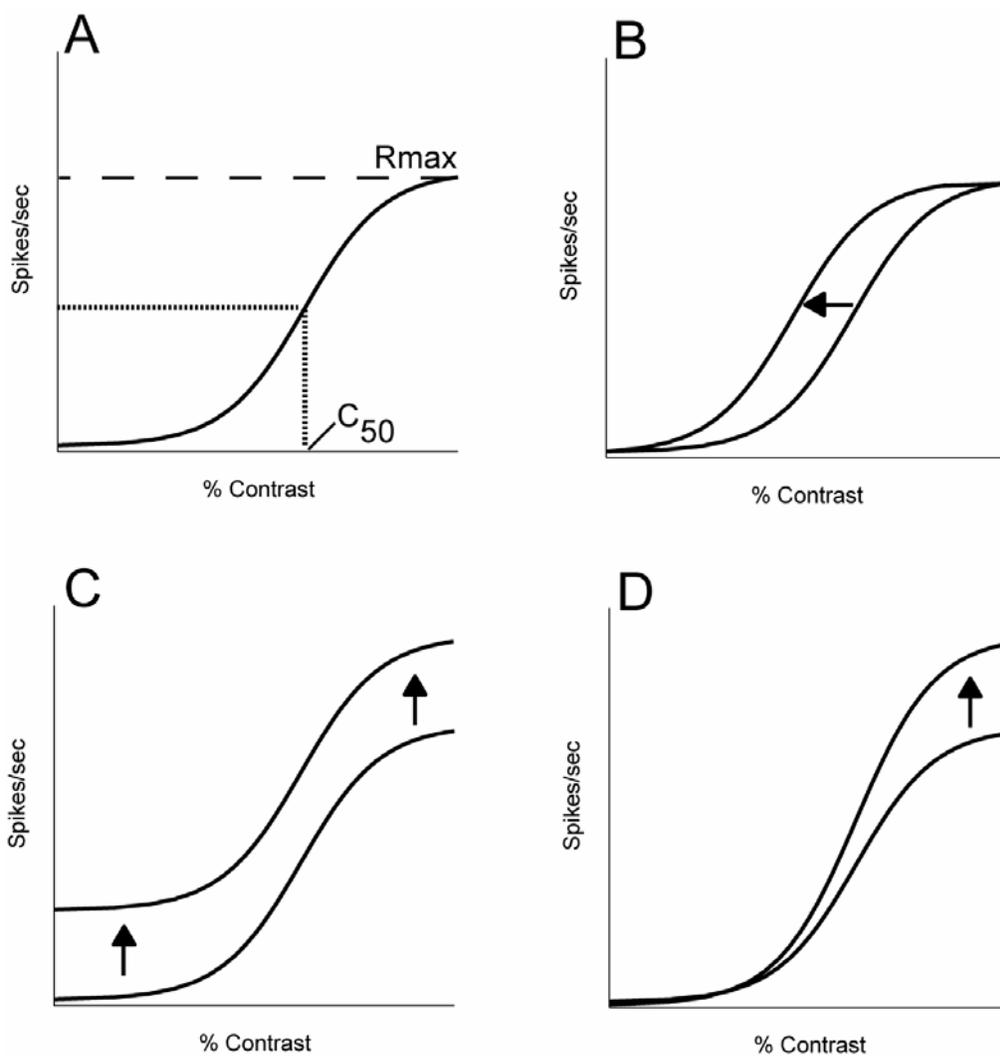


Gain Modulation by Nicotine in Macaque V1

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Supplementary Figure 1: Models of visual gain change. The relationship between stimulus contrast and neuronal spike rate is often modelled parametrically using a hyperbolic ratio function

$$R = R_{\max} \frac{C^n}{C^n + C_{50}^n} + sFR$$

Fitting data in this way provides values for 4 parameters; R_{\max} , C_{50} , slope (n) and function offset (sFR). The R_{\max} parameter (dashed line in **A**) describes the peak neuronal response (highest firing rate observed). The C_{50} parameter (dotted line in **A**) gives the contrast value at which half this maximum firing rate was observed. The relationship between contrast and response – the gain - can change in a number of ways (Sengpiel et al., 1998; Williford and Maunsell, 2006). **(B)** illustrates a pure ‘contrast gain’ change. The fitted function has shifted along the x-axis (in this case moving the entire function to the left). Contrast gain changes appear as changes in the fitted value for the C_{50} parameter. In the case of pure contrast gain increase, the dynamic range of the neuron remains the same, but the neuron responds to lower contrast stimuli and reaches an asymptotic firing rate earlier. **(C)** illustrates an ‘activity gain’ change. In this case the entire function is shifted upward along the y-axis and both the R_{\max} parameter and the function offset have changed. **(D)** illustrates a ‘response gain’ change. In this the dynamic range of the neuron has increased. The function offset and C_{50} are unchanged while the value of the R_{\max} parameter has increased.

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Supplemental Methods

Animals:

Adult male cynomolgous monkeys (*Macaca fascicularis*) were used in these experiments. Those in the anatomy studies had been used previously for unrelated electrophysiology experiments (Solomon et al., 2004). Procedures were approved by the Institutional Care and Use Committee for NYU, in accordance with NIH guidelines.

Immuno-electron microscopy:

To localise cholinergic receptors in the thalamic recipient layer of V1, we used tract-tracing from the thalamus combined with single-label immunocytochemistry for m1 and m2 muscarinic receptors and the $\beta 2$ subunit of nicotinic receptors. Layer 4c was identified using cytochrome oxidase histochemistry and data were collected using an electron microscope.

Tracer injection surgery: Tracer injections were made in three animals on the first day of unrelated physiology experiments. Initial anaesthesia was induced using ketamine hydrochloride (10mg/kg i.m.) and the saphenous veins cannulated. Then under thiopental sodium (animals 7 and 15) or isoflurane (animal 17) anaesthesia, the animals were intubated (animals 7 and 15) or tracheotomised (animal 17), mounted in a

stereotaxic frame and transferred to sufentanil citrate anaesthesia. Three injection tracks (with three tracer deposits per track; nine deposits in total) were made in each animal, at 8mm antero-posterior/11mm medio-lateral (with respect to interaural zero), 9mm antero-posterior/11mm medio-lateral and at a point just posterior to the central sulcus at 11mm medio-lateral. Injections of a tracer solution containing both 10% biotinylated dextran-amine (3000 MW, BioDesign) and 10% micro-ruby (BioDesign) in sterile saline were made using a Hamilton syringe. After craniotomy and reflection of the dura, the syringe was lowered to 28mm below the cortical surface. After a rest of 30 minutes, three 0.25 μ L injections were made at depths of 27, 26 and 25mm below the cortical surface, with 10 minute rests after each injection and a 15 minute rest at 24mm after all injections had taken place. After removal of the injection syringe the dura was then laid back over the cortex, the bone plug replaced and the exposure 'sealed' using agar. At this point the physiology experiments were continued as per the usual physiology protocol. After occipital craniotomies, the animals were paralysed using pancuronium bromide and anaesthesia continued with sufentanil. They were maintained in this state for approximately 96 hours and, following a dose of 60 mg/kg pentobarbital that resulted in a flat EEG, sacrificed.

Histological preparation: Animals were transcardially perfused with heparinised lactated Ringer followed by 4 litres of chilled, freshly prepared 4% paraformaldehyde with 0.25% glutaraldehyde, pH 7.4. The fixative was run for at least 40 minutes. The visual cortex (V1) was removed by a coronal cut at the level of the lunate sulcus. The LGN was removed by coronal cuts at the anterior tip and midpoint of the intraparietal sulcus. Both blocks were post-fixed at 4°C in 4% paraformaldehyde. Within 48 hours, the blocks

were vibratome-sectioned at 40µm (in the sagittal plane for V1, coronal plane for the LGN) and reacted for 30 minutes in 1% sodium borohydride in 0.1 M PB, to stop glutaraldehyde fixation. After 0.1 M PB rinses, every third section of the visual cortex tissue was set aside for the cytochrome-oxidase (CO) reference set. Remaining sections were stored at 4°C in 0.01M phosphate-buffered saline (PBS) with 0.05% sodium azide added. Cytochrome oxidase histochemistry was commenced no more than 72 hours after perfusion as described elsewhere (Disney et al., 2006).

'Double DAB' procedure for light microscopic identification of thalamic injection sites and V1 terminal fields: All LGN sections and every second section of the remaining V1 set (after removal of sections for the CO reference) were first incubated in 1% hydrogen peroxide in PBS for 30 minutes to block endogenous peroxidase activity. They were then rinsed and placed into an avidin-horseradish peroxidase (avidin-HRP) complex (Vectastain Elite ABC Kit, Vector Laboratories) with 0.3% Triton x-100 added. This incubation proceeded at room temperature overnight on a shaker. The next day, following PBS rinses, the sections were reacted for 15 minutes using the ABC-DAB technique (Hsu et al., 1981). After rinsing, the V1 set were placed into a fresh avidin-HRP solution for a further three hours and the DAB reaction repeated. We have found that repeating the ABC-DAB procedure in this way produces better labelling of the tracer in fine axons and terminals. All sections were then mounted and dried overnight before a 10 minute, on-slide exposure to 0.1% osmium tetroxide in 0.1M PB to further enhance the DAB staining. The slides were then rinsed and the tissue dehydrated and coverslipped.

Single-label immunocytochemistry for electron microscopy: Using the V1 ABC-DAB

reference set, sequential sections with large labelled thalamic terminal fields were selected. A 'freeze-thaw' technique (Wouterlood and Jorritsma-Byham, 1993) was used to improve antibody penetration in a manner that minimises ultrastructural damage. The tissue was cryoprotected in a series of 10 minute washes through 5%, 10%, and 20% dimethylsulfoxide (DMSO) in 0.1M PB, then run through eight freeze-thaw cycles, each comprising a brief dip in partially frozen isopentane followed by a thaw in room temperature 20% DMSO. The sections were then rinsed in PBS and endogenous peroxidases blocked (see Double-DAB procedure above) before being placed in a blocking solution of 1% IgG-free bovine serum albumin (BSA, Molecular Probes) with .05% sodium azide (Sigma), 0.04% Triton x-100 (Triton) and 0.1% Photoflo (Kodak) in 0.01 M PBS for 30 minutes.

Primary antibodies were diluted in PBS with 1% BSA and .05% sodium azide (Standard Blocking Solution). Added to this buffer were a rabbit anti- β 2 nicotinic subunit (45 μ g/mL; directed against amino acids 493-502 of the C-terminal, BioDesign # Q4A665R, lots 2L35700, 13B03702 and 12J29502), a rabbit anti-m1AChR (1:200; directed against amino acids 227-353 of the i3 intracellular loop, Chemicon #AB5164, lots 22060712 and 22060716) or a rat anti-m2AChR (1:250; directed against amino acids 225-359 of the i3 intracellular loop, Chemicon # MAB367, lots 22090231 and 21120073). Free-floating sections were incubated in one of these primary antibodies for 72 hours at room temperature on a shaker.

The silver-intensified immunogold method was used for visualisation. After the primary antibody incubation and six hours of PBS rinsing, the tissue was incubated overnight at room temperature in a 0.8nm gold-conjugated secondary antibody (goat anti-rabbit IgG

or goat anti-mouse IgG; Aurion), diluted 1:100 in Standard Blocking Solution. The next day, after brief PBS rinses, the sections were post-fixed with 2% glutaraldehyde in PBS for 10 minutes and then rinsed again before silver enhancement. After 3 brief washes in 0.2M citrate buffer (pH 6.0) to remove phosphate buffer salts, the gold particles were enhanced for 5 to 12 minutes using the Amersham IntenSE silver enhancement kit, followed by citrate buffer and then 0.1M phosphate buffer rinses.

Following the silver autometallography, sections were processed to visualise the tract-tracer using the 'double DAB' procedure described above, omitting the Triton x-100 in the overnight incubation. The now dually-labelled sections were rinsed in 0.1M PB and fixed with 0.5% osmium tetroxide in 0.1M PB for 30 minutes, followed by dehydration in 50% ethanol and an overnight incubation in 4% uranyl acetate in 70% ethanol at 4°C on a shaker. The next day the dehydration series proceeded through 70%, 90% and 100% ethanol and three 30-minute acetone rinses before overnight infiltration of 1:1 EMBED 812 (EM Sciences)/acetone and then 100% EMBED 812. Following embedding in EPON capsules and 48 hour curing at 60°C, the tissue was re-sectioned at a thickness of 80-90 nm, mounted on formvar-coated nickel grids and counter-stained with Reynold's lead citrate ready for inspection under a JEOL 1200 XL transmission electron microscope.

Data collection: Using tissue maps obtained by camera lucida drawings, data were collected from regions in which a tissue/EPON interface passed through fields containing labelled thalamic terminals in layer 4c. The area at the tissue-EPON interface, being the region of maximum antibody penetration, was searched and images of labelled terminals taken at 20-40,000x magnification using either film or a

Hamamatsu CCD camera controlled by ATM software. Image analysis was undertaken offline.

Immunofluorescence:

To determine the degree of nicotinic receptor expression by inhibitory neurons of different classes we used dual immunofluorescence cytochemistry. The antibody directed against the $\beta 2$ nicotinic receptor subunit was combined with each of the following antibodies; anti-GABA, anti-parvalbumin, anti-calbindin D-28K or anti-calretinin. Layers were identified using CO histochemistry and data collected using a confocal microscope. Brief methods are outlined below, for further details see Disney et al (2006).

Tissue preparation: Seven animals were transcardially perfused and visual cortex blocked, removed, post-fixed, sectioned and stored as described above, following unrelated physiology experiments.

GABA/ $\beta 2$ subunit dual immunofluorescence: Three animals were used for this experiment. Following freeze-thawing, randomly selected sections were blocked in Standard Blocking Solution and then exposed to two antibodies in a single, co-incubation step. The polyclonal rabbit anti- $\beta 2$ nicotinic subunit antibody (described above) was combined with a monoclonal mouse anti-GABA (1:100; directed against purified GABA, Sigma #A0310, lot 042K4817). The tissue was incubated in both antibodies for 72 hours at room temperature on a shaker.

Secondary antibodies were diluted 1:50 in Standard Blocking Solution. GABA-immunoreactive sites were visualised using the Alexa 594nm fluorophore (Alexa 594 chicken anti-mouse IgG, Molecular Probes # A21201, various lots) and nAChR

immunoreactive sites with the Alexa 488 fluorophore (Alexa 488 chicken anti-rabbit IgG, Molecular Probes #A-21441, various lots). This second incubation proceeded in the dark, at room temperature, for 4-6 hours. Sections were then rinsed, mounted, coverslipped and stored in the dark at 4°C.

Confocal microscopy: Image montages from pia to white matter were taken of the opercular surface of V1 (2-8° parafoveal visual field representation) using a Zeiss LSM 310 confocal microscope. Counting within each colour channel was undertaken individually, offline, using carefully co-registered cytochrome oxidase references images for identifying laminar boundaries.

Calcium-binding protein/nAChR dual immunofluorescence: Four animals were used for this experiment. The protocol followed was identical to that described above for GABA/nAChR dual labelling, with the exception that 0.3% Triton X-100 (Triton) was added to the blocking solution in place of the freeze-thaw process. Calcium-binding proteins were detected using antibodies directed against parvalbumin (goat anti-parvalbumin 1:5000, Swant, directed against rat muscle parvalbumin, #PVG-214, lot 3.6), calbindin (monoclonal mouse anti-calbindin D-28K 1:5000, directed against calbindin purified from chicken gut, #300, lot 18F) and calretinin (goat anti-calretinin, 1:1000, Swant, directed against human recombinant calretinin, #CG1, lot 1§.1).

Antibody controls:

Primary antibodies: The antibody directed against the $\beta 2$ subunit has not been described before in the literature and so to check for non-specific binding, we performed control experiments using preadsorbed antibodies for light microscopy and western blot analysis, using protocols described in detail elsewhere (Disney et al 2006). The

antigens used for preadsorption were synthetic peptides (Cys-493-502 of C-terminus) corresponding to the immunogen and were provided by the antibody manufacturer. Preadsorption abolished immunolabelling in tissue sections. In the Western blot analysis, the antibody directed against the $\beta 2$ nicotinic subunit produced a single band at 60kD, the expected molecular weight of the receptor (manufacturer specifications). Details of secondary antibody controls are detailed in Disney et al, (2006).

Physiological Recordings:

Details of surgical preparation, maintenance and of stimulus delivery and recording appear elsewhere (Xing et al., 2005). Animals were anaesthetised (sufentanil citrate 6 $\mu\text{g}/\text{kg}/\text{hr}$) and a craniotomy made over V1. After surgery, animals were paralysed (vencuronium bromide 100 $\mu\text{g}/\text{kg}/\text{hr}$) and anaesthesia maintained with sufentanil (6-24 $\mu\text{g}/\text{kg}/\text{hr}$). Extracellular recordings were made using a carbon fibre combined iontophoresis/physiology electrode with 2,3 or 6 glass iontophoresis barrels (Carbostar, Kation Scientific). The electrode was advanced through the cortical layers using a motorised microdrive (Narishige, Japan). The signal from the carbon fibre was amplified differentially (Dagan, Minnesota) with band-pass filtering (300 Hz to 10 kHz). The signal was then digitised using an A/D signal processing board (SGI). Spikes were discriminated and time-stamped by custom software running on a Silicon Graphics computer. Spike waveforms were selected for data collection using a window discriminator and spike times were stored for offline analysis.

Nicotine hydrogen tartrate (0.25 M), sodium chloride (0.25 M, adjusted to pH 3), mecamylamine hydrochloride (0.1 M) and Chicago Sky Blue 6B (2%), all dissolved in water, were applied using multibarrel pipettes (tip diameter 1-2 μm). All drug ejection

currents were below 160 nA and in between ejection periods, 10 nA holding currents were applied to prevent barrel leak. A multi-channel, nanoampere-range iontophoresis pump (Dagan) was used for current delivery.

After a qualitative characterisation of receptive field properties, quantitative measures of orientation, spatial frequency, temporal frequency and area were made using drifting grating stimuli (Xing et al., 2005). Then, a drifting grating - optimised for orientation, spatial and temporal frequency and area - was selected and the contrast response measured in 12 logarithmic steps from 2 to 96% contrast. The contrast response across these same values was then measured accompanied by iontophoretic ejection of nicotine. Constant current ejection began 10 seconds before visual stimulation, and ejection times never exceeded 90 seconds without a period of recovery (minimum 5 minutes). For the antagonist studies, ejection of the antagonist preceded agonist ejection by 60-120 seconds (and thus preceded visual stimulation by 70-130 seconds). A baseline contrast response was recorded before each ejection period and two or three 'recovery' contrast responses were recorded after drug application. This series of recordings was repeated with varying ejection currents up to a limit of 160 nA.

At the end of physiological recording, animals were sacrificed and perfused and blocks of V1 tissue removed and sectioned in the sagittal plane, as described in the 'Immunoelectron microscopy' section above. Laminar reconstruction was done by camera lucida aided by CO histochemistry with Nissl counterstaining to identify layer boundaries.

Parametric analysis:

For each condition (baseline, nicotine at varying ejection currents, recovery and controls), responses (R) were averaged across between 2 and 5 repeats of the

increasing sequence of stimulus contrasts (C). A hyperbolic ratio (Naka-Rushton) function,

$$R = R_{\max} \frac{C^n}{C^n + C_{50}^n} + sFR$$

was fit to these data and the parameters Rmax (maximum response), c50 (contrast value at half-maximum response) and n (slope) obtained, along with a parameter (sFR) capturing the offset of this equation attributable to spontaneous firing.

For each cell the nicotine ejection current which produced the maximal change in the response was determined by visual inspection of the fitted functions. A parametric bootstrap was then performed on the data for this condition (and the immediately preceding baseline) by sampling from a Gaussian distribution defined by the mean and standard deviation of the response to each contrast (Efron and Tibshirani, 1993). This procedure produces a contrast response function that reflects the statistics of the originally recorded responses; the sampled data were then fit with the Naka-Rushton function. 1000 iterations of this process were performed to obtain estimates of the Naka-Rushton parameter distributions. For each parameter, statistical analyses of the difference in the generated parameter distributions (between the nicotine condition and its immediately preceding baseline) were conducted adopting an α level of .05.

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