7235.3 Da (av. isotope composition) Obsd. 7235.4 ± 0.2 Da. The MS data shown were collected across the entire UV-absorbing peak.
Figure S3. Optically-induced currents in DRG neurons are linear with respect to both laser power and membrane potential. (A) Under voltage-clamp at -80 mV, optical stimulation produces an inward current. (B) The laser-induced currents are linear with respect to laser power. (C) A family of voltage-clamp traces at different potentials shows that the amplitudes of the optically-induced currents depend on the membrane potential. (D) The laser-induced currents are linearly dependent on voltage with a reversal potential near 0 mV. Green bars show laser stimuli.
Figure S4. Consecutive optical stimuli can produce paired-pulse facilitation in hippocampal slices. The brain slice was stimulated with two identical 532 nm laser flashes (225 mW, 10 ms each – green bars) spaced 82 ms apart. The second stimulus produces around 40% more depolarization than the first stimulus.
Figure S5. AuNP-TRPV1ab clusters sensitize DRG cells to light but often lead to laser-induced damage. (A) As with other AuNP-antibody labeling strategies, AuNP-TRPV1ab clusters render DRG cells optically excitable and do not wash out with active perfusion. Electrical stimuli (500 pA, 1 ms – blue bars) and optical stimuli (48 mW, 1ms – green bars) are shown. (B) Laser pulses (48 mW, 1ms – green bars) often cause a persistent depolarization following the action potential when using AuNP-antibody clusters. These are interpreted as membrane damage caused by excessive heating, and the membrane potential usually returns to the initial resting potential with a time course of seconds to minutes. These five traces show the range of damage observed, from near-normal behavior (top) to large and prolonged depolarization (bottom). By contrast, electrical stimuli (500 pA, 1 ms – blue bars) do not cause these pathological depolarizations. (C) The amount of abnormal depolarization remaining 15 ms after a laser pulse (taken as a readout of membrane damage) is strongly correlated with the magnitude of laser-induced depolarization at the beginning of the pulse. Essentially, stronger heating causes larger depolarizing effects, but also leads to greater membrane damage when using AuNP clusters. Interestingly, there seems to be a threshold stimulus size below which no damage is observed. Damage increases sharply with stimulus size above the threshold level.
Figure S6. AuNP-P2X$_3$ab clusters behave similarly to AuNP-TRPV1ab clusters. (A) AuNP-P2X$_3$ab clusters sensitize DRG cells to light and do not easily wash out. Electrical stimuli (500 pA, 1 ms – blue bars) and optical stimuli (48 mW, 1ms – green bars) are shown. (B) As with AuNP-TRPV1ab clusters, a wide range of laser-induced damage is often seen following stimulating pulses of light (top – least damage; bottom – most damage). (C) Again, membrane damage depends strongly on stimulus size and seems to exhibit a similar threshold effect. Laser-induced depolarizations below the threshold magnitude (approximately 25 mV in this particular cell) do not lead to abnormal and persistent depolarizations 15 ms after optical stimuli.
Figure S7. Using the one-dimensional heat diffusion model (see Supplemental Experimental Procedures), changes in temperature were calculated as a function of time and distance from the heating slab. As distance from the heating layer increases, there is an increasing delay before the temperature begins to rise, the kinetics of the temperature increase are slower, and the time to reach peak temperature becomes larger. For this particular simulation, parameters were chosen to model the case of a DRG cell being perfused with 20 nM AuNPs. Accordingly, the heating layer was chosen to be 4 μm thick, the pulse duration was 1 ms at 50% of full laser power, and the fractional volume of the slab occupied by nanoparticles was set as 1 part in 20,000. See also Figure S8.
Figure S8. The one-dimensional heat diffusion model qualitatively recapitulates experimental results. (A) The results from DRG cells were modeled for the case where they are being perfused with 20 nM AuNPs as well as after the excess particles are washed out (compare to Figure 2F in the main text). Both simulations used a pulse duration ($t_1$) of 1 ms and a “measurement location” ($x$) close to the heating slab, since the measuring pipette was very close to the cell under investigation during the experiment (approximately 2 μm from the edge of the cell, thus perhaps 5 μm from the laser spot). The power used during these experiments was one-half of maximum laser power ($I_0/I_{max}$). For the case of perfusion with 20 nM AuNPs, the fractional volume ($V_{frac}$) of particles in solution can be calculated to be 1 part in 20,000. We found that given these parameters, a 4 μm-thick heating layer ($2 \times b$) most accurately recapitulated the observed data. For the attached nanoparticles case, all parameters were kept the same, except that slab thickness ($2 \times b$) was set at 20 nm to model a single-particle-thick heating layer, and $V_{frac}$ was varied to best reproduce the experimental data. In this case, the value of 1/650 corresponds to having approximately 1500 AuNPs attached to a single neuron. While this number is not quantitatively rigorous, it nonetheless is of a very plausible order of magnitude given the number of sodium channels in an average DRG neuron soma. (B) Similar to (A), but simulating the planar lipid bilayer experiment (compare to Figure 7D in the main text). In this case, the measurement location was a much larger and hard-to-estimate distance from the bilayer (since the measurement pipette was vertically above the bilayer rather than lateral to it); we thus estimated 50 μm for this value. Additionally, a relatively large bolus of AuNPs was added via pipettor to the bilayer chamber, likely resulting in a thick heating layer (estimated at 18 μm). The AuNP concentration was again 20 nM, allowing easy calculation of $V_{frac}$. Finally, the full laser power was used, but through a microscope objective which was 4X less powerful than with the DRG neurons, resulting in $I_0/I_{max} = (4)^2$. Keeping these parameters identical and varying only pulse duration produced two traces which recapitulate the experimental data well. All traces in this figure were filtered at 1 kHz to better compare with the experimental data (which was identically filtered).
Supplemental Experimental Procedures

Experimental Equipment

Both patch clamp of cells and clamp of painted bilayers were performed using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, California). The amplifier output was passed through an 8-pole Bessel filter (Frequency Devices, Ottawa, Illinois, model 950L8L) and digitized with an Innovative Integration SBC-6711-A4D4 data acquisition board (Simi Valley, CA). The DAC of the data acquisition board supplied the command voltage to the clamp amplifier. Preparations were mounted on a Zeiss IM 35 microscope (Carl Zeiss Microscopy, Thornwood, New York) and visualized through objective lenses ranging from 10x/0.25NA to 40x/0.55NA. Nanoparticles were stimulated with a 532 nm DPSS laser (UltraLasers, Ontario, Canada), and the beam was modulated with an acousto-optic modulator (NEOS Technologies, Gooch & Housego, PLC., Melbourne, Florida). For patch clamp experiments, pipettes were pulled on a Sutter Instruments P-2000 CO2 laser micropipette puller (Novata, California). Patch pipettes were flame-polished using a custom microforge to produce approximately 2 MΩ resistances when filled with internal pipette solution (see ‘Solutions’ below for all solution compositions). Nanoparticles were delivered via a theta capillary tube pulled to approximately 20 μm tip diameter on a Flaming/Brown pipette puller (Sutter Instruments, model P-87). One side of the theta capillary was filled with an AuNP solution while the other half was filled with bath solution for washing. Each side of the tube was connected to independently-controlled pressurized air. Temperatures were measured by monitoring the resistance in a micropipette with an OC-725A amplifier (Warner Instruments, Hamden, Connecticut). The temperature pipette was pulled to about 2 MΩ and filled with bath solution. Sinusoidal command voltages for measuring membrane capacitance were produced by a function generator (Krohn-Hite, Brockton, Massachusetts, model 1200A).

Brain slices were mounted on an IX71 inverted microscope (Olympus, Center Valley, Pennsylvania) and imaged with an Evolve 128 EMCCD camera (Photometrics, Tucson, Arizona), containing a 128 x128 pixel CCD capable of acquiring images at 500 Hz full-frame. Excitation of ICG was accomplished with a World Star Technologies 780 nm solid-state laser (Toronto, Canada) and the Chroma Technology Corp. Indocyanine Green filter set (Cat #: 49030 ET, Bellows Falls, Vermont), along with a 20X/0.45NA objective lens in the normal inverted microscope position. Stimulation of the nanoparticles was achieved by mounting a 532nm DPSS laser (Laserglow Technologies, Toronto, Canada) and 32X/0.4NA objective on the sample stage and illuminating the brain slices from above.

Materials and Solutions

All concentrations are in mM unless specified otherwise.

Bath solution: NaCl 132, KCl 4, MgCl2 1.2, CaCl2 1.8, HEPES 10, glucose 5.5 pH 7.4

Internal pipette solution: NaCl 10, KF 130, MgCl2 4.5, HEPES 10, EGTA 9, ATP 2, pH 7.3

Artificial cerebrospinal fluid (aCSF): NaCl 125, KCl 2.5, NaHCO3 26, NaH2PO4 1.25, MgCl2 1.5, CaCl2 2.5, glucose 10

Bilayer recording solution: KCl 90, HEPES 10, pH 7.4

EBSS: NaCl 132, KCl 5.3, HEPES 10, NaH2PO4 1, glucose 5.5, pH 7.4

DMEM: Gibco DMEM, high glucose, HEPES, no phenol red (Life Technologies, Grand Island, New York, Cat #: 21063-029)

PLL: Poly-L-lysine solution, Sigma-Aldrich, St. Louis, Missouri, Cat #: P8920

Trypsin: Worthington, Lakewood, New Jersey, Cat #: TRL3
FBS: Fetal bovine serum, ATCC, Manassas, Virginia, Cat #: 30-2020
Asolectin: Soybean polar lipid extract, Avanti Polar Lipids, Alabaster, Alabama, Cat #: 541602C
Penicillin: Sigma-Aldrich, Cat #: 13750
Streptomycin: Sigma-Aldrich, Cat #: S6501

20 nm AuNP-Streptavidin: Nanopartz, Loveland, Colorado, Cat #: C11-20-TS-50-Buffer
Indocyanine green (ICG): Sigma-Aldrich, Cat #: I2633 (sold as Cardiogreen); stored at 20 mM in DMSO at -80°C
Tetramethylrhodamine-5-maleimide (TMRM): Life technologies, Cat #: T-6027; stored at 20 mM in DMSO at -20°C
Azide-PEG3-Biotin: Sigma-Aldrich, Cat #: 762024
Anti-Rat TRPV1 (extracellular) Antibody, Alomone Labs, Jerusalem, Israel, Cat #: ACC-029
Anti-P2X3 Receptor (extracellular) Antibody, Alomone Labs, Cat #: APR-026
Slide-A-Lyzer MINI Dialysis Devices 20K MWCO, Thermo Scientific, Waltham, Massachusetts, Cat #: 87734
Pierce Antibody Clean-up Kit, Thermo Scientific, Cat #: 44600
EZ-Link NHS-PEG12-Biotin, Thermo Scientific, Cat #: 21312
BupH Phosphate Buffered Saline packs, Thermo Scientific, Cat #: 28372

Methods

Cell Culture Protocol

Glass-bottomed culture dishes were thoroughly cleaned, rinsed with water, dried, and sterilized with UV light. They were then incubated with PLL for 20 min at room temperature, after which they were rinsed with sterile water and stored until use.

Dorsal root ganglia were excised from P1-P3 Sprague-Dawley rats following decapitation and were immediately placed in DMEM on ice. Ganglia were rinsed multiple times with EBSS then digested with EBSS + 0.25% Trypsin for 20 min at 37°C with shaking. Following digestion, the cells were centrifuged and the supernatant removed and replaced with EBSS + 10% FBS to inhibit remaining trypsin. The digested ganglia were then extruded through three glass pipettes of decreasing size to mechanically disperse the cells. The cells were centrifuged a final time and the supernatant replaced with DMEM + 5% FBS. Cells were seeded into the previously prepared PLL-treated culture dishes and allowed to sit undisturbed for 30 min to facilitate DRG cell adhesion to the glass. Finally, the dishes were flooded with DMEM + 5% FBS + 100 U/ml penicillin + 100 μg/ml streptomycin and incubated at 37°C with 5% CO₂ until use.

Hippocampal Slice Preparation Protocol

C57BL/6 mice 65 to 102 days old were anesthetized with isoflurane and decapitated. Their skulls were opened, the cerebella excised from the rest of the brain, and the brains removed from the skull and immediately placed in partially-frozen aCSF. After 5 to 10 minutes, the brains were placed in a vibratome and 350 μm hippocampal slices were cut and immediately placed in aCSF continuously bubbled with carbogen (95% O₂ / 5% CO₂). A mixture was made of 20 nM AuNP-
Ts1 and 2 μM tetramethylrhodamine-5-maleimide (TMRM), and this was loaded into a glass micropipette. Under a microscope, approximately 50 nL of the AuNP-Ts1 solution was injected into the CA1 region of a hippocampal slice immediately before use. This injection was done to bypass the layer of dead cells on the exterior of an acute brain slice. Following the injection, slices were placed in a perfusion chamber on the imaging microscope and stained for 5 minutes with 40 μM indocyanine green (ICG) dissolved in carbogen-bubbled aCSF. Following the staining, carbogen-bubbled aCSF was continuously perfused through the chamber. The 532 nm laser was manually positioned to focus on the nanoparticles by looking for TMRM fluorescence at the injection site excited by the 532 nm light.

**Electrophysiology**

Neurons were patched under voltage-clamp to monitor the seal resistance. When the giga-seal was achieved, a whole-cell patch clamp configuration was reached by applying a pulse of negative pressure inside the pipette. The ideal series resistance at this point was to be less than three times the initial pipette resistance before touching the neuron. At this point, the amplifier mode was changed to current clamp and the cell excitability was tested with 1 ms current injection pulses of increasing amplitudes. The minimal amplitude needed to trigger action potentials with 70% probability ranged from 300 pA to 700 pA for different cells. The membrane voltage was filtered at 5 kHz and digitized at 20 kHz.

For the voltage clamp experiments, both the membrane capacitance and series resistance were compensated as well as possible. The amplifier current output was filtered at 50 kHz and digitally sampled at 200 kHz.

**Painted Bilayer Formation and Electrophysiology**

Asolectin lipids were dried in a clean test tube by evaporating the chloroform under a stream of dry nitrogen. The resulting lipid films were placed in a desiccator for at least 1 hour and then dissolved in n-decane to produce a final concentration of 25 mg/ml.

The lipid bilayers were painted in home-made acetal chambers using a horizontal bilayer configuration. A top chamber, containing a 300 μm orifice, was mounted inside a glass-bottomed lower chamber to allow for laser illumination. The n-decane lipid solution was deposited around the orifice of the top chamber and dried for 10 minutes. Finally, both chambers were filled with bilayer recording solution (see ‘Solutions’). Bilayers were painted with an air bubble in a pipettor tip, and their formation and growth monitored under voltage clamp by observing a current response to a voltage ramp. The current data were filtered at 50 kHz and digitized at 200 kHz.

**Capacitance Measurement**

Capacitances of lipid bilayers were measured by calculating the impedance of the bilayers in response to sinusoidal input voltages. Since the impedance of a capacitor depends inversely on its capacitance, an increasing bilayer capacitance will result in lower impedance and thus an increase in current through the system. Conversely, a decrease in capacitance will cause a decrease in current. Sinusoidal signals were processed by rectifying the waves and then digitally low-pass filtering the result with a Gaussian filtering algorithm. This produced a readout of amplitude versus time while removing the sine wave itself. For these experiments with a 5 kHz carrier sinusoidal applied voltage, we chose a filter cutoff frequency of 1 kHz to strongly attenuate the sinusoid while maintaining features of interest in the signal.

**Temperature Measurement**

For temperature measurement, pipettes of approximately 2 MΩ were filled with the same solution as in the current bath to avoid an ionic gradient across the pipette tip. They were then placed in as close proximity as possible to either the DRG neuron under investigation or the planar bilayer. Pipette resistances were monitored using a voltage-clamp amplifier.
After each experiment, pipettes were calibrated by placing them in solution next to a thermocouple tip and changing the solution temperature to build a calibration curve between temperature and pipette resistance. Since each pipette had a different shape and resistance, they were calibrated individually after use.

**Brain Slice Image Processing**

Brain slice data were acquired as movies recorded at 500 Hz (2 ms per frame) on an EMCCD camera. To produce time-dependent traces of activity (Figure 4B in the main text and Figure S4), all pixel values in each frame were averaged together. The resulting fluorescence traces were normalized by taking the average of a region of data from before the laser stimulus ($F_0$), subtracting this value from all other data points to produce a $\Delta F$, and then finally dividing this value by $F_0$. This produced the commonly-used normalized fluorescence metric of $\Delta F/F_0$.

The activity images (Figure 4C in the main text) were produced by creating five different averaged images. The first was the average of a group of frames spanning a 180 ms interval before the stimulus. This was the baseline image. The other four were averages of all frames from four different, consecutive 80 ms windows shortly following the stimulus. For instance, the first of these images was formed by averaging all frames from 20 ms to 100 ms post-stimulus. To reduce pixel noise, all five of these averaged images (pre-stimulus baseline image and four post-stimulus images) were then filtered with a Gaussian low-pass image filter using a standard deviation of five pixels. Finally, the left-most panel in Figure 4C was formed by subtracting the baseline image from the first post-stimulus image and dividing all resulting pixel values by the average fluorescence value of the baseline image. This produces $\Delta F/F_0$ pixel values completely analogous to the time-dependent traces as described above. The other three panels in Figure 4C were formed similarly, but using post-stimulus images starting at 100 ms, 180 ms, or 260 ms post-stimulus. All four panels in Figure 4C use the same color scale and no additional image processing was performed beyond the filtering and normalization described above.

**Software**

Data acquisition, analysis, and modeling were performed using programs written in-house, as well as MATLAB (The MathWorks, Inc.), Mathematica (Wolfram Research), and Origin (OriginLab Corp.).

**Mathematical Modeling**

**Modeling gold nanoparticles**

Understanding how the absorption of light by gold nanoparticles leads to membrane depolarization required modeling two distinct but related phenomena. The first step was to model laser-induced heating of the gold nanoparticles and the subsequent diffusion of the heat to a membrane. The final output of this step was a time-course of membrane temperature during and after a laser pulse. Following this, the temperature profile was fed into a second model, described in detail previously (Shapiro et al., 2012), which calculated membrane capacitance as a function of temperature. This capacitance time-course could then be directly converted into membrane currents by elementary electronics calculations.

The first question faced in these models was how to treat the nanoparticles as heat sources. Femtochemistry experiments have shown that the plasmonic absorption of photons and subsequent conversion to heat in gold nanoparticles is a complicated process, involving details such as transiently-differing temperatures between the electron gas of the particle and the crystal lattice of the particle (Link and El-Sayed, 1999). Fortunately, most of these complex dynamics occur on picosecond timescales, vastly faster than neuronal activity. Therefore, at the biologically-relevant timescales for this work (hundreds of microseconds to milliseconds), we treated the particles as simple constant heat sources during laser illumination and assumed that they were always in thermal equilibrium with their immediate aqueous environment. These assumptions greatly simplified the model and likely introduce little error into the final results.
The initial time points for our heat simulations were chosen to correspond to the beginning of a laser pulse. At this time, the system was assumed to be in thermal equilibrium, so there were constant-temperature initial conditions. Additionally, the system contained internal, time-dependent heat sources (the nanoparticles). During the laser pulses, these behave as constant heat sources as discussed previously; after the end of the pulses they generate zero heat. When added to the heat equation, these sources represent a discontinuous forcing function. This makes the equation most tractable to solving via Laplace transforms, which easily handle forcing functions of this kind.

The next decision was how to model the geometry of the nanoparticles on a membrane. We simulated two distinct scenarios: an idealized case which used symmetry to limit heat diffusion to a single dimension, and a more realistic and general (but more computationally intensive) case with three-dimensional heat diffusion.

**One-dimensional heat diffusion**

The first scenario considered was a situation where nanoparticles cover an infinitely-large planar membrane. In this scenario, the membrane is treated as being covered in a homogenous layer of “nanoparticle material” of a constant thickness. This entire layer uniformly generates heat during the laser pulse, at a rate dictated by the density of the nanoparticles near the membrane. This set of conditions possesses a 2-dimensional symmetry, and thus there is effectively no lateral heat diffusion or temperature variation in the direction parallel to the membrane. Accordingly, the problem reduces to one of heat diffusion in a single dimension: the direction normal to the plane of the membrane (see Figure below). While such a model is a great simplification of reality and incorporates several assumptions that are not strictly true, such as infinitely-large membranes and particles spread into a homogenous layer, it also greatly reduces both computation times and the number of free parameters in the system. This technique allowed for a rapid survey of many conditions and provided insight into some of the observed features of our data.

![Diagram of 1D heat diffusion](image)

The equation describing this problem is:

\[
\frac{\partial^2 T}{\partial x^2} + \frac{A_0}{k} = \frac{1}{\alpha} \frac{\partial T}{\partial t}
\]

Where \( T \) is the temperature, \( A_0 \) is the heat generated during the laser pulse by the particles contained in the slab between \( x = -b \) and \( x = b \), \( \alpha \) is the thermal diffusivity of water (0.143e-6 \( \text{m}^2 \text{s}^{-1} \)), \( k \) is the thermal conductivity of water (0.6 \( \text{W} \text{m}^{-1} \text{K}^{-1} \)), \( x \) is distance in \( \text{m} \) and \( t \) is time in \( \text{s} \). The pulse of light is applied at \( t = 0 \) and is removed at \( t = t_1 \). By applying the Fourier and Laplace transforms we obtained the solutions (for \( x > b \)):

For \( t \leq t_1 \):
\[ T(x, t) = \frac{A_0 \alpha}{k} \left[ \frac{t}{\alpha \pi} \left( x \exp \left[ \frac{-x^2}{4at} \right] - (x - b) \exp \left[ \frac{-(x - b)^2}{4at} \right] \right) + \left( t + \frac{(x - b)^2}{2\alpha} \right) \text{erfc} \left[ \frac{x - b}{2\sqrt{\alpha t}} \right] - \left( t + \frac{x^2}{2\alpha} \right) \text{erfc} \left[ \frac{x}{2\sqrt{\alpha t}} \right] \right] \]

and for \( t > t_1 \):

\[ T(x, t) = \frac{A_0 \alpha}{k} \left[ \left\{ \frac{t}{\alpha \pi} \left( x \exp \left[ \frac{-x^2}{4at} \right] - (x - b) \exp \left[ \frac{-(x - b)^2}{4at} \right] \right) + \left( t + \frac{(x - b)^2}{2\alpha} \right) \text{erfc} \left[ \frac{x - b}{2\sqrt{\alpha t}} \right] - \left( t + \frac{x^2}{2\alpha} \right) \text{erfc} \left[ \frac{x}{2\sqrt{\alpha t}} \right] \right\} - \left\{ \frac{(t - t_1)}{\alpha \pi} \left( x \exp \left[ \frac{-x^2}{4a(t - t_1)} \right] - (x - b) \exp \left[ \frac{-(x - b)^2}{4a(t - t_1)} \right] \right) + \left( t + \frac{(x - b)^2}{2a(t - t_1)} \right) \text{erfc} \left[ \frac{x - b}{2\sqrt{a(t - t_1)}} \right] - \left( t - t_1 + \frac{x^2}{2a(t - t_1)} \right) \text{erfc} \left[ \frac{x}{2\sqrt{a(t - t_1)}} \right] \right\} \right] \]

To compute \( A_0 \), we first computed the heat \( Q \) generated per unit volume by spherical nanoparticles using the formula of Govorov and collaborators (Govorov et al., 2006).

\[ Q = \text{Re} \left[ i \omega \frac{\varepsilon(r) - 1}{c \sqrt{\varepsilon_0}} I_0 \left| \frac{3\varepsilon_0}{2\varepsilon_0 + \varepsilon_m} \right|^2 \right] \]

Where \( c \) is the speed of light, \( r \) is the distance from the center of the spherical particle, \( \varepsilon \) is the complex dielectric constant, \( \omega \) is the angular frequency of the light, \( I_0 \) is the light intensity, \( \varepsilon_m \) is the dielectric constant of the metal and \( \varepsilon_0 \) is the dielectric constant of the water. We took the \( Q \) produced by each nanoparticle and multiplied it by a factor to account for the fractional volume of the heating slab occupied by nanoparticles (as opposed to non-absorbing aqueous medium) to obtain \( A_0 \):

\[ A_0 = Q \ast V_{frac} \]

This process assumes that whatever nanoparticles are in the slab are homogenously spread throughout the volume of the slab. The only difficulty in this process is the determination of the fractional volume occupied by nanoparticles. In the case where a known concentration of particles is present in solution, this value can be calculated directly from the known nanoparticle radius and Avogadro’s number. However, in the case where a thin layer is attached to a cell surface, the value must be estimated.

Figure S7 shows an example of a temperature surface output by this model. The gross behavior is as expected, with temperature increasing during the pulse and largely decreasing after. However, it shows that at distances far from the heating layer, the temperature can continue to rise long after the laser pulse ends. Also, it shows that much higher temperatures are reached near to the heating slab than far from it, as one would predict. We used this model to attempt to recapitulate experimental temperature recordings from both DRG neurons and planar lipid bilayers (Figure S8). The model does a good job of qualitatively reconstructing the observed details, although it is not accurate enough to make useful quantitative predictions beyond order-of-magnitude accuracy.

We then computed the capacitance change using the equations of Shapiro et al. (2012). Finally, to compute the currents, we solved the membrane circuit under voltage clamp to take into account the limited clamp speed imposed by the series resistance. Like the modeled temperatures, the modeled capacitance changes broadly reflect the experimental results, although they differ from experiment in the fine details.
Three-dimensional heat diffusion

The second scenario we considered was to assume that individual nanoparticles are located at specific locations in an infinite three-dimensional medium. This simulates each particle individually and allows for control over both the density of particles on the surface and the number of layers of particles piled on each other. The solution to this system for a single point-source that turns on at \( t = 0 \) had been previously derived (Ozisik, 1968), and a modification of this solution allowed for a single point source which also turned off at \( t = t_1 \). Extending this solution to account for the effects of many individual nanoparticles at specific locations simply amounts to summing the individual contributions of each particle’s heat output to the time-dependent temperature at a fixed location, \( T(x, y, z, t) \):

For \( t \leq t_1 \):

\[
T(x, y, z, t) = \frac{Q V_{NP}}{k} \sum_i \frac{1}{4\pi d_i(x, y, z)} \text{erfc} \left[ \frac{d_i(x, y, z)}{2\sqrt{\alpha t}} \right]
\]

And for \( t > t_1 \):

\[
T(x, y, z, t) = \frac{Q V_{NP}}{k} \sum_i \frac{1}{4\pi d_i(x, y, z)} \left( \text{erfc} \left[ \frac{d_i(x, y, z)}{2\sqrt{\alpha t}} \right] - \text{erfc} \left[ \frac{d_i(x, y, z)}{2\sqrt{\alpha(t-t_1)}} \right] \right)
\]

Where \( d_i(x, y, z) \), given by:

\[
d_i(x, y, z) = \sqrt{(x - X_i)^2 + (y - Y_i)^2 + (z - Z_i)^2}
\]

is simply the Euclidean distance between the point of observation at the coordinate \((x, y, z)\) and the position of the \( i \)th nanoparticle, located at \((X_i, Y_i, Z_i)\). All other constants and variables are defined as in the one-dimensional case, with the addition of \( V_{NP} \), the volume of a gold nanoparticle (4.19e-24 [m³]).

Although the equations describing this model appear simpler than the equations in the one-dimensional model, the summation over all nanoparticles in the system quickly becomes very time-consuming once more than a few thousand particles are being modeled. Practically speaking, this limits the utility of this three-dimensional diffusion model to simulating the case of the DRG neurons after washing, where only a single layer of attached AuNPs is present. From the one-dimensional simulation, we expect the number of particles in this scenario to be on the order of 1,500 (Figure S8), a tractable quantity. We modeled this system a number of times with a variety of specific nanoparticle geometries, and the results are all in broad agreement with the one-dimensional case, although the quantitative specifics vary from simulation to simulation. This corroborates our belief that the one-dimensional model is accurate enough to facilitate qualitative understanding of AuNP heating dynamics, but that neither model is sufficient to support precise quantitative predictions.

As a final test, we modeled the AuNPs as heat-generating spheres of finite size rather than as infinitesimal point-sources of heat using the method of Goldenberg and Tranter (1952). The results were nearly identical to the case of point-sources, suggesting that this additional computational burden is unnecessary for the purposes described here.

Chemical Synthesis

Reagents

Azide-PEG3-Biotin was obtained from Sigma-Aldrich; [W50Pra]Ts1.CONH₂ was prepared as previously described (Dang et al., 2014). 20 nm AuNP-Streptavidin was obtained from Nanopartz. Anti-Rat TRPV1 (extracellular) Antibody and Anti-P2X3 Receptor (extracellular) Antibody, were obtained from Alomone Labs. Slide-A-Lyzer MINI Dialysis Devices 20K MW cut-off,
Pierce Antibody Clean-up Kit, EZ-Link NHS-PEG12-Biotin, BupH Phosphate Buffered Saline packs, were obtained from Thermo Scientific.

Bath solution was prepared as described above.

**Reverse phase HPLC and LC-MS analysis**
Analytical reversed phase HPLC and LC-MS were performed using an Agilent 1100 series HPLC system equipped with an online LCQ-Advantage ion trap. Column used was Phenomenex Aeris WIDEPORE 3.6 μm C4, 150 x 4.6 mm. Chromatographic separations were performed using a linear gradient of 20-60% acetonitrile (0.08% TFA) versus water (0.1% TFA) over 20 min with column temperature 40 °C. Flow rates were controlled at 0.9 mL/min. Peptide detection was by UV absorption at 214 nm, and masses were obtained by online electrospray mass spectrometry.

**Preparative HPLC**
The product from the click reaction was purified using a Phenomenex Aeris WIDEPORE 3.6 μm C4, 150 x 4.6 mm column. A shallow gradient of acetonitrile (0.08% TFA) versus water (0.1% TFA) was used. Flow rates were controlled at 0.9 mL/min. Fractions containing the desired product were collected, identified by analytical LC and mass spectrometry, then combined and lyophilized.

**Labeling [W50Pra]Ts1.CONH₂ with Azide-PEG3-Biotin.**
In a typical reaction, to 2.0 mL of degassed buffer (1 M Guanidine hydrochloride, 200 mM Tris, 40 mM TCEP hydrochloride) was added 80 μL 1 M CuSO₄ to generate Cu(I) in-situ; the buffered solution was heated up to ensure the TCEP hydrochloride was fully consumed. After cooling to room temperature, the click reaction buffer pH was adjusted to 8.7 before use. [W50Pra]Ts1.CONH₂ (0.2 mg, 29.4 nmol) was dissolved in 240 μL degassed water then Azide-PEG3-Biotin (40 μL of 2.5 mg/mL in DMF) and 120 μL click reaction buffer were added to the solution. The reaction was left for 30 minutes, then another 90 μL of freshly prepared click reaction buffer was added to the reaction mixture. After a further 10 minutes, the reaction product was purified on a C4 analytical column (90 μg, 12.4 nmol, 42.2% yield. Obsd. 7235.4 ± 0.2 Da, Calc. 7235.3 Da (av. isotope composition)). The amount of protein product was determined from the OD 280nm measured on a NanoDrop spectrophotometer.

**Conjugation of Ts1-PEG3-Biotin with 20 nm Streptavidin-AuNP:**
Ts1-PEG3-Biotin (4 μM, 50 μL in bath solution) and 20 nm AuNP-Streptavidin (54 nM, 50 μL in bath solution) were mixed and shaken well. After 5 hours incubation at 4°C, the mixture was transferred to the 20 MW cut-off MINI dialysis tube and dialyzed against 700 mL bath solution 3 times at 4 °C (4 hours, overnight, 4 hours) with gently stirring to remove non-bound Ts1-PEG3-Biotin. This dialyzed AuNP-Streptavidin-Biotin-PEG3-Ts1 conjugate was used directly for assays.

**Biotinylation of Antibodies:**
Anti-P2X3 Receptor antibody was reconstituted by adding 100 μL water to give ~2.6 μM antibody solution with 0.5% BSA, 0.025% sodium azide. Sodium azide was removed by dialysis, and BSA was removed by using Pierce Antibody Clean-up Kit. After BSA was removed, 200 μL of 260 nM antibody solution was dialyzed against amine-free phosphate buffered saline (pH 7.5) to exchange the antibody buffer. The antibody solution was then transferred to 0.5 mL Eppendorf tube, and EZ-Link NHS-PEG12-Biotin (10 μL, 50 μM in DMSO) was added, then the mixture was shaken at room temperature for 30 minutes. The mixture was then transferred to a 20 MW cut-off MINI dialysis tube and dialyzed against water for 1 hour at room temperature, then dialyzed against bath solution twice at 4 °C (3 hours, 10 hours) to exchange buffer and to remove non-bound NHS-PEG12-Biotin.
Anti-Rat TRVP1 antibody was reconstituted by adding 100 μL water to give ~2.8 μM antibody solution with 0.5% BSA, 0.025% sodium azide. Sodium azide was removed by doing dialysis, BSA was removed by using Pierce Antibody Clean-up Kit. After BSA was removed, 200 μL of 280 nM antibody solution was dialyzed against amine-free phosphate buffered saline (pH 7.5) to exchange the antibody buffer. The antibody solution was then transferred to a 0.5 mL Eppendorf tube, and EZ-Link NHS-PEG12-Biotin (10 μL, 50 μM in DMSO) was added, then the mixture was shaken at room temperature for 30 minutes. The mixture was then transferred to a 20 MW cut-off MINI dialysis tube and dialyzed against water for 1 hour at room temperature, then dialyzed against bath solution twice at 4 °C (3 hours, 10 hours) to exchange buffer and to remove non-bound NHS-PEG12-Biotin.

**Antibody-Biotin conjugation with 20 nm AuNP-Streptavidin:**
Each biotinylated antibody (50 μL, 260 nM in bath solution) was mixed with 20 nm AuNP-Streptavidin (50 μL, 54 nM in bath solution) and incubated at 4°C for 1.5 hours. The mixture was then transferred to a 0.5 mL Eppendorf tube. The sample was centrifuged at 12.8 × 1000 rpm for 3.5 minutes, supernatant ~90 μL was removed, and 90 μL bath solution was added to re-suspend the AuNP. Centrifugation was repeated 3 times more to further purify the antibody-AuNP conjugate. The final purified AuNP-Streptavidin-Biotin-Antibody was used for assays.
Supplemental References:


