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In Vitro Application of Gold Nanoprobes in Live Neurons for Phenotypical Classification, Connectivity Assessment, and Electrophysiological Recording

Karl C. Mendoza
College of William & Mary - Arts & Sciences

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In Vitro Application of Gold Nanoprobese in Live Neurons for Phenotypical Classification, Connectivity Assessment, and Electrophysiological Recording

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A Thesis presented to the Graduate Faculty of the College of William and Mary in Candidacy for the Degree of Master of Science

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Master of Science

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Thermoregulatory neurons in the preoptic area of the anterior hypothalamus (POA) form synaptic networks, which affect responses that regulate body temperature. In order to characterize these pathways of activation, projections to effector control areas, like the dorsomedial hypothalamus (DMH) or raphe pallidus (RPa), require labeling in live tissue slices. Traditional fluorescent dyes label axon terminals near an injection site, and unfortunately, also that of nearby fibers of passage. Here, we describe a novel methodology for retrograde labeling of neurons in vitro, while also allowing for further electrophysiological recording. To determine if POA neurons project to either the DMH or RPa, we have used nanometer-sized, gold nanoprobes, which provide for specific neuronal entry, via synapses in close proximity to the injection site. Upon neuronal entry, these nanoprobe complexes transport to the soma, where they are readily visualized and quantified. We found that conjugation of these gold nanoprobes with VGLUT-2 antibodies and polyethyleneimine (PEI) facilitate neuronal entry among glutamatergic neurons, with high levels of labeling efficacy with the POA. Probes with VGAT and PEI, on the other hand, which are designed to preferentially enter GABAergic neurons, show modest labeling of the POA. This novel method, adapted from emerging cancer therapy technologies, is highly specific for determining axon terminal projections within particular neuronal populations, while simultaneously maintaining neuronal viability for targeted live cell electrophysiological recording.
# TABLE OF CONTENTS

Acknowlegments .................................................. ii

List of Figures .................................................... iii

List of Tables ..................................................... iv

List of Abbreviations and Probe Configurations .......... v

Chapter I. Thermoregulation and the Hypothalamus ...... 1

Chapter II. In Vitro Application of Gold Nanoprobes in Live Neurons for Phenotypical Classification, Connectivity Assessment, and Electrophysiological Recording ........................................ 20

Vita ....................................................................... 67
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# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Hammel’s Model</td>
<td>10</td>
</tr>
<tr>
<td>2. Anatomical and distance orientation in injected tissue</td>
<td>28</td>
</tr>
<tr>
<td>3. Schematic diagram of Probe A or Z’s mechanism</td>
<td>29</td>
</tr>
<tr>
<td>4. Efficacy of Probe A of DMH vs. non-DMH Tissue</td>
<td>36</td>
</tr>
<tr>
<td>5. Microscope images of labeled neurons</td>
<td>37-8</td>
</tr>
<tr>
<td>6. Probe A spatial distribution</td>
<td>41</td>
</tr>
<tr>
<td>7. Labeling efficacy by probe type</td>
<td>44</td>
</tr>
<tr>
<td>8. Labeling efficacy and spatial distribution of Probe Z</td>
<td>47-8</td>
</tr>
<tr>
<td>9. Effect of colchicine on POA labeling and spatial distribution</td>
<td>50-1</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Figures

1. Labeling efficacy by probe configuration 42
2. Chi-square results by probe configuration or injection sit 46
LIST OF ABBREVIATIONS

aCSF  artificial cerebrospinal fluid
AHA  anterior hypothalamic area
AuNP gold nanoparticle
DMH  dorsomedial hypothalamus
LH  lateral hypothalamus
PEI  polyethyleneimine
PGE$_2$ prostaglandin E$_2$
POA  preoptic area
PVN  paraventricular nucleus
RPa  raphe pallidus
VGAT  vesicular GABA transporter
VGLUT-2 vesicular glutamatergic transporter – type 2
VMH  ventromedial hypothalamus
VMPO  ventromedial preoptic area of the hypothalamus

PROBE CONFIGURATIONS

A  AuNP + VGLUT-2 + PEI
B  AuNP + VGLUT-2
C  AuNP + PEI
D  AuNP (~5 nm)
DX  AuNP (~50 nm)
Z  AuNP + VGAT + PEI
CHAPTER I:

Thermoregulation and the Hypothalamus
THERMOREGULATION

Homeostasis involves the dynamic interaction between an organism’s internal processes and its external environment to maintain physiological stability. One such homeostatic process is thermoregulation. Among animals, there are thermoformers and thermoregulators. A thermoconformer’s body temperature changes based on external temperature, whereas a thermoregulator maintains its body temperature within a particular range, in response to various external, environmental stimuli. Among thermoregulators, failure to achieve thermoregulatory balance may result in physiological damage, metabolic irregularities, or even death (Boulant, 2000).

In order to maximize heat loss in warm environments, mammals typically employ a number of adaptations. These include physiological strategies such as evaporative cooling (i.e. panting, perspiration), highly vascularized extremities, and compartmentalized fat storage to reduce insulation (e.g. camels). Other strategies involve behavioral adaptations such as daytime burrowing and increased nocturnal activity. Conversely, mammals utilize a variety of strategies to retain heat in cold environments. Physiologically, vascular countercurrent exchange systems mitigate heat escaping through the extremities, by
transferring heat from outgoing arterial to incoming venuous blood. Metabolic adaptation includes the ability to store metabolic energy in the form of fat. Through thermogenesis, energy can be released rapidly (e.g. shivering) or gradually (e.g. liver). Furthermore, evolutionary pressures also select for advantageous anatomical features, such as shortened extremities, or increased body size to more easily facilitate a steady core body temperature (Hammel and Pierce, 1968).

Humans are thermoregulators and have thus been able to adapt to a diverse set of climates. In cooler conditions, the human body engages in thermogenesis (e.g. liver processes and muscle contractions) to produce heat, and vasoconstriction to retain heat. In warmer environments, humans engage in vasodilation and sweating to transfer excess heat through evaporation. In addition to physiological mechanisms, humans also employ other social (e.g. huddling), cultural (e.g. clothing), and technological adaptations to produce or retain heat.

Thermometers provided the means to accurately measure temperature in animals. Because rates of heat loss and heat production vary in different parts of the body, local temperature
readings may differ considerably. Although circulating blood tends to standardize body temperature, the identification of body parts that closely match those of the internal organs have proven useful in ascertaining core temperature. These body parts include the rectum, uterus, bladder, mouth, ear, and groin (Hammel and Pierce, 1968).

Heat Loss

When environmental temperature is above core body temperature, humans lose heat through evaporation by secreting sweat. Furthermore, dermal hairs lie flat to prevent air retention between them, and thus complement convective heat loss. Vasodilation is another mechanism by which humans lose heat. Relaxation of smooth muscle in arteriole walls increases arterial blood flow. By redirecting blood to distal parts of the body, such as the skin, heat is lost through radiation and convection. In contrast to humans, only few other mammals lose heat primarily through sweating (e.g. horses). Rats, mice, cats, and dogs possess sweat glands exclusively on their foot pads. Instead, many mammals (e.g. dogs) pant to cool their bodies. Highly vascularized lungs, with a large surface area, facilitate heat exchange between the air and their bodies.
Heat Retention/Production

In contrast to heat loss, humans apply the opposite mechanisms to retain heat whenever environmental temperature dips below core body temperature. Sweat pathways are deactivated. Vasoconstriction is achieved by constricting subdermal capillaries and thus, shunting blood away from the skin and the body’s periphery. In extremely cold conditions, prolonged rerouting of blood away from the extremities causes numbness and cellular damage (e.g. frostbite). To augment heat retention, humans contract miniscule subdermal muscles (erector pili) to erect dermal hair follicles. These erect hairs form an insulating layer capable of trapping heat. Furthermore, humans and some animals produce heat in an exothermic process called thermogenesis. Effector signal from the hypothalamus reach peripheral muscles to cause shivering. Humans engage in short-term shivering, where heat is produced through high-intensity glucose metabolism in a relatively short period of time. Other animals, such as bears, are capable of long-term shivering, often for months, by gradually and steadily metabolizing fat stores.
Circadian Rhythm

Daily variations in body temperature have been observed in mammals, particularly humans and rats. The daily peak of body temperature rhythm in rats was found to consistently follow the daily peak of heat production rhythm and to precede the daily peak of heat loss rhythm. Furthermore, the daily rhythm of heat balance is 180 degrees out of phase with the rhythm of body temperature. Animals with lesions in the dorsomedial nucleus of the hypothalamus (DMH) maintained a normal circadian variation, though body temperature remained 0.5°C lower. Rat studies have shown that the circadian rhythm may be inverted by restricting food access to daylight hours. When only permitted to eat during the latter half of the light period, these animals awaken and increase body temperature a few hours before food administration. Lesion studies have determined that the ability to alter rhythms in body temperature, as well as the wake-sleep cycle, feeding, activity, and corticosteroid rhythms, correlate with DMH activity (Refinetti, 2003).
Sleep

Mammals sleep to conserve energy, during which decreased body and brain temperatures are observed. Small mammals tend to sleep the most, since they also have higher energy demands for thermoregulation and locomotion, while possessing relatively lower energy reserves. Disruption of normal sleep cycles has been associated with thermoregulatory irregularities. For example, chronically sleep-deprived rats show increased preference for cooler ambient temperatures (~10°C), which suggests sleep has cooling function. Conversely, rats sleep deprived for two weeks show a significant decrease in body temperature despite a doubling in metabolic rate, suggesting sleep’s role in heat retention. In humans, increased hypothalamic temperature induces sleep, and body heating prior to sleep increases subsequent slow wave sleep (Saper et al., 2005).

HYPOTHALAMUS

Found in all mammalian brains, the hypothalamus is located ventral to the thalamus, where it coordinates certain metabolic processes and various autonomic nervous system activities, as well as, synthesizes and secretes particular hormones. Stimulation and ablation studies
have determined that the preoptic anterior part of the hypothalamus (POA) is crucial for thermoregulation (Hammel et al., 1963). Approximately 40% of POA neurons are temperature-sensitive, while the rest are insensitive. Local warming of POA neurons evoked heat loss responses, whereas local cooling induced heat production. This observation led to the idea that the POA as an area responsible for thermoregulatory control, i.e. the body’s “central thermostat” (Boulant, 2000).

Hammel’s Model

In the 1960s, the six-neuron Hammel’s model, named after its developer, proposed that set-point temperature was determined by the integration of afferent signals with neuronal interactions within POA (Fig. 1). The model proposed that warm-sensitive neurons positively synapse on warm effector neurons, whereas temperature-insensitive inhibit them. The temperature where excitation equals inhibition determines is the set-point. Above the set-point temperature, warm effector neurons receive more stimulation than inhibition, thus leading to heat loss behavior. Conversely, temperature-insensitive neurons positively synapse on cold effector neurons, whereas warm-sensitive inhibit them. Below the
set-point temperature, cold effector neurons receive more stimulation than inhibition, thus initiating heat retention/production behavior (Hammel et al., 1963).

**Neuroanatomical Correlates**

Observed heat loss behaviors include vasodilation, panting, and sweating, while heat production behaviors involve vasoconstriction, shivering, breakdown of brown adipose tissue, and huddling. Further studies have determined that heat loss and production are triggered by the actual ambient temperature, and not by the rate of change in temperature, since no burst of neuronal firing rates were detected during rapid temperature change. Hypothalamic temperature does not vary considerably (range: ± 1°C) even when ambient temperature is changed drastically. However, drops in hypothalamic temperature below this range often induce a coma and other neuroprotective mechanisms (Hammel et al., 1963).
Figure 1. Hammel’s Model. A modified six-neuron model explaining the hypothalamic determination of set-point temperature. Key: FR, firing rate; W, warm-sensitive neuron; I, temperature-insensitive neuron; w, heat loss effector neuron having synaptically derived-warm sensitivity; c, heat production effector neuron having synaptically-derived cold sensitivity; SP, dorsal horn spinal neuron; OC, optic chiasm; MB, mammillary body. Adapted with permission from Boulant, J. A. (2006) Neuronal basis of Hammel’s model for set-point thermoregulation.
Changes in the activity of neurons in the POA have been correlated with initiation of thermoregulatory mechanisms. Nearby areas, such as the DMH, may include thermoeffector neuron. Electrical stimulation of the DMH induced an increase in heat production behavior. Retrograde labeling show dye beads transported to the lateral hypothalamus (LH), POA, ventromedial preoptic (VMPO), and paraventricular area (PVN). It is thought that the POA may have direct control of the DMH (Nakamura et al., 2005).

**Neuronal Thermosensitivity**

Various *in vivo* and *in vitro* electrophysiology studies have identified four types of thermoregulatory neurons: warm-sensitive, cold-sensitive, temperature-insensitive, and silent. Among these types, the vast majority of the POA neuronal population comprise of warm-sensitive (~30%) and temperature-insensitive (~60%) neurons. One main difference between these two types is that warm-sensitive neurons not only inherently respond to local thermal input, but also to hypothalamic, skin, and/or spinal temperature. Conversely, temperature-insensitive neurons do not exhibit temperature-related changes in activity (Boulant et al., 1997).
Thermal Classification

Historically, temperature sensitivity has been ascertained in one of two ways: either through the determination of a $Q_{10}$ thermal coefficient, or through calculation of the neuronal firing rate regression line slope. The slope reflects the number of action potentials as a function of hypothalamic temperature (Boulant et al., 1997).

Using the latter criteria, temperature-sensitive neurons can be divided into two subgroups: warm- (35% POA) and cold-sensitive (5%). Warm-sensitive neurons fire at $\geq 0.8$ impulses/sec/°C, when the POA temperature is changed by ± 2-3°C (Boulant et al., 1997). Warm-sensitive neurons respond to both direct changes in hypothalamic temperature and peripheral thermal stimulation. They also have a distinct lateral/medial orientation in dendritic morphology, which is consistent with their roles as integrators of thermal information (Griffin et al., 2001). Cold-sensitive neurons, on the other hand, fire at $\leq -0.6$ impulses/sec/°C when POA temperature is changed by ± 2-3°C. Unlike warm-sensitivity, cold-sensitivity is not an inherent property, and may be a result of synaptic input from nearby warm-sensitive and insensitive neurons (Boulant et al., 1997).
In contrast, temperature-insensitive neurons show little change in firing rate, when temperature deviates from the normal physiological condition (~37°C). Temperature-insensitive neurons fire at ≤ 0.79 impulses/sec/°C and ≥ -0.6 impulses/sec/°C when the POA temperature is changed by ± 2-3°C. Essentially, this range is between the firing rates of warm- and cold-sensitive neurons (Boulant et al., 1997). Unlike their warm-sensitive counterparts, temperature-insensitive neurons extend their dendrites in rostral/caudal directions, consistent with their roles as indicators of baseline of hypothalamic activity (Griffin et al., 2001).

The characterization of physical differences between temperature-sensitive and insensitive neurons is an active area of research. These differences in firing rates may be determined by various ionic conductances, such as tandem-pore K+ leak channels (TASK, TREK, and TRAAK), transient K+ A-current channels, hyperpolarization-activated cyclic nucleotide-gated channels (HCNs), and vanilloid transient receptor potential (TRP) channels. The proportion of these channels within the hypothalamus may explain the difference among warm-, cold-sensitive, and temperature-insensitive neurons (Wechselberger et al., 2006). It has been observed that increased temperature positively correlated with both firing rate
thermosensitivity and A-current inactivation, resulting in a higher probability of reaching firing threshold (Griffin et al., 1996).

**MEDICAL IMPLICATIONS**

**Fever**

Several studies estimate that the average core temperature for healthy adults is approximately 98.2 °F or 36.8 °C. When presented with an immunological challenge, the human body undergoes a cascade of factors that signal the brain. The resulting rise in body temperature of 1-4°C is defined as a fever. Metabolically, specific cytokines cause production of prostaglandin (PGE$_2$) in the brain, which in turn affects neuronal activity in the ventralmedial preoptic (VMPO; Blatteis and Sehic, 1998). Introduction of PGE$_2$ to this region or stimulation of afferent pathways causes significant rise in body temperature (> 1°C). According to Hammel’s model, an increase in the firing rate of temperature-insensitive neurons and/or a decrease in the firing rate of warm-sensitive neurons result in a new, higher set-point. The presence of PGE$_2$ causes warm-sensitive neurons to decrease their firing rate and temperature-insensitive neurons to be unaffected or increase their firing rate (Ranels and Griffin, 2003).
**Thermal Dysregulation**

Because of the hypothalamus’ importance in thermoregulation, injuries, tumors, genetic abnormalities, and its exposure to pyrogenic and other exogenous compounds may cause thermal dysregulation. Because the POA can be influenced by factors besides temperature, (e.g. glucose levels, testosterone, estrogen, osmotic balance, etc.), dysfunctions in maintenance of these factors may be comorbid with thermal dysregulation. Furthermore, metabolic and physiologic conditions, such as hormonal imbalance and poor blood circulation, may contribute to abnormal body temperature.

**CONCLUSION**

To summarize, thermoregulation is a dynamic, homeostatic interaction between an organism’s internal processes and its thermal environment. To maintain physiological stability, many organisms employ processes such as heat loss, retention, and production. These thermoregulatory mechanisms influence other aspects of life, such as sleep and circadian rhythms. Among mammals, the hypothalamus (located deep within the brain) coordinates certain metabolic processes, regulates various autonomic nervous system activities, and
synthesizes/secretes particular hormones critical to thermoregulation. Through thermal dysregulation and protective mechanisms like fever, we are reminded of thermoregulation’s importance in maintaining life.

**Hypothesis:**
Synaptic entry of novel nanoprobes within the DMH will result in somatic labeling in the POA, confirming connectivity. Probe conjugation, with either VGLUT-2 or VGAT antibodies, will facilitate preferential entry into either glutamatergic or GABAergic synapses.

**SPECIFIC AIM:**
Although recent evidence has suggested that POA neurons project to the DMH, direct demonstration of these connections and the distinct phenotypic populations responsible for the activation of thermoregulatory responses remains to be elucidated.
REFERENCES


Hypothalamic Proportional Control with an Adjustable Set Point.

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CHAPTER II:

*In Vitro* Application of Gold Nanoprobes in Live Neurons for Phenotypical Classification, Connectivity Assessment, and Electrophysiological Recording
1. INTRODUCTION

Thermoregulatory neurons in the preoptic area of the anterior hypothalamus (POA) form synaptic networks, which affect responses that regulate body temperature around a set-point (Boulant, 1997; Hammel, 1965). Changes in this area’s neuronal activity have been correlated with the initiation of thermoregulatory mechanisms (Griffin et al., 1996; Imbery et al., 2008; Saper and Breder, 1994). Recent studies also suggest that certain thermoregulatory phenomena, such as hyperthermia, can be elicited by activation of neurons in the dorsomedial hypothalamus (DMH) and the nucleus raphe pallidus (RPa), which receive direct input from neurons in the POA (Nakamura and Morrison, 2008; Nakamura et al., 2009). Therefore, in order to produce these distinct changes in thermoregulatory control, POA neurons may have direct axonal connections to the DMH or RPa. Although recent evidence has suggested that POA neurons project to these areas (Nakamura et al., 2009), direct demonstration of these connections and the distinct phenotypic populations responsible for the activation of thermoregulatory responses remains to be elucidated.
In order to determine the thermoregulatory role of POA neurons that project to the DMH or the RPa, their phenotypes and connectivity must be characterized. Past studies have attempted to phenotype these neurons through their synaptic projections. For example, certain techniques, such as glutamate decarboxylase staining, resulted in localization of not only the synapses of interest, but also nearby axonal fibers (Schaffer and Griffin, 2006). While the majority of POA neurons are thought to be inhibitory (Nakamura and Morrison, 2008; Nakamura et al., 2009) and therefore GABAergic, the literature describes the glutamatergic system in more detail due to its abundance in the mammalian brain (Meldrum, 2000). This made the glutamatergic system more accessible for initial investigation. Once proof of principle was established, investigation of the GABAergic system subsequently followed.

Recent developments in the field of bionanotechnology have yielded new approaches to supplement molecular studies at the subcellular level (Bergen et al., 2008; Grimm et al., 2004; Kukowska-Latallo et al., 2005; Merrifield et al., 2005). For example, metallic nanoparticles, through their biocompatibility and low toxicity, are gradually expanding drug delivery and cancer treatment options (Kukowska-Latallo et al., 2005; Sperling et al., 2008; Vasey et al., 1999). The
potential uses for nanoparticles, however, are not confined to these applications. In addition to therapeutic applications as vehicles, nanoparticles may expand bioimaging techniques as contrast agents, by functionally assessing and localizing specific molecular signatures or physiological systems (Portney and Ozkan, 2006; Shamsaie et al., 2007; Thurn, 2007). Furthermore, because of the nanoparticles’ metallic properties, the photoelectric effect neutralizes photobleaching concerns associated with conventional fluorescent dyes (Thurn, 2007).

Cellular metallic nanoparticle studies have recently come into existence, and literature involving neuronal applications are still evolving (Olivier, 2005). Here, we report an adaptation of this technology as a potential tagging system for neuronal pathway identification *in vitro*. Whereas previous studies involved gold nanoparticle vehicles delivering genetic or drug payloads to the nucleus of a cell (Olivier, 2005), we have adapted the original intent of these nanoparticles, whereby the payload (subsequently referred to as ‘conjugates’) facilitate delivery of the vehicle (i.e. gold nanoparticle) to the neuronal soma. This has allowed us to characterize both glutamatergic and GABAergic neurons in a tissue slice preparation, which synapse in the DMH and originate from within the POA. Since the nanoparticles used are non-lipid soluble, and are specifically
conjugated to enter and escape the synaptic vesicular machinery, these nanoparticles allow us to probe for a neuron’s somatic origin, via the synapse, by retrograde diffusion or facilitated transport.

Since thin neuronal tissue slices remain viable in vitro for up to 12 hours under proper conditions, an appropriate retrograde labeling technique must work within this time period to allow for the targeted electrophysiological recording of these neurons in the POA. Other high-contrast, “gold standard” methods, such as Fluorogold (Llewellyn-Smith et al., 1990; Moore et al., 1995; Tong et al., 1999), cholera toxin B-gold, and immunogold silver staining exist. However, only our adapted gold nanoprobe (AuNP) technique simultaneously offers the appropriate time course, specificity, and low biotoxicity (Allen et al., 1988; Chan et al., 1990; Cowan et al., 1994; Gillitzer et al., 1990; Naumann et al., 2000) required to leave neurons of interest suitably intact for live-cell recording. Finally, techniques lacking actual metallic components, like Fluorogold, exhibit limitations found in fluorescent dyes, such as potential labeling of nearby fibers of passage (Chen and Aston-Jones, 1995; Dado et al., 1990) and photobleaching.
2. EXPERIMENTAL PROCEDURES

2.1 Hypothalamic Tissue from Rat Brain

Extraction procedures of hypothalamic tissue have been previously described in detail (Griffin et al., 1996; Griffin et al., 2001; Imbery et al., 2008; Olivier, 2005). Briefly, brain tissue sections containing the DMH and POA were prepared from male Sprague-Dawley rats (Harlan; 100-150 g), that were housed under standard conditions and provided food and water ad lib. Before each session, a rat was anesthetized using isoflurane and promptly decapitated, following procedures approved by the Animal Care and Use Committee of the College of William and Mary. After dissection of the brain, a tissue block containing the hypothalamus was mounted on a vibratome and bathed in artificial cerebral spinal fluid (aCSF). Sagittal plane, 400 μm-thick tissue sections were produced and then placed in a submersion recording chamber.

2.2 Tissue Perfusion and Probe Injection

Tissue sections were continually perfused with normal aCSF, which consisted of (in mM): 124 NaCl, 26 NaHCO₃, 10 glucose, 5 KCl, 2.4
CaCl\(_2\), 1.3 MgSO\(_4\), and 1.24 KH\(_2\)PO\(_4\). After gentle aeration (95% O\(_2\), 5% CO\(_2\)), the aCSF (300 mOsM; pH 7.5) was allowed to gravity flow at 1-2 ml/min into the recording chamber (volume = 2 ml).

Approximately 1-5 μL of probe solution (AuNPs in bovine serum albumin suspension) were backfilled into glass microelectrodes pulled to a tip diameter of ~ 2-5 μm. The solution was pressure-injected into the tissue area of interest, using a nitrogen puffer system (Fig. 2). Successful injection sites aimed at the DMH, regardless of probe type, were designated as ‘target’ tissue (n = 57), while non-DMH were ‘control’ (n = 7). Tissue classified as non-DMH included injection sites in the anterior hypothalamic area (AHA) or the ventromedial hypothalamus (VMH). Probes were allowed to diffuse through the tissue for an average of 5.9 ± 0.1 h at a mean temperature of 35.5 ± 0.18°C. A thermocouple was placed adjacent to the tissue slices to constantly monitor the temperature.

Upon tissue placement into the chamber, a small concentration of gold nanoprobes was pressure-injected into the DMH (Fig. 3). These AuNPs are spherical, colloidal nanoparticles composed of a dielectric silica core and an ultra-thin metallic (gold) coating, providing them with a strong reflectivity (peak wavelength: glutamatergic = 524 nm,
GABAergic = 510 nm), and an extremely low biotoxicity. For our purposes, the glutamatergic AuNPs were conjugated with a polyclonal antibody for the rat vesicular glutamate transporter type-2 (VGLUT-2; Millipore) and polyethyleneimine (PEI; Nanopartz, Inc.).
Figure 2. Anatomical and distance orientation in injected tissue. Tissue-level image (4x) of injection site in the DMH, labeled soma in the AHA/POA, and other anatomical landmarks.

DMH: dorsomedial hypothalamus
Fx: fornix
AC: anterior commissure
AHA: anterior hypothalamus, which contains the POA
A, B: arrows pointing to labeled soma
Figure 3. Schematic Diagram of Probe A or Z’s Mechanism.
Probe injection in the DMH allows vesicular uptake at the axon terminal. (a) Probe conjugates, such as VGLUT-2 or VGAT antibodies (curvy line) and PEI molecules (Y-shape), are surface-conjugated on gold nanoshells (circle). (b) These conjugates facilitate membrane protein attachment during vesicular formation and, later, (c) endosomal escape from the vesicular lumen into the cytosol. Intracellular diffusion and possible retrograde axonal transport machinery facilitation result in nanoaggregate deposition in the neuronal soma.
This fully-conjugated nanoprobe complex, consisting of both V-GLUT-2 antibodies and PEI polymers attached onto a gold nanoshell (diameter = 5 nm), was designated as Probe A. GABAergic probe complexes were similarly constructed, only that the VGLUT-2 antibodies were replaced with polyclonal antibodies for the rat vesicular GABA transporter (VGAT; Millipore; peak wavelength = 510 nm). The VGAT and PEI double-conjugated probe was designated Probe Z. From this point onwards, administration methodology and experimental conditions for the VGAT probes were similar to the VGLUT-2 probes.

After injection into the tissue slice, specific antibody binding of Probe A to nearby VGLUT-2s (which are exposed to the terminal surface during vesicle formation) facilitates AuNP uptake exclusively into synaptic vesicles at axon terminals (Jung et al., 2006). Once inside a newly-formed vesicle, “endosomal escape” occurs when the attached, cationic PEI conjugates sequester surrounding protons. To maintain chemiosmotic balance, vesicular ATPases continue to pump protons into the vesicular space. To maintain electrochemical balance, chloride ions follow into the vesicle, through their own channels. Increasing concentrations of both ions attract water molecules into the vesicular lumen, initiating a progressive “proton sponge effect” (Bergen et al., 2008; Guerra-Crespo et al., 2003; Suk et al., 2007; Wu et al., 2004).
Upon hydrosaturation, the vesicles lyse and a significant number of AuNPs diffuse into the cytosol. Over the next several hours, while the tissue slice equilibrates to the chamber environment, some AuNPs will retrogradely transport from the synaptic terminal region to the neuronal soma (Bergen et al., 2008; Suk et al., 2007).

2.3 Histology

Upon removal from the recording chamber, the tissue sections were allowed to soak in 30% paraformaldehyde/sucrose solution at 12°C for a minimum of 3 h. Tissue was frozen and sliced into 50 μm thick sections using a microtome (Leica SM-2000R). After suspension in phosphate-buffered saline, the tissue sections were mounted on slides and were given 12 h to dry at room temperature before beginning the staining process. Slides were first placed in a 50/50 chloroform/alcohol solution for 3 h at room temperature. Afterwards, they were carried through a series of rehydration steps, for five minutes each in 300 mL of, successively, 95.5% reagent alcohol, 70% reagent alcohol, and distilled water, followed by a 5 min submersion in phosphate buffer monobasic solution and 4 min in giemsa stain. Excess stain was then removed via 2 min agitation in 95.5% reagent alcohol before the slides
were placed in 300 mL of xylene (Sigma, 98.5+%) for 12 h. Slides were coverslipped and allowed 12 h to set before microscopy.

2.4 Probe Visualization and Quantification

The location and confirmation of each injection site were noted on a section diagram adapted from a rat brain atlas (Pellegrino et al., 1979). Quantification of the probes involved tallies of labeled neurons and their location. Labeled neurons were readily identified by the presence of AuNPs in their giemsa-stained somas, which can be visualized under normal bright field illumination, and then confirmed through their reflective properties under dark field illumination. To reduce bias, undergraduate counters were blinded to the tissue sample’s probe treatment. Microscopy images were obtained using an Olympus CCD camera (DP11) at 4x, 10x, and 40x magnification. Full-image brightness and contrast were non-destructively adjusted using Adobe Photoshop.

2.5 Control Probe Configurations

To determine the differential contribution of the complex’s components, isolation of individual component effects was required. To accomplish this, progressively simpler, control probes were designed
by removing individual modifications. After testing the performance of the complete probe (Probe A; AuNP + VGLUT2 antibody + PEI), we studied the properties of Probe B (AuNP + VGLUT2 antibody only), Probe C (AuNP + PEI only), and finally Probe D (AuNP shell only, diameter = 5 nm). All probes were synthesized by Nanopartz, Inc.

2.6 Colchicine Treatment

To determine whether passive diffusion or facilitated transport through the axonal machinery is the primary mode by which the probes reach the soma, 100 nM of the microtubule formation inhibitor colchicine (Sigma-Aldrich; dissolved in distilled water) was co-administered with Probe A (n=8). Both Probe A and colchicine solution were backfilled onto the same electrode prior to pressure injection.

2.7 Statistical Analysis

Mann-Whitney U-tests (nonparametric data) or Student’s T-tests (parametric data) were conducted when comparing two treatment groups, whereas a Kruskal-Wallis test was utilized for three or more. Chi-square tests were used to compare between probe efficacy results. To determine which factors influence the quantity of labeled neurons
within the POA, a principal components analysis (PCA) was used to develop a multiple regression model. Robust regression confidence intervals and standard errors were generated using a replacement-and-resampling bootstrap method (Efron, 1977; Efron, 1994). These estimates were bias-corrected and validated using a separate jackknife procedure (Efron, 1977). Unless otherwise noted, P-values < 0.05 were stated as statistically significant. Statistical analyses were performed using Stata 10 (StataCorp. LP).
3. RESULTS

Trials assessing the efficacy of Probe A yielded results dependent on initial probe injection location. Significant differences in somatic labeling were observed between tissues injected in the DMH as compared to non-DMH tissues (Fig. 4), whereby probe injection into the DMH resulted in significantly higher POA somatic labeling. DMH-injected samples also displayed a relatively higher variance in POA labeling compared to non-DMH, with at least one sample exhibiting over 60 labeled soma. Discrete reflecting units within clearly-marked somas were observed (Fig. 5 a,b,c). Reflectance observed does not represent individual nanoprobe complexes, since individually, these particles have insufficient surface area to reflect the necessary amount of light perceivable to the human eye. Instead, what we are likely seeing are collections of several probes that can be best described as nanoaggregates (Kneipp et al., 2006). An adjusted density map was constructed to visualize Probe A’s spatial distribution in DMH-injected tissue (n = 20). In tissue slices with confirmed injection sites in the DMH, the majority of labeled cells were located near the POA. Not surprisingly, the highest densities (excluding areas immediately surrounding the injection site) were observed in the rostral sections of
Figure 4. Efficacy of Probe A of DMH vs. non-DMH Tissue. The number of labeled POA neurons is significantly higher, if the injection site is located in the DMH.

**Boxplot Notation**

- **middle bar:** median
- **shaded rectangle:** interquartile range
- **whiskers:** upper/lower adjacent values
- **dot:** outlier
Figure 5 a, b, and c. Microscope images of labeled neurons. Images (40x) in visible light (a), dark field (b), and superimposed composite (c) show distinct probe nanoaggregates within giemsa-stained POA soma. Arrows indicate labeled soma.
the POA -- the area closest to the DMH (Fig. 6). In contrast, control tissue with injection sites to the mammillary peduncle or AHA, showed significantly lower POA somatic labeling. Outside the POA, probes were relatively scattered. Higher probe accumulations reported in soma closer to the injection site (i.e. labeled, non-POA neurons) may reflect shorter traveling times due to smaller distances. Longer perfusion times may result in increased labeling within the POA proper, though this speculation may require further testing. Another possibility is that Probe A is terminally labeling somas in the area rostral to the POA. If that is the case, this may provide evidence that this area may also provide effector signals to the DMH. Due to time limitations, we were not able to investigate this possibility further. Future experiments may be warranted to provide answers.

A linear, multiple regression model was developed to determine the incremental effect of probe type, location of injection site, diffusion time, and perfusion temperature respectively, on the number of POA neurons labeled. All explanatory variables were assessed for collinearity, and all showed sufficient independence. The regression model significantly explained approximately 50% of the POA labeling variation.
Bootstrapped linear regression models (n = 45, iterations = 10,000) indicate that, overall POA labeling was significantly affected by probe configuration and injection site (Table 1). Injection in the DMH yielded, on average, significantly more POA labeling, after controlling for probe type and other factors. Although diffusion time differences did not show significance at the $\alpha < 0.05$ level (most tissue slices were perfused for approximately 6 hours with minimal variation), future investigation and manipulation of this variable may be informative.
Figure 6. Probe A spatial distribution. Normalized spatial distribution of Probe A in DMH-injected tissue (n=20) show higher labeling density in the anterior POA and areas immediately rostral to the POA. Grid: The origin represents the DMH injection site. Higher axis numbers indicate a more dorsal direction, while latter axis letters indicate a more caudal direction. Legend: Number of labeled neurons observed averaged over 20 slices.
Table 1. Labeling Efficacy by Probe Configuration. Estimated incremental contributions of each conjugate type and injection site to overall labeled POA neurons are reported as beta coefficients and 95% confidence intervals in the regression results.

<table>
<thead>
<tr>
<th></th>
<th>Beta</th>
<th>SE</th>
<th>Sig.</th>
<th>95% Conf. Interval</th>
<th>lower</th>
<th>upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neither</td>
<td>0</td>
<td>5.4</td>
<td>1</td>
<td>-10.5</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td>PEI only</td>
<td>-6</td>
<td>4.9</td>
<td>0.21</td>
<td>-15.6</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>VGLUT-2 only</td>
<td>2.3</td>
<td>5.1</td>
<td>0.65</td>
<td>-7.6</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>Both</td>
<td>11.6</td>
<td>5.4</td>
<td>0.03</td>
<td>1</td>
<td>22.3</td>
<td></td>
</tr>
<tr>
<td>Tissue type</td>
<td>11.7</td>
<td>3.4</td>
<td>0.001</td>
<td>5</td>
<td>18.5</td>
<td></td>
</tr>
<tr>
<td>Time (min)</td>
<td>-0.2</td>
<td>0.1</td>
<td>0.07</td>
<td>-0.3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Temp (°C)</td>
<td>0.7</td>
<td>1.4</td>
<td>0.65</td>
<td>-2.1</td>
<td>3.4</td>
<td></td>
</tr>
</tbody>
</table>

N: 45
Replications: 10000
Wald chi-square: 31
P-value: 0.0001
R-squared: 0.56
Adj. R-squared: 0.49
The regression model described above (Table 1) also estimated the incremental effect of individual conjugates on POA labeling efficacy, where deconjugated probe configurations exhibited decreased overall labeling efficiency (Fig. 7). When compared to baseline performance of the “naked” 5 nm probe, the average increase/decrease in POA neurons labeled were calculated by conjugate type, statistically controlling for other factors. While adding either VGLUT-2 or PEI individually may affect the efficacy of the probe, only the attachment and interaction of both conjugates significantly improved POA labeling over baseline (Table 1). It was also noted that, in tissues treated with control probes (especially unconjugated ones), somatic labeling was generally more sporadic and less specific to any particular region of the tissue. Because unconjugated, gold nanoprobes (like Probe D) are, by design, biocompatible and inert, they are unlikely to initiate the cell’s immunological response and thus, be subject to degradation. This property, when coupled with their miniscule size, makes “naked” nanoprobes more likely to pass through the synaptic machinery, than their larger, conjugated counterparts (Verma et al., 2008). To determine the effect of nanoprobe size, we enlarged the “naked” shells tenfold and compared the performance of these extra-large probes (Probe DX, diameter ~ 47 nm; peak reflectance = 530 nm; Nanopartz, Inc.) with their 5 nm counterparts. Fifty nanometers was chosen,
Figure 7. Labeling Efficacy By Probe Type. The median number of labeled POA neurons is significantly higher, when both VGLUT-2 and PEI conjugates are present, with Probe A reporting a bootstrapped population median estimate (iterations = 10,000) between 18-30 labeled neurons per 50-nm slice.

Boxplot Notation

middle bar: median
shaded rectangle: interquartile range
whiskers: upper/lower adjacent values
dot: outlier

Probe Configurations

A  AuNP + VGLUT-2 + PEI
B  AuNP + VGLUT-2
C  AuNP + PEI
D  AuNP (~5 nm)
DX AuNP (~50 nm)
because it is at the uppermost limits of synaptic vesicle diameters; most synaptic vesicles do not grow to be nearly this size (Harris and Sultan, 1995). Chi-square comparisons between Probe D and DX yielded a significant difference (Table 2) where Probe DX showed lesser POA labeling efficacy. Probe DX performed even less efficiently than all its 5-nm, deconjugated/unconjugated counterparts, registering a similar POA labeling efficacy to non-DMH Probe A samples. Besides initial injection site and conjugate type, size may be another major factor affecting probe uptake (Chithrani et al., 2006). Differences between Probe D and DX’s spatial distribution were not apparent in the anatomical position data.

Subsequent trials assessing the efficacy of the GABAergic probes (Probe Z; n = 23; Fig. 8a) yielded significantly lower POA labeling when compared to fully-conjugated glutamatergic probes (p < .001), despite statistically similar perfusion temperature, diffusion time, and initial injection volume. Spatial distribution patterns are similar to that of Probe A, where the densest labeling occurs in the anterior POA. It is dissimilar to Probe A, however, in that the lower overall labeling is reflected by lighter density shades (Fig. 8b). Potential explanations for the labeling disparity between the glutamatergic and GABAergic probes are explored in the Discussion section.
Table 2. Chi-square results by probe configuration or injection site. Overall, comparisons between probe configurations, based on injection site, conjugate type, and probe size, indicate significant efficacy differences.

<table>
<thead>
<tr>
<th></th>
<th>A (non-DMH)</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>DX</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (DMH)</td>
<td>15.03</td>
<td>5.68</td>
<td>14.57</td>
<td>11.8</td>
<td>--</td>
</tr>
<tr>
<td>B</td>
<td>--</td>
<td>--</td>
<td>6.58</td>
<td>3.71</td>
<td>--</td>
</tr>
<tr>
<td>C</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.29</td>
<td>--</td>
</tr>
<tr>
<td>D</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>5.14</td>
</tr>
</tbody>
</table>

Bold values are significant at the .05 level. Bold and italicized values are significant at the .01 level or less.

**Probe Configurations**

A             AuNP + VGLUT-2 + PEI  
B             AuNP + VGLUT-2  
C             AuNP + PEI  
D             AuNP (~5 nm)  
DX            AuNP (~50 nm)
Figure 8, a and b. Labeling Efficacy and Spatial Distribution of Probe Z. (a.) Probe Z labels POA neurons at a significantly lower efficacy rate than Probe A (p < .001).

Boxplot Notation

middle bar: median
shaded rectangle: interquartile range
whiskers: upper/lower adjacent values
dot: outlier

Probe Configurations

A                      AuNP + VGLUT-2 + PEI
Z                      AuNP + VGAT + PEI
(b.) Spatial distribution of Probe Z in target tissue. Grid: The origin represents the DMH injection site. Higher axis numbers indicate a more dorsal direction, while lower axis letters indicate a more caudal direction. Oval denotes POA area. Each grid cell is approximately 100 microns long. Key: Number of labeled neurons observed averaged over 23 slices.
To determine whether passive diffusion or facilitated axonal transport contributes primarily to somatic probe deposition, control tissues were treated with colchicine. Colchicine inhibits microtubule formation, and consequently, shuts down axoplasmic transport. POA labeling is significantly (p < .01) and substantially decreased upon colchicine administration (Fig. 9a), when compared to the non-colchicine treatment. Spatial distribution analysis also confirms that in colchicine-treated tissue, probes visualized outside the injection site tend to remain proximal to their synaptic entry points (Fig. 9b). From this we conclude, that nanoprobe deposition in the soma is substantially facilitated by the axonal transport machinery. The precise mechanism, by which these probes “hitch a ride” to the soma, remains unknown and may warrant further study.
Figure 9, a and b. Effect of Colchicine on POA Labeling and Spatial Distribution. (a.) Simultaneous administration of 100 nM colchicine and Probe A significantly curtails POA labeling ($p=.007$), suggesting facilitated axonal transport of nanoprobe.
(b.) Spatial distribution of visualized probes in tissue treated with 100 nM colchicine. Grid: The origin represents the DMH injection site. Higher axis numbers indicate a more dorsal direction, while lower axis letters indicate a more caudal direction. Oval denotes POA area. Each grid cell is approximately 100 microns long. Key: Number of labeled neurons observed averaged over 8 slices.
The mechanisms, by which the nervous system maintains or adjusts body temperature in response to thermal stressors, remain a fundamental area of study in physiology. Recent studies suggest that responses, such as hyperthermia, can be elicited by activation of neurons in the DMH and RPa, which receive direct input from thermally-responsive neurons in the POA. Therefore, in order to produce distinct changes in thermoregulatory control, these POA neurons may have direct axonal connections to the DMH or RPa. Phenotypical and connectivity assessments of neuronal populations within these areas could provide insight to their functional physiology.

Since VGLUT expression acts as a specific biomarker of a neuron’s glutamatergic phenotype (Santos et al., 2009), it is a sufficient indicator of the glutamatergic machinery’s presence within the terminal. Of the three known neuronal VGLUT isoforms, hypothalamic synapses predominantly express VGLUT-2 (Varoqui et al., 2002). Therefore, it is reasonable to conclude that probes with the VGLUT-2 antibody are preferentially binding to and being endocytosed in glutamatergic axon terminals. By observing and quantifying neurons labeled with Probe A, we were able to simultaneously ascertain a likely
glutamatergic phenotype, while confirming connections between the DMH and the POA.

Although a median of approximately 20+ labeled neurons (out of a population of thousands within the POA) may seem small, this number only represents labeling from intact neurons within a single 400 μm section, during a time course of only a few hours. Furthermore, when considering the number of oblique, axonal fibers severed during the slicing process, labeling of 60 or more neurons (like the outlier in Fig. 4) is still possible.

To understand the incremental contribution of the probe’s constituent parts, we need to elaborate on the mechanisms by which they affect efficacy rates. Terminal uptake and endosomal escape can be classified in two ways: facilitated and passive. During vesicular uptake, probes with the VGLUT-2 antibodies (Probe A and B) were more likely to enter and remain with the vesicles, since they are more likely to bind to exposed VGLUT-2s and be co-transported into the lumen upon vesicular formation. Though still possible through passive diffusion, non-VGLUT-2 probes (C and D) are less likely to be actively taken into vesicles.
Once inside the vesicular lumen, the probe’s fate is co-determined with that of the vesicle. Excluding degradation, three proposed, post-uptake vesicular mechanisms may affect probe efficacy. Vesicles may exist in a transient kiss-and-run cycle, a short-term recycling pool, or a long-term reserve pool (Ceccarelli et al., 1973; Heuser and Reese, 1973; Wu et al., 2007). Although the molecular processes underlying the kiss-and-run recycling mechanism are still unclear, this pathway is too transient for any appreciable probe vesicular entry; it may even return probes into the synaptic cleft. It is therefore unlikely to be a starting point for probes that reach the soma. Vesicles that follow the short-term recycling pool or long-term reserve pool pathway exist long enough to be reasonable probe entry points. Probes that display PEI conjugates (Probe A and C) take advantage of the “proton sponge effect”, which facilitates their escape into the cytosol. Non-PEI probes (Probe B and D) are less likely to exit intact vesicles, since the vesicular plasma membrane presents a significant barrier. Due to physical obstruction, non-PEI probes can presumably exit into the cytosol, in appreciable amounts, only when the vesicle itself and integral membrane proteins, are lysed and degraded by the cellular machinery.
As a consequence of the relative absence of lysosomes within the presynaptic terminal, integral vesicular membrane proteins are retrogradely transported to the soma, where they are destroyed by lysosomes (Li and Dahlstrom, 1997; Tsukita and Ishikawa, 1980). In this sense, we can explain the near similarities in Probe B and C’s efficacy rates in the following way: while probes with the VGLUT-2-antibody are more likely to remain within the vesicles, and therefore be present in larger numbers post-formation, only a small percentage escape passively into the cytosol and reach the soma, presumably through vesicle degradation. For PEI-conjugated probes, the reverse is true: while relatively fewer of them passively remain in the vesicle post-formation, those that do are more likely to actively escape into the cytosol, through vesicle osmolysis, and transport to the soma.

Furthermore, the absence of either the VGLUT-2 or VGAT antibody renders these particular probes to enter neurons nonspecifically, with regards to phenotype. While it is clear that possessing either conjugate may be sufficient to reach the soma, efficacy rates are significantly much higher and more specific, when both are concurrently present, to take advantage of as many vesicular pathways as possible.

Although conjugate type plays a significant role, overall size may also influence probe efficacy. While adding the appropriate conjugates
facilitates probe specificity, it also adds to the probe complex’s size. The larger the size, the more likely steric effects (such as blockage or entanglement) may influence efficacy. We are likely seeing this effect when comparing the relatively larger Probe C complex with that of Probe D (Table 2). Despite Probe D’s lack of any facilitative conjugates, tissue injected with this “naked” probe shows a comparable efficacy rate to its deconjugated counterpart. Concurrently, we also observe that merely increasing the probe shell size, as in the case of Probe DX, is sufficient to diminish efficacy rates. When coupled with its biocompatibility, the unconjugated, 5-nm probes take advantage of various mechanisms to enter neurons, which their conjugated counterparts cannot, such as occasionally passing interstitially through the plasma membrane (Verma et al., 2008). From this, we observe that the trade-off in specificity is mobility.

As for the GABAergic probes, it seems unusual that Probe Z labels POA neurons at a significantly lower efficacy rate, especially when the GABAergic phenotype comprises the majority of hypothalamic neurons (~60%). Several mitigating factors, however, may explain the lower than expected rate.
From a chemical standpoint, VGAT’s binding potential to its endogenous cargo (GABA), is different from VGLUT-2’s interaction with its endogenous ligand (glutamate). If the principle of a "molecular velcro" does apply to the facilitated vesicular entry of these probes, then it is reasonable to assume that the differential binding kinetics between these two transporters may affect the binding interactions with their respective probes. This, in turn, alters the likelihood of vesicular uptake and therefore, somatic labeling. Furthermore, at physiological temperatures (~36°C), glutamate is endocytosed at faster rates and in larger vesicular compartments (approximately 4-5 fold; Roskoski, 1978). These differences may be related to glutamate’s general metabolic role in neuronal cells, as opposed to GABA’s more specialized, neurotransmitter capacity.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Several validation studies, to further assess the technique’s viability, are currently under way. For example, direct histochemical techniques are being applied to verify and establish the accuracy of each probe’s phenotypic-tagging capabilities. In another study, we are attempting to patch clamp from live, labeled neurons and then ascertain attenuations (or enhancements) from recorded electrical signals, if
Preliminary indications from patch clamp attempts, suggest that labeled neurons do remain viable and are still capable of displaying firing patterns.

The possibilities for future studies are numerous. As the body of nanobiotechnological knowledge grows, miniaturization technologies mature, and novel materials are introduced, future refinement of this technique is inevitable. This methodology has already demonstrated its wide applicability, by allowing conjugation of glutamatergic and GABAergic antibodies, and thereby targeting the two most abundant neurotransmitter systems in the central nervous system. Countless conjugate permutations could allow for specific visualization, customized characterization, connectivity assessment, and electrophysiological recording of hypothalamic cells and various other neuronal populations (e.g. cortical, hippocampal, interneuronal, etc.) within the brain and its periphery.
REFERENCES


Chen, S., Aston-Jones, G., 1995. Evidence that cholera toxin B subunit (CTb) can be avidly taken up and transported by fibers of passage. Brain Res. 674, 107-11.


Harris, K.M., Sultan, P., 1995. Variation in the number, location and size of synaptic vesicles provides an anatomical basis for the nonuniform probability of release at hippocampal CA1 synapses. Neuropharmacology. 34, 1387-95.


Imbery, T.E., Irdmusu, M.S., Speidell, A.P., Streer, M.S., Griffin, J.D., 2008. The effects of Cirazoline, an alpha-1 adrenoreceptor agonist, on the firing rates of thermally classified anterior


Naumann, T., Hartig, W., Frotscher, M., 2000. Retrograde tracing with Fluoro-Gold: different methods of tracer detection at the ultrastructural level and neurodegenerative changes of back-


VITA

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