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Brain region specific single-molecule fluorescence imaging

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Abstract

We developed an approach utilizing nanoscale vesicles extracted from brain regions combined with single molecule imaging to monitor how an animal's physiological condition regulates the dynamics of protein distributions in different brain regions. This method was used to determine the effect of nicotine on the distribution of receptor stoichiometry in different mouse brain regions. Nicotine induced upregulation of $\alpha 4\beta 2$ nicotinic acetylcholine receptors (nAChRs) is associated with changes in their expression, trafficking, and stoichiometry. The structural assembly of nAChRs has been quantified in cell culture based systems using single molecule techniques. However, these methods are not capable of quantifying biomolecule assembly that takes place in a live animal. Both nicotine induced upregulation and changes in nAChR stoichiometry differ across brain regions. Our single molecule approach revealed that nicotine acts differentially across brain regions to alter assembly in response to exposure and withdrawal.

Graphical Abstract

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Author Contributions

C.I.R., B.J.H., and J.R.P. supervised the research and designed the experiments. X.F., F.H.M., and A.M.L. performed experiments. F.H.M. wrote the data analysis scripts. All authors contributed to writing the manuscript and given approval to the final version of the manuscript.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

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Neuronal nicotinic acetylcholine receptors (nAChRs), the target of nicotine's rewarding and reinforcing effects, functionally assemble as pentamers composed of alpha (α 2–10) and beta (β 2–4) subunits. Chronic exposure to nicotine increases the expression level of some nAChR subtypes and influences their trafficking. Nicotine also alters the assembly of α 4 β 2 nAChRs, the predominate subtype in the central nervous system, resulting in a shift in their stoichiometry. The two stoichiometries, (α 4)₂(β 2)₃ and (α 4)₃(β 2)₂, exhibit distinct pharmacological properties¹. Nicotine induced upregulation and changes in receptor stoichiometry are believed to play a critical role in several processes related to nicotine addiction^{2–4}. However, the long-term changes in the structural assembly of nAChRs during nicotine exposure and withdrawal are not fully understood.

While nicotine-induced upregulation has been demonstrated both through *in vitro* and *in vivo* studies^{2, 5–7}, quantitative measurements of changes in the stoichiometry of α 4 β 2 nAChRs have been restricted to *in vitro* studies in cells^{8–10}. Studies using heterologous expression in isolated cellular systems provide insight into receptor assembly, but they lack the context of the complex environment present in an animal. This is particularly true in the central nervous system (CNS) where neuronal and glial interactions shape processes ranging from protein expression to cellular function and communication¹¹. Thus, observations *in vitro* may not correspond to that of native protein *in vivo*.

Single-molecule imaging techniques enable the study of complex biological dynamics, including molecular structure, protein interactions, and functional activity^{12–13}. One of the primary advantages of single molecule imaging is the elimination of ensemble averaging seen with traditional techniques, thus providing detailed information of population distributions and dynamic interactions. This provides a way to delineate protein populations that exhibit different conformational states, functional states, and assemble into multiple stoichiometries^{14–15}. Single molecule imaging has been extensively applied to purified proteins providing new insights into protein-proteins interactions, conformational changes, complex assembly, and functional activity of a wide variety of biomolecules^{16–17}. Outside of single molecule measurements, studies of changes in receptor stoichiometry have been limited to indirect methods that are not capable of quantifying changes in population distributions between different α 4 β 2 nAChRs assemblies. Despite inherent challenges such as fluorophore labeling, sensitivity, and complex biological environments, single molecule studies are now regularly used to study protein dynamics in cells in culture^{18–20}. Recent technological advances such as single-molecule pull-down (SiMPull) along with other techniques has also helped extend quantitative studies of protein stoichiometry, interactions, and activity to a wider array of biomolecules.^{21–22}

However, single molecule techniques have not been routinely used to study the regulation of protein dynamics using whole animal approaches. Complex tissue architecture, cell heterogeneity, low light penetration, and lack of imaging sensitivity makes the extension of single molecule techniques *in vivo* more challenging than previously encountered in transitioning single molecule studies to live cell imaging. The ideal technique would quantify proteins of biological processes within an animal at specific time intervals with single molecule resolution. Nicotine has been shown to act differentially across areas of the brain leading to varying levels of upregulation in different brains regions such

as the mid-brain, cortex, and hippocampus. Nicotine also acts in a cell specific fashion inducing upregulation to a higher degree in GABA neurons compared to dopaminergic cell bodies^{23–24}.

Here we developed a technique that uses the rapid extraction of nAChRs trapped in nanoscale vesicles composed of their original cellular membrane within the brain. Vesicles are generated either from a whole brain preparation or specific microdissected mouse brain regions. Brain region specific vesicles are produced by fractioning cell membranes from isolated target regions. Receptors remain in their endogenous membrane, thus maintaining their structural integrity. This approach was used to study the assembly profile of the $\alpha 4\beta 2$ nAChRs extracted directly from animals at specific time points.

Experimental Section

Animal Care

The $\alpha 4$ -GFP knock-in mouse strain was acquired from Dr. Jerry Stitzel's Lab (Institute for Behavioral Genetics University of Colorado) and maintained in Dr. James Pauly's Lab (University of Kentucky, Department of Pharmaceutical Science). The study used male mice that were 2–5 months old at study initiation. All experiments were conducted within the guidelines set forth by the National Institutes of Health and were approved by the University of Kentucky's Institutional Animal Care and Use Committee.

Nicotine treatment

Chronic nicotine was administrated using implanted osmotic pumps (model 1002, Alzet) at a dosage of 0.7mg/kg/hr (free base) for 12 days. Sterile saline was filled in osmotic pumps as a control. For all surgeries, mice were anesthetized by isoflurane and pumps were implanted subcutaneously. After 12 days of treatment, nicotine administration was stopped by removing the osmotic pumps. Some animals were euthanized immediately following pump removal and others were housed for another 7 and 21 days for nicotine withdrawal studies.

Vesicle isolation

Mice were euthanized with Fatal-Plus (Vortech) followed by cardiac perfusion with 1x PBS. Fresh mouse brain tissue was obtained and kept in cold homogenization buffer (0.32M sucrose, 10mM HEPES, pH 7.4, 2mM EDTA, and protease inhibitor). For whole brain single molecule imaging studies, the entire brain tissue was used to produce $\alpha 4\beta 2$ nAChRs containing vesicles. For brain region specific single molecule imaging study, the regions of interest were initially separated using a 2mm mouse brain matrix (Zivic) followed by additional micro-dissection. The same brain regions from 3 to 5 mice were combined together to generate nanovesicles. Fresh brain tissue was homogenized immediately using a dounce homogenizer (PYREX) with 2 mL cold homogenization buffer. 3 mL of additional buffer was added to this mixture and centrifuged at 200xg for 15 min to remove large tissue. The supernatant of brain lysate was centrifuged at 1,000xg for 15 min at 4 °C to remove the pelleted nuclear fraction. Then, the supernatant was collected and subjected to ultra-centrifugation at 10,000xg for 20 minutes at 4 °C to remove mitochondria. The

supernatant was again centrifuged at 100,000xg for 2 hours at 4 °C. This yielded a pellet containing vesicles which was resuspended in 1x PBS buffer and aliquots were stored at –80 °C until use.

TIRF Imaging

Before imaging, samples were immobilized on clean functionalized glass bottom dishes. A glass bottom dish was cleaned by sonicating in 5 M NaOH solution for 1 hour and then in 0.1 M HCl solution for 1 hour at 45 °C. After rinsing with water and ethanol 3 times each, the cleaned dish was dried by compressed air and cleaned by oxygen plasma (Harrick Plasma PDC-32G). To functionalize the dish, 1mg/mL Saline-PEG-Biotin in 95% ethanol, 0.1 mg/mL NeutrAvidin in 1x PBS, and 1µg/mL biotinylated anti-GFP antibody in 1x PBS were treated to the dish for 30 minutes sequentially. Between each step, the dish was rinsed 3 times with 1x PBS. Before binding vesicles, empty cleaned functionalized glass bottom dishes were imaged to confirm the absence of background in each dish. Finally, single vesicles were spatially isolated and immobilized on this functionalized dish by incubating at room temperature for 30 minutes. The dish was again rinsed with 1x PBS 3 times to remove unbound vesicles. Immobilized vesicles were maintained in 1mL 1x PBS during imaging.

Brain derived nanovesicles were imaged using total internal reflection fluorescence (TIRF) microscopy. TIRF imaging is a powerful technique that reduces background fluorescence but increases resolution by focusing on a single, optical plane. Only fluorescent molecules within 100–200nm of the glass surface could be efficiently excited. A 488nm DPSS laser (~60 W/cm²) was used to excite green fluorescent protein (GFP). The beam traveled through the appropriate dichroic and filter to the objective (1.49NA, 60x oil immersion) wherein the angle was adjusted to gain total internal reflection (TIR) using a stepper motor. The emission light was detected by an electron multiplying charge coupled device (EMCCD) (Andor). An auto focus module (Olympus ZDC2) was used to diminish focal drift. A TIFF stack containing 800 to 1000 continuous frames with 100 ms exposure time was acquired for each field of view.

Confocal Imaging

Mice were treated with nicotine (0.7mg/kg/hr) or saline using osmotic pumps (model 1002, Alzet). Following drug administration, mice were euthanized with Fetal Plus and subjected to cardiac perfusion with 1xPBS. The brain was then quickly removed and frozen using isopentane in dry ice and then stored at –80 °C. Later, brains were sectioned at 20 µm using a cryostat (Lecia CM1850). Brain slices were mounted with Vectashield (Vector labs, H-1000) in the day of imaging. A Nikon A1Rsi laser scanning confocal microscope equipped with a 20X 0.9NA Plan Apo water objective was used for confocal imaging. 20X images were collected by using 5×5 large imaging combination.

Receptor Autoradiography

To corroborate the single molecule findings, quantitative receptor autoradiography was used to measure α4β2 nAChRs in a subset of animals. Following animal euthanasia, the brains were immediately removed and frozen in isopentane that was chilled in dry ice. Brains were sliced using a Lecia CM1850 cryostat to make a series of 16-micron

thick sections. Receptor density was assessed using [³H]-Epibatidine autoradiography (100 pM incubation concentration) and previously established methods. RayMax Beta High Performance Autoradiography Film was used to visualize the areas of ligand binding following a 3-day exposure. All films were processed using Kodak D-19 developer and binding data were analyzed using NIH imageJ and expressed as uncalibrated optical density in arbitrary units.

Dynamic Light Scattering

Brain nanovesicle preparation is the same as described above. All samples for DLS measurement were filtered by a standard syringe filter (0.45μm) A ZetaPALS potential Analyzer (Brookhaven Instruments) was used to collect the DLS measurements of vesicle size.

Western Blot Analysis

Western blots were used to determine α4-GFP isolation from brain lysate. Denatured whole brain nanovesicle fraction were loaded into a pre-packaged NuPAGE 4–12% Bis-Tris gel (Life Technologies). Bands were transferred to a nitrocellulose membrane after electrophoresis. The membrane was first blocked at room temperature for 1 hour with a PBST solution (5% non-fat milk, 0.1% Tween in PBS). Anti-GFP antibody was then added in a 1:1000 dilution and incubated overnight at 4 °C. The following day, primary antibody was removed by rinsing the membrane with PBST solution 4 times 5-minute incubation. Secondary anti-rabbit antibody (Jackson ImmunoResearch) was added to the membrane in a 1:5000 dilution and incubated for 1 hour at room temperature. Secondary antibody was removed in the same way by rinsing the membrane with PBST solution 4 times 5-minute incubation. After removal of PBST solution from the membrane, bands were visualized by chemiluminescent detection (Clarity, Bio-Rad) using Chemi-Doc system (Bio-Rad).

Photobleaching Step Analysis

Customized Matlab scripts were written to generate time traces from image sequences and analyze photobleaching steps. Briefly, the first 10 frames of the TIFF stack were combined to a composite image, which was employed to find peaks by comparing with the user defined threshold value. A 3-pixel × 3-pixel region of interest (ROI) was selected at each peak position to read the intensity and a 5-pixel × 5-pixel region around peak was used to measure the background. The background was subtracted from the intensity of each ROI. Changes in the intensity occurring at each peak over the length of this stack, a time trace, was determined and saved as a text file. This file was then used to plot the time trace and count the number of photobleaching steps. Finally, results from different TIFF stacks were accumulated to generate a distribution of photobleaching steps.

Photobleaching steps were determined manually according to the stepwise decrease of fluorescence decay. A single molecule was accepted with the following criteria: (1) the time trace of a single molecule should have at least one clear bleaching step (but on more than 3 steps); (2) the GFP molecule fluorescence (each bleaching step) had to last at least 10 frames (1 second); (3) the mean intensity needed to be 3 times higher than the standard deviation of the mean count of the background. All data sets were analyzed blindly at least twice,

and the results were only accepted after comparison of results from each analysis round. For statistical analysis see supporting information.

Results and Discussion

Chronic, low dose nicotine induces modest but significant levels of nAChR upregulation *in vivo*

Chronic administration of nicotine increases the density of neuronal nAChRs *in vivo*. To examine this phenomenon, we first measured binding of [³H]epibatidine in different brain regions for both nicotine and saline treated $\alpha 4$ -GFP Knock-In (KI) mice. The density of neuronal nAChRs was visualized and measured by nearly saturated [³H]epibatidine binding sites. As shown in Fig. 1A and 1B, at the low dose of chronic nicotine administration (0.7mg/kg/hr via osmotic minipump), clear up-regulation was observed in the cortex (~10%) and the hippocampus (dentate gyrus temporoammonic path and medial perforant path) (~25%) compared to the saline treated group (Supporting table 1).

To specifically examine nicotine induced up-regulation of $\alpha 4^*$ nAChRs, the fluorescence intensity of the GFP signal of the confocal microscopic images from the 20 μ m-brain slices was analyzed. Two confocal images of the representative cortical and hippocampus brain slice section from $\alpha 4$ -GFP KI mice are shown in Fig. 1 C and 1 D. Higher fluorescence intensities in the hippocampus were observed in the mice exposed to nicotine (0.7mg/kg/hr), which agrees with the radioligand binding studies and verifies the chronic nicotine administration upregulation of $\alpha 4^*$ nAChRs in the $\alpha 4$ -GFP KI mice used for single molecule studies.

These experiments confirm that chronic, low dose nicotine only moderately increased the high affinity binding of [³H]epibatidine and the expression level of nAChRs. This agrees with previous *in vivo* studies showing that upregulation scales with the amount of nicotine delivered. These studies also verify the selectivity of nicotine induced upregulation *in vivo* across different brain regions, with the greatest increase in nAChR expression in the hippocampus.

Generation and characterization of the $\alpha 4^*$ brain nanovesicles

To determine correlations between upregulation and receptor stoichiometry, we performed single molecule experiments to examine the changes in receptor assembly in response to the animal's physiological condition. Here, we developed a novel technique to perform brain region specific single molecule analysis via the rapid extraction of receptors isolated in nanovesicles. The receptor isolation strategy for the entire mouse brain and specific brain regions is shown in Fig 2. Nanoscale vesicles are generated directly from brain tissue obtained from $\alpha 4$ -GFP KI mice. These vesicles encapsulate single receptors within the physiological membrane where they resided at the time of extraction. A total internal reflection fluorescence microscopy (TIRFM) image of isolated vesicles containing $\alpha 4\beta 2$ nAChRs is shown in Figure 3A. To further characterize brain derived nanovesicles, we employed dynamic light scattering (DLS) to determine the size of vesicles in our preparations. Vesicles range in size from 90 to 150 nm in diameter (Fig 3B). The size of

vesicles is small enough that they spatially isolate nAChRs at the single molecule level. The isolation of GFP containing receptors in vesicles was verified via western blot using an anti-GFP antibody (Fig 3C).

In order to determine the assembly of $\alpha 4\beta 2$ nAChRs, we recorded the fluorescence intensity versus time traces for each single vesicle to show a stepwise decay of fluorescence (Fig 3D and 3E). Photobleaching steps within each time trace correspond to the number of GFP labeled subunits. Since nAChRs assemble as pentamers, the observed distribution of bleaching steps arises from the combination of $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ stoichiometries. The observed population distributions were fit to binomial distributions to extract the ratio of the two stoichiometries (see methods and supporting figures).

Stoichiometric distribution $\alpha 4\beta 2$ nAChRs in whole brain preparations

Cell culture based single molecule experiments have tied upregulation of nAChRs to changes in stoichiometry.⁸ However, the native population distribution of each stoichiometry and changes in this distribution upon exposure to nicotine within the brain is still unknown. In order to correlate *in vivo* upregulation with changes in receptor stoichiometry, our single molecule approach was used to examine the regulation of $\alpha 4\beta 2$ nAChR expression in native brain tissue.

We first determined the baseline distribution of the two populations of $\alpha 4\beta 2$ nAChR stoichiometries. The effect of chronic nicotine treatment on receptor assembly in the entire mouse brain was then evaluated. In the saline treated group, the distribution of observed photobleaching steps were fit to binomial distributions weighted to 48% of $(\alpha 4)_2(\beta 2)_3$ and 52% of $(\alpha 4)_3(\beta 2)_2$. This matches previous *in vitro* reports that suggest an equal ratio of the two $\alpha 4\beta 2$ nAChR stoichiometries²⁵. After 12 days of chronic nicotine exposure (0.7mg/kg/hr), the $\alpha 4\beta 2$ nAChRs exhibited a distribution of $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ of 55% and 45%, respectively which was significantly different than the saline group ($\alpha=0.05$) (Fig 4). The result of the whole brain studies gives a slightly higher fraction of the $(\alpha 4)_2(\beta 2)_3$ stoichiometry than observed in cells, which is in agreement with the existence of low numbers of various subtypes of neuronal $\alpha 4$ containing nAChRs, such as $\alpha 4\alpha 5\beta 2$, $\alpha 4\alpha 6\beta 2$.^{26–27}

Brain region specific stoichiometric distribution of $\alpha 4\beta 2$ nAChRs

We then determined the population distribution of $\alpha 4\beta 2$ nAChR stoichiometries across different brain regions. These brain region specific single molecule studies were used to determine whether the selectivity of nicotine-induced upregulation in different brain regions correlates with changes in the distribution of receptor stoichiometries. We separated and performed nanovesicle extraction from the cerebellum, cortex, hippocampus, hypothalamus, midbrain, striatum, and thalamus (Fig 5). Receptors encapsulated in both cortex and hippocampus derived nanovesicles showed that, in the absence of nicotine, the distribution of $\alpha 4\beta 2$ nAChRs fit a binomial weighted for 41% $(\alpha 4)_2(\beta 2)_3$ and 59% $(\alpha 4)_3(\beta 2)_2$. A clear nicotine-induced shift in stoichiometry was observed in these two regions with 12 days of chronic nicotine treatment (0.7mg/kg/hr). In the presence of nicotine, single-molecule analysis of isolated $\alpha 4\beta 2$ nAChRs derived from the cortex showed the majority of

receptors assemble as the high sensitivity stoichiometry of $(\alpha 4)_2(\beta 2)_3$, weighted to 60% of $(\alpha 4)_2(\beta 2)_3$ and 40% of $(\alpha 4)_3(\beta 2)_2$. Similarly, vesicles generated from the hippocampus were fitted to 61% of $(\alpha 4)_2(\beta 2)_3$ and 39% of $(\alpha 4)_3(\beta 2)_2$ (Fig 5B). Distributions of observed photobleaching events and their fitting results are shown in supporting information (Fig S1 and S2). While statistically significant shifts in stoichiometry were observed in the hippocampus and cortex, $\alpha 4\beta 2$ nAChRs encapsulated in nanovesicles originating from the remaining five brain regions, cerebellum, hypothalamus, midbrain, striatum, and thalamus, showed no statistically significant shifts due to chronic nicotine treatment. Two stoichiometries of $\alpha 4\beta 2$ nAChRs weighted to similar, near equivalent proportions of $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ subtypes in all five brain regions with or without nicotine exposure (Fig 5C). Distributions of observed photobleaching events and their fitting results are shown in supporting figures (Fig S3–S7). The absence of a shift for $\alpha 4\beta 2$ nAChR expression in these regions could provide a new explanation of selectivity in nicotine induced upregulation in the brain.

Comparing the distribution of low sensitivity and high sensitivity $\alpha 4\beta 2$ nAChRs between whole brain preparations and previous cell based studies^{8, 10}, we found a higher fraction of high sensitivity stoichiometry receptors in the whole brain preparations. This further emphasizes the need for single molecule stoichiometry studies from animals as the homogeneous cell culture environment lacks the complexity present *in vivo*. Here low levels of nicotine exposure led to undetectable changes in receptor stoichiometry in the cerebellum, hypothalamus, mid-brain, thalamus, and striatum. Higher levels of nicotine exposure have routinely been shown to lead to upregulation in variety of studies^{28–29}. However, upregulation has not previously been correlated to *in vivo* changes in stoichiometry. This new single molecule approach was used to observe robust changes in $\alpha 4\beta 2$ nAChR assembly in the cortex and the hippocampus even at these moderate levels of upregulation. It suggests that structural changes in receptor assembly are more sensitive and responsive to nicotine than other commonly observed physiological changes such as nAChR upregulation. Thus, even low levels of nicotine appear to have significant consequences.

Changes in $\alpha 4\beta 2$ nAChR stoichiometric distribution during withdrawal

To capture changes in receptor stoichiometry that occur during nicotine withdrawal, our method was utilized to examine the stoichiometry of $\alpha 4\beta 2$ nAChRs for time points after exposure to nicotine. Animals with no nicotine, at 12 days of nicotine exposure, and after withdrawal from nicotine were compared at time points of 1 and 3 weeks (Fig 6). Single-molecule bleaching event analysis was applied to study the stoichiometric assembly of $\alpha 4\beta 2$ nAChRs captured in brain regions specific vesicles. The distributions of $\alpha 4\beta 2$ nAChRs in 5 different brain regions, in the presence of nicotine, were analyzed (Fig 6B, left). Single-molecule analysis of photobleaching events from nanovesicles extracted from the cortex after day 7 of withdrawal showed 44% $(\alpha 4)_2(\beta 2)_3$ and 56% $(\alpha 4)_3(\beta 2)_2$. After a 21-day nicotine withdrawal, nanovesicles encapsulating single $\alpha 4\beta 2$ nAChRs from the cortex were distributed as 42% $(\alpha 4)_2(\beta 2)_3$ and 58% $(\alpha 4)_3(\beta 2)_2$, which represented the same stoichiometric distribution as the control group (Fig 6B and Fig S8). Analysis of hippocampus associated receptors showed a distribution of 49% $(\alpha 4)_2(\beta 2)_3$ and 51% $(\alpha 4)_3(\beta 2)_2$ at 7-day withdrawal, and 41% $(\alpha 4)_2(\beta 2)_3$ and 59% $(\alpha 4)_3(\beta 2)_2$ at 21-day

withdrawal (Fig 6B and Fig S9). However, single $\alpha 4\beta 2$ nAChRs isolated from the cerebellum, midbrain, and thalamus, exhibited no significant differences in their assembly in the presence of nicotine or after nicotine withdrawal (Fig 6B and Fig S10–12). This demonstrated that nicotine induced upregulation of $\alpha 4\beta 2$ nAChRs was brain region specific and that stoichiometry returns to the original population distribution after withdrawal.

These studies reveal a reversal of the upregulation and demonstrate that our single molecule approach is capable of following the time course of changes in protein populations taking place within live animals. The longer period of withdrawal that was observed for the hippocampus to return to baseline stoichiometry also further suggests differential effects of nicotine across brain regions.

Conclusion

We have developed a new technique that allows us to perform single molecule measurements of proteins synthesized and modified in the CNS of an animal. This technique relies on the generation of nanoscale vesicles from microdissected brain regions. Vesicles are composed of the identical membrane where the receptors resided immediately prior to formation. This maintains the vesicles in an environment that supports their structural integrity but still provides spatial isolation for single molecule imaging. This novel approach was used to determine the proportion of $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ nAChR stoichiometries in native brain tissue. Our approach takes snap shots in time of the receptor population at multiple time points during modifications to receptor assembly that occurred during nicotine exposure and withdrawal.

These studies confirm that low dose nicotine induced up-regulation is associated with stoichiometric changes with high selectivity in different regions of the brain. Our novel technique, provides insight into how an animal's physiological environment alters assembly of nAChRs in the brain. For the first time, single molecule changes in receptor assembly that occurred within a live animal were observed. These studies provide a general method to use quantitative single molecule measurements of biomolecular processes occurring in an animal. This technique should be directly applicable to the study of other systems. The observation of robust stoichiometry changes in response to low levels of nicotine with only modest upregulation points to a prominent role for nAChR structural changes in nicotine addiction and provides further evidence of the differential behavior between brain regions in response to nicotine

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Upregulation of nAChRs. A and B, Representative autoradiographic comparison of [^3H]-Epibatidine binding from brains of the $\alpha 4$ -GFP knock-in mice pretreated with nicotine (0.7mg/kg/hr) and saline, respectively, showing upregulation in the hippocampus. **C and D**, Representative confocal imaging comparison from brains of the $\alpha 4$ -GFP knock-in mice pretreated with nicotine (0.7mg/kg/hr) and saline. Arrows indicate upregulation in the hippocampus.

Figure 2. Isolation of $\alpha 4 \beta 2$ nAChR containing nanovesicles from mouse brain tissue.

Schematic depicting the generation of brain-derived nanovesicles containing single $\alpha 4 \beta 2$ nAChR. The whole brain (top) from $\alpha 4$ -GFP KI mice is homogenized to form small fragment of brain tissues. The resulting lysate is purified to acquire nanoscale vesicles which maintain single $\alpha 4 \beta 2$ nAChRs in the same physiological membrane they resided in prior to extraction from the brain. The vesicles are sorted from the cell debris resulting in isolated nanoscale vesicles. Individual brain regions (bottom), such as the cerebellum and hippocampus, are dissected and then separated to generate region specific nanovesicles. Vesicles are prepared from brain regions specific tissue in the same fashion as described for whole brain.

Figure 3. Isolation and characterization of brain derived vesicles.

Characterization of brain $\alpha 4\beta 2$ nAChR nanovesicles. **A**, A representative image of isolated vesicles containing $\alpha 4\beta 2$ nAChRs. **B**, Size distribution from DLS measurement of brain derived nanovesicles. **C**, Western blot verifying the isolation of $\alpha 4$ -GFP from brain preparations. **D**, Time traces of photobleaching steps from isolated single vesicles showing 2 bleaching steps caused from two GFP molecules which suggest a $(\alpha 4)_2(\beta 2)_3$ stoichiometry in theory. Similarly, three photobleaching steps **E** corresponds to a $(\alpha 4)_3(\beta 2)_2$ stoichiometry.

Figure 4. Distributions of $\alpha 4 \beta 2$ nAChR assembly whole brain preparations.

A. Schematic illustration of the generation of whole brain-derived nanovesicles containing single $\alpha 4 \beta 2$ nAChR. Observed (blue) and fitted (red/black) distributions of one, two, or three bleaching steps for the $\alpha 4 \beta 2$ nAChRs encapsulated in brain derived nanovesicles from the saline treated group **B**, and the nicotine treated group **C**. Error bars are the square root of the number of vesicles counted. These expected distributions fit a binomial of 48% of $(\alpha 4)_2(\beta 2)_3$ and 52% $(\alpha 4)_3(\beta 2)_2$ in the saline group and 55% of $(\alpha 4)_2(\beta 2)_3$ and 45% $(\alpha 4)_3(\beta 2)_2$ in the nicotine group **D** with statistical significance between saline and nicotine conditions. Error bars are 95% confidence intervals. A 2 proportion Z test was used to determine significance at $\alpha=0.05$.

Figure 5.

Nicotine treatment caused stoichiometric changes of $\alpha 4\beta 2$ nAChRs in different brain regions. **A.** Schematic illustration of the generation of brain region specific nanovesicles containing single $\alpha 4\beta 2$ nAChR. **B.** The fitted populations of the 2 possible $\alpha 4\beta 2$ nAChR stoichiometries from the hippocampus and the cortex in saline (left) and nicotine (right). **C.** The distribution of stoichiometries in brain regions showing no changes when animals are exposed to nicotine (right) as compared to saline (left). Error bars are 95% confidence intervals. There was a significant difference at $\alpha=0.05$ for the cortex and hippocampus regions between saline and nicotine treated groups using a 2 proportion Z test.

Figure 6. Nicotine withdrawal caused stoichiometric changes of $\alpha 4\beta 2$ nAChRs in different brain regions.

A. Schematic illustrating the nicotine withdrawal study. **B.** The population distribution of the two possible stoichiometries of $\alpha 4\beta 2$ in five different brain regions with nicotine treatment (left), 7-day withdrawal (middle), and 21-day withdrawal. Nicotine altered the stoichiometry of receptors in the cortex and hippocampus but not in the other regions. The cortex fully returned to baseline distributions after 7 days of withdrawal while the hippocampus returned to baseline levels after 3 weeks of withdrawal. The remaining brain regions showed no change in distribution during nicotine administration or after withdrawal. Error bars are 95% confidence intervals. There are significant differences ($\alpha=0.05$) for the cortex and hippocampus regions between nicotine treated and 1-week withdrawal groups. There is also a significant difference for the hippocampus between 1-week withdrawal and 3-week withdrawal groups using a 2-proportion Z test.