

# A method to assess the functional impact of cerebral connections on target populations of neurons

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## Abstract

We describe an innovative and tested approach combining two individually potent techniques to visualize simultaneously the functional impact of multiple projections on target populations of neurons in the brain. The rationale is simple: silence a defined set of efferent projections from one cortical region using cooling deactivation and then measure the impact of the deactivation on activities in multiple target regions using 2-deoxyglucose (2DG). This is a straightforward and sound approach because 2DG uptake by neurons reflects levels of underlying neural activity. All distant modifications evoked by the silencing of the set of efferent projections are examined in anatomical tissue and simultaneously for the multiple target sites to provide a global view of the functional impacts of the set of projections on the targets. With this method, downward adjustments of 2DG uptake levels identify removals of net excitatory signals, whereas upward adjustments identify net removals of suppressive influences. Future possible uses and modifications of the technique, including optical imaging, are discussed. Overall, the technique has the potential to provide fundamental, new measures on cerebral network interactions that both complement and extend current static models of cerebral networks and electrophysiological measures of functional impacts on individual neurons. © 1999 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

Over the past 30 or so years a massive effort has been exerted to elucidate the connectivity of the cerebral cortex and countless other brain and spinal cord systems using anatomical methods. These studies have culminated in a static anatomical description of cerebral and other brain networks, which usually resemble a ladder-like web of multiple parallel forward, lateral and backward sets of connections, and countless examples can be readily found in research papers and text books. These static 'wiring diagrams' have been pivotal in guiding discoveries of sequential modifications in signal processing on successively higher rungs of the ladder. However, little beyond inference can be gained from these descriptions of signal transformations be-

cause the majority of network portrayals are static and firmly rooted in anatomy, and descriptions of functional interactions are neither identified nor specified. The most conspicuous absences from the descriptions are measures of functional impact and sign of influence. Surprisingly, measures of anatomical strength, the fundamental measure against which functional measures can be gauged, are also absent.

Attempts to redress the functional deficiencies are noble yet onerous at best and tedious or impossible at worst. The typical approach adopted by the small band of investigators prepared to take on the challenge to win the data has relied on reversible deactivation (cooling or chemical; Payne et al., 1996a; Hupé et al., 1999; Malpeli, 1999; Villa et al., 1999) and assessments of deactivation-induced modifications on neurons known to be normally in receipt of signals from the deactivated region. This approach has a sound theoretical base, but is limited in scope because target neurons are studied

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one-at-a-time through one or more deactivation cycles comprised of pre- and post-deactivation control data bracketing the test data acquired during the deactivation. In recent years the number of neurons that could be studied has increased by the application of stereotrode, tetrode and spike sorting software and more comprehensive measures on respectable populations of neurons can be acquired (e.g. Payne et al., 1991; Hupé et al., 1998). Even so, the pace of data accumulation is painstakingly slow compared to many other branches of the neuroscientific endeavor, because multiple measures must be taken for each neuron after its properties have been well characterized. Moreover, with chemical or improperly applied cooling methods, post-deactivation recovery is rarely complete (e.g. Maunsell et al., 1990), which can further limit the approach. Even so, important conclusions have been reached. In broad terms anatomically-defined forward projections usually excite neurons, lateral projections may either drive or modulate target neurons, whereas backward projections only modulate existing signals, apparently being incapable of activating target neurons alone (Payne et al., 1996a).

Here we describe an innovative and tested approach combining two individually potent techniques to visual-

ize the functional impact of multiple projections in the brain, and simultaneously, for populations of neurons. The rationale is simple: silence a complete set of efferent projections from one cortical region using cooling deactivation (or some other method) and then measure the impact of the deactivation on activities in multiple target regions using 2-deoxyglucose (2DG). This is a straightforward and sound approach because 2DG uptake by neurons reflects levels of underlying neural activity (e.g. Sokoloff et al., 1977; Sokoloff, 1981a,b). All distant modifications evoked by the silencing of the set of efferent projections are examined in post-mortem anatomical tissue and simultaneously for the multiple target sites to provide a global view of the functional impacts of the set of projections. Importantly, the greatly reduced uptake of 2DG at the primary, deactivated site provides an accurate measure of the extent of silenced tissue (Figs. 1 and 2). With this method, downward adjustments of 2DG uptake levels identify removals of net excitatory signals, whereas upward adjustments identify net removals of suppressive influences.

We tested our approach in cat cerebral cortex with the assistance of Wim Vanduffel and Guy Orban for the 2DG aspect of the work (Vanduffel et al., 1997b).

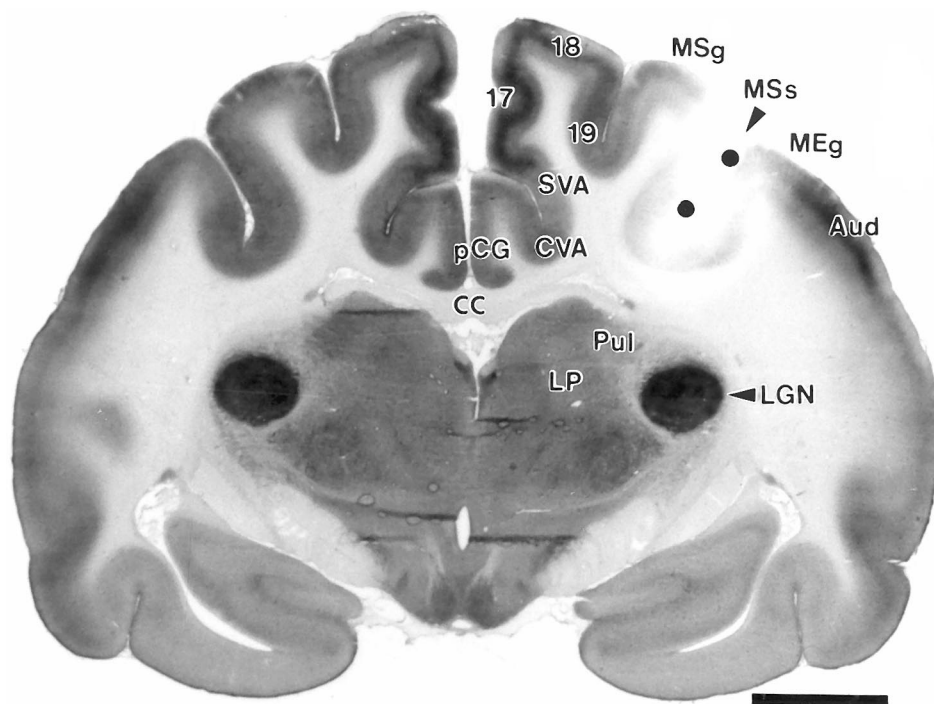


Fig. 1. Coronal section to show diminished 2DG uptake in the vicinity of a cooling loop (black dots) in MS sulcus cooled to 1°C. Note: (1) very low 2DG uptake throughout all or most of the thickness of cortex bounding the MS sulcus; (2) high 2DG uptake in LGN, and middle layers of areas 17 and 18, and auditory cortex (Aud); and (3) diminished uptake, compared to homotopic equivalents in the opposite hemisphere in superficial and deep layers of areas 18 and 19; through the cortical thickness of fundal area 19; areas SVA, CVA and pCG; and within the lateral-posterior (LP) and pulvinar (Pul) nuclei in thalamus. Tissue was derived from study described by Vanduffel et al. (1997b). Abbreviations: 17, 18 and 19, areas 17, 18 and 19; CC, corpus callosum; CVA, cingulate visual area; LGN, lateral geniculate nucleus; MEg, middle ectosylvian gyrus; MSg, middle suprasylvian gyrus; MSs, middle suprasylvian sulcus; pCG, posterior cingulate gyrus; SVA, splenial visual area. Scale bar = 5 mm.

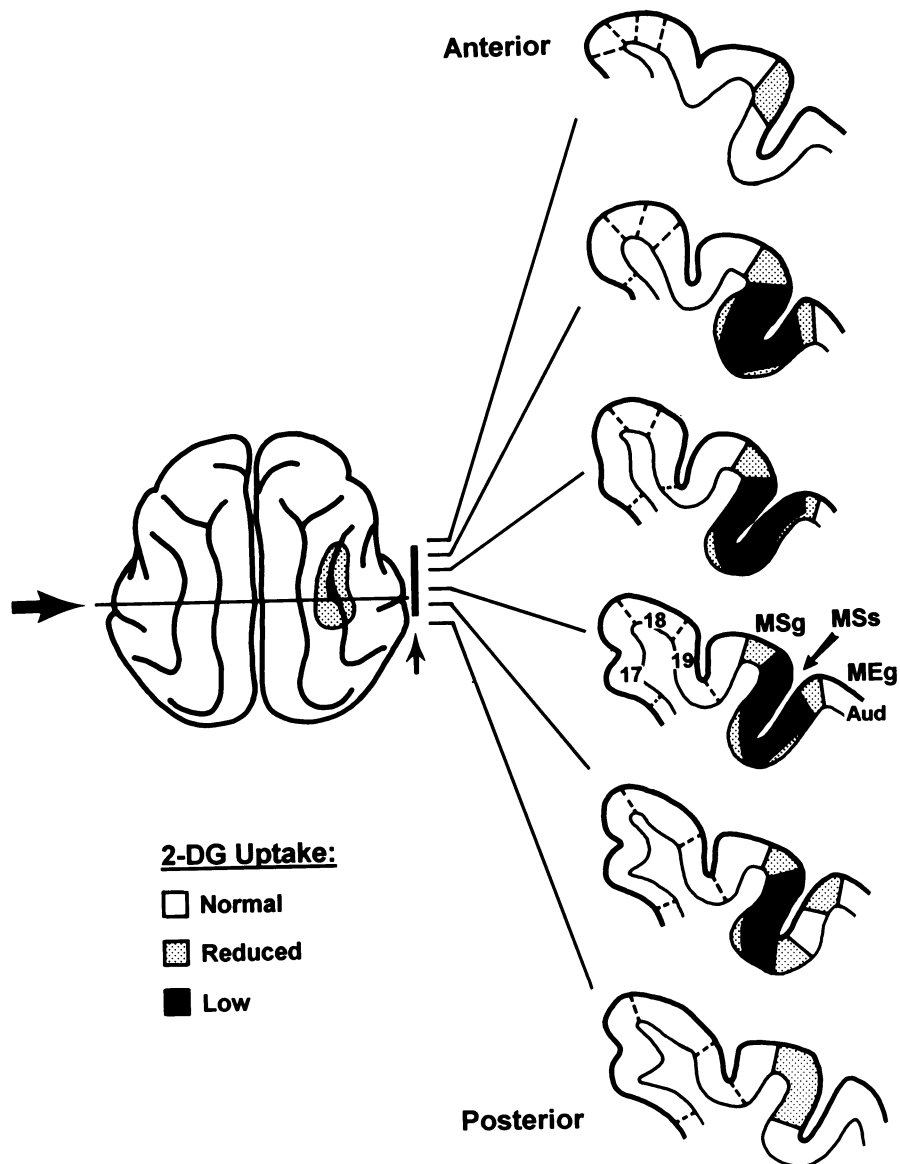


Fig. 2. Reconstruction from a series of coronal sections showing cooling-induced diminution of 2DG uptake. The deactivated zone extends from coronal level A3–A13 (small arrow and line). Large arrow indicates position of the section shown in Fig. 1. The containment of cooling-induced effects to the banks of the MS sulcus shows that the continuous and prodigious supply of warm blood to brain counteracts and contains the spread of cooling. The mediolateral asymmetry in the spread of cooling reflects the medial to lateral flow of blood in the terminal branches of the middle cerebral artery. The cooling spreads further medially because cooled blood flows away from the cooling probe and exports the cooling a short distance, whereas on the lateral side blood flows towards the probe and tends to counteract the spread of cooling. For abbreviations see legend to Fig. 1.

Our initial study shows that the approach is pragmatic. Moreover, when compared to data gathered on the anatomical strength of connections, important inferences on the structure-function relationships of brain pathways can be conceived. For example, in broad terms, anatomically-defined forward projections have the greatest functional impact and exceed those predicted from the magnitude of anatomical projections; lateral projections have a lesser, but usually significant functional influence that is more closely matched to anatomical strength; and backward projections have

functional influences that are markedly weaker than predicted from the scale of the projection system. Gratifyingly, these observations are largely in accord with hard won electrophysiological data on single neurons summarized above.

The electrophysiological influences cited are of considerable significance because they validate the use of the 2DG/cooling approach on neuron populations, and show that the method is a logical, global and economical method to probe interactions between neuron populations. This feature has been noticeably absent from

studies carried out so far, and in this regard the cooling/2DG approach can be viewed as a new analytical tool to probe neural circuits. Moreover, when data are compared to anatomical connectional strength, valuable inferences on structure/function linkages can be extracted for projection systems that were heretofore unavailable. We anticipate that measures of interactions between populations of neurons will free networks from the static anatomy and permit important conceptual forward steps to be taken that lead to the emergence of functionally-based and functionally dynamic models of networks.

## 2. Method

In this section we provide a summary of the cooling method, a description of the 2DG metabolic and the amino acid-pathway tracing techniques and, finally, the strategies to analyze and interpret acquired data.

### 2.1. Cooling loops

#### 2.1.1. Assembly and implantation

The area of cortex to be cooled is selected and several cryoloops are prepared to conform to different gyral shapes in a collection of fixed and rubber-replica brains. Each cryoloop is fashioned out of 23G hypodermic tubing and has a copper/constantin microthermistor attached at the union of the inlet and outlet tubes (Lomber et al., 1999). The limbs of the completed loops are separated by 2–4 mm and are designed to fit snugly, after final adjustment, in contact with the selected area of cortex. The loops are positioned in sulci or in contact with the cortical surface and secured to the skull with screws and acrylic. Small arrays of four or eight microthermocouples (Omega Engineering, Stamford, CT) can also be implanted into cortex near and far from the cooling loop to verify reproducible cooling near to the loops and absence of significant cooling at more distant positions (e.g. Payne et al., 1996a; Vanduffel et al., 1997b). The dura and bone pieces are replaced. With care, neither surgical nor repeated coolings alter cortical structure or function (e.g. Payne et al., 1996a,b; Lomber et al., 1999).

#### 2.1.2. Operation

Cooling of cortex is effected by pumping chilled methanol through the cryoloop tubing (Horel, 1991). Cryoloop temperature is monitored and accurately governed to within 1°C of the desired value and can be maintained for the duration of a cooling by controlling the temperature and rate of methanol flow.

### 2.2. Administration of 2DG and functional impact numbers (FINs)

Functional impact numbers (FINs) of the effects of deactivating a set of efferent connections are obtained by administering 2DG. One of two strategies is followed.

One strategy, and one we have used, is to administer a single tracer ( $^{14}\text{C}$ -2DG) and compare uptake at homologous sites in the two hemispheres, using the non-cooled hemisphere as a standard against which the cooling effects are measured. We have used this strategy in our initial study (Vanduffel et al., 1997b). However, it has the drawback that the cooling of an area in one hemisphere may modify, via commissural connections, 2DG uptake at reference loci in the opposite hemisphere. In this event, interhemispheric comparisons may lead to underestimates of functional impact numbers (FINs). This drawback can be overcome by disconnecting the two hemispheres by prior section of crossing fibers in the callosal and anterior commissures to create non-cooling-modified standard areas in the hemisphere opposite to the cooling. However, this surgical procedure in itself may modify cerebral activity, but this modification is equal in both hemispheres. Given these factors, the design and objectives of the experiment require careful forethought. One significant advantage of the single label approach is the absence of need to calculate absolute amounts of 2DG uptake.

A second, and experimentally sounder, strategy is to apply a technique using two differently tagged 2DG molecules to assess activity before and during cooling deactivation. The two label-signals are derived from  $^3\text{H}$  and  $^{14}\text{C}$  isotopes of 2DG. This double labeling strategy has worked well in with differential activation paradigms (e.g. Friedman et al., 1987, 1989; Geesamen et al., 1997), but is untested in conjunction with the cooling technique. Even so, it has substantial potential for large gains in experimental data.

According to Geesamen et al. (1997) unambiguous signals between the two isotopes are obtained when the concentration of  $^3\text{H}$  exceeds that of the  $^{14}\text{C}$  by a factor of 800:1. The high bias towards  $^3\text{H}$  is necessary to counteract the high energy of the  $^{14}\text{C}$  emission. Based on these ideal proportions and the need to inject 17  $\mu\text{Ci}$  of  $^{14}\text{C}$ -2DG per kg to obtain adequate  $^{14}\text{C}$  signals, 13.6 mCi of  $^3\text{H}$ -2DG must be injected per kg in each animal (Geesamen et al., 1997). While scientifically ideal, the approach has generally poor economic value because the cost of the two 2DG labels for one 3 kg animal can exceed \$12 000. Moreover, significant protective measures need to be taken in handling of large quantities of  $^3\text{H}$ , and there is considerable additional expense disposing of highly radioactive perfusate fluids and carcass because only about 5% of the 2DG remains in brain. Even so, for some studies the potential gain in knowl-

edge by adopting this strategy is worth the economic cost.

### 2.3. Detailed methods of 2DG application, and preparation of tissue and radiograms

We first describe the single tracer 2DG technique in some detail, and second the aspects of the double label 2DG technique that either differ from the single label methods or are specific to the double label technique. Precaution in handling radioisotopes and radioactive tissues and fluids are exercised at all times. Clean-up is scrupulous and checks for radioactive contamination are carried out at the conclusion of all procedures.

#### 2.3.1. Single tracer ( $^{14}\text{C}$ )

We adopt the procedures summarized in Vanduffel et al. (1997a). Prior to any administration of 2DG the animal is accustomed to a holding apparatus. This is a bag or box with baffles for cats or a primate chair for monkeys. The animal is accustomed in several sessions to the cooling of the cryoloop (s) for periods up to 1 h. In addition, one or several days prior to administration a venous catheter is inserted into the cat's cephalic or saphenous vein (Sherwood Medical, Ireland, 20GA, 5 cm long indwelling catheter) or the monkey's external jugular vein and secured to the cooling probe implant with dental acrylic (bilumen pediatric catheter, Becton-Dickinson # 388292). The catheter is periodically flushed with heparin (100 U/ml) to ensure patency, and it is used for the application of 2DG and anesthetics.

For vascular flushes and tissue fixatives we use 15% sucrose in 0.1 M phosphate buffer, pH 7.4 for the flush and 2% paraformaldehyde in the same sucrose/phosphate solution for the fixative. The countertops in the room for the application of 2DG and the necropsy hood are covered with plastic-backed disposable absorbent paper in case of spillages.

Animals are fully conscious during administration of 2DG to maximize uptake (e.g. Löwel et al., 1987; Vanduffel et al., 1995). Their bodies are comfortably restrained in a box with form fitting baffles or in a primate chair, and the inlet and outlet tubes of the cryoloop are connected to the cooling circuit. Chilled methanol is circulated through the cryoloop and the desired temperature reached. After a period of 5 min for complete temperature stabilization, the first of four boluses of 100  $\mu\text{Ci kg}^{-1}$  of 2-deoxy-D-[U- $^{14}\text{C}$ ]glucose (Amersham, specific activity 55 mCi  $\text{mmol}^{-1}$ ) is administered. The remaining boluses are injected at 5 min intervals, after which a further 10 min is allowed for uptake of the final 2DG dose. Heparin (2500 units) is injected to prevent the blood from clotting, 2 ml of a 1% sodium nitrite is injected to dilate arteries, and 35–50 mg  $\text{kg}^{-1}$  sodium pentobarbital administered as an overdose. Once the animal is unconscious it is

transferred to a nearby necropsy area and the vascular system is perfused for 1 min with the sucrose solution to flush out the blood, and then with the paraformaldehyde solution for 5 min to fix the brain. The rate of pump flow is 400 ml  $\text{min}^{-1}$ . The brain is exposed as quickly as possible (2–4 min), blocked, removed from the skull, and photographed. The blocks are coated with egg albumin, and lowered into in  $-35^\circ\text{C}$  methylbutane contained in a bath of dry ice and methanol. As the brain is immersed the methylbutane temperature rises to the desired temperature of  $-30^\circ\text{C}$ . Thirty minutes later the brain blocks are transferred without thawing prior to a  $-80^\circ\text{C}$  freezer for subsequent tissue processing.

#### 2.3.2. Double tracer ( $^3\text{H}$ and $^{14}\text{C}$ )

Largely identical procedures to those described above for the single tracer are followed with the following modifications. The  $^3\text{H}$ -2DG (Amersham, specific activity: 90 Ci/mmol; in 1 mCi/ml water) is injected as a 13.6 mCi  $\text{kg}^{-1}$  bolus through the catheter over a three minute period (Geesamen et al., 1997) prior to any cooling to provide baseline measures. Thirty minutes later, cooling of the cryoloop is initiated and it is cooled to the desired temperature. After temperature stabilization ( $\sim 5$  min) a bolus of the second 2DG (17  $\mu\text{Ci kg}^{-1}$   $^{14}\text{C}$ -2DG; Amersham, specific activity: 230–330 mCi/mmol) is injected (Geesamen et al., 1997) to provide experimental measures.

### 2.4. Administration of $^3\text{H}$ -amino acids

Measures of anatomical connective strength (ACS) are very useful for interpreting the meaning of functional impact numbers (FINs), and the procedures to collect these measures are summarized below. The most useful method to acquire data on ACSs is by injection of  $^3\text{H}$ -amino-acids into specific cortical regions. Typically, 100  $\mu\text{Ci}$  of a mixture of  $^3\text{H}$ -proline and  $^3\text{H}$ -leucine (Amersham, Life Sciences, Arlington Heights, IL.) is injected at multiple sites to approximate the region of cortex to be deactivated by cooling. To maximize data, tracers are injected into two regions, one in each hemisphere of commissurotomed animals. With this strategy two uncontaminated hemispheres are obtained from each animal, at the expense of data on commissural projections. One week later, brains are fixed with 4% paraformaldehyde in 0.1 M phosphate buffer.

#### 2.5. Preparation of tissue sections

The brain blocks are mounted onto a cold cryostat chuck using a frozen-tissue embedding medium (Histo-prep<sup>TM</sup>, Fisher Scientific, Fairlawn, NJ, # SH75-125D) and stored in the  $-80^\circ\text{C}$  freezer. The cryostat is stabilized at a temperature of  $-23^\circ\text{C}$  for 45 min. The brain

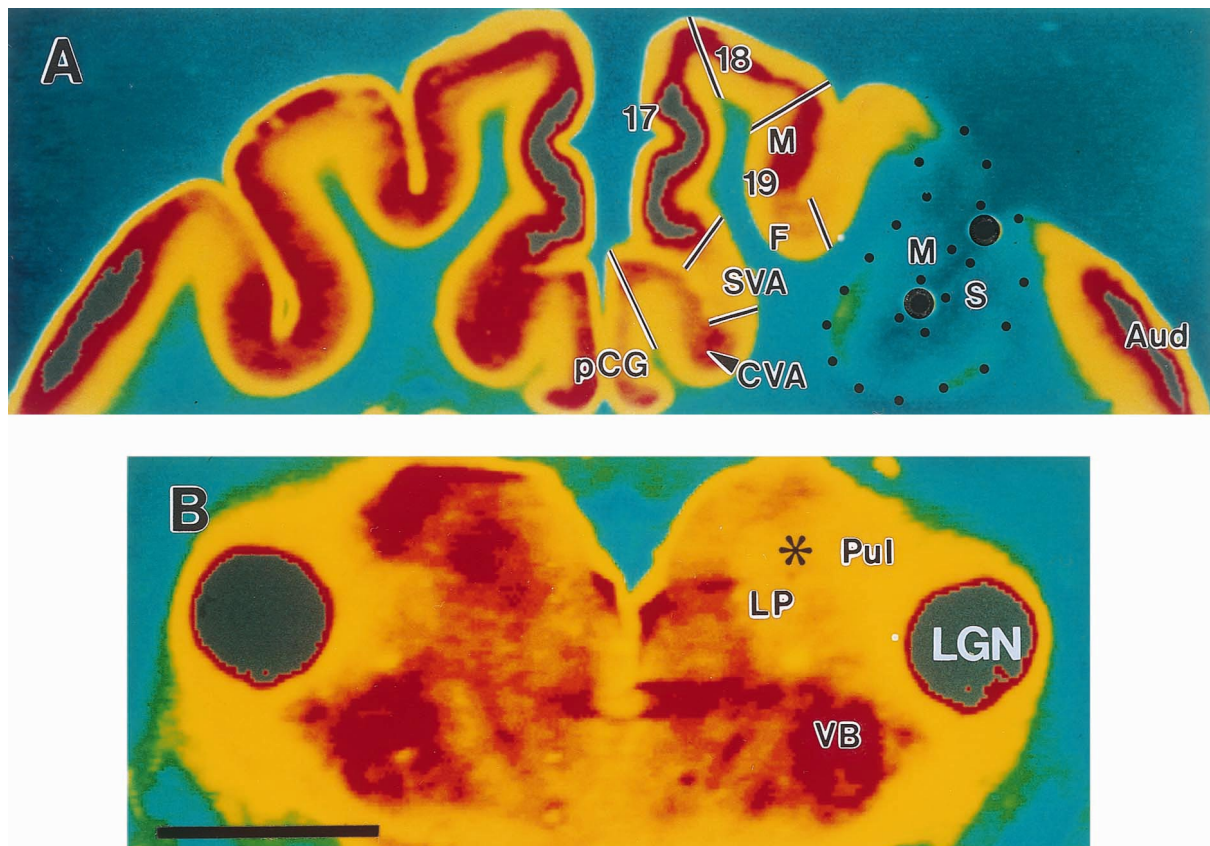


Fig. 3. Color-coded image of 2DG concentration in coronal sections of cerebral cortex (A) and dorsal thalamus (B) during cooling deactivation of cortex lining the middle suprasylvian (MS) sulcus. 2DG densities are color coded along a linear scale ranging from blue (zero to negligible 2DG uptake) green low uptake, to yellow (modest uptake) to red (high uptake) to black (very high uptake). In (A) large dots in MS sulcus represent position of the cooling loop, and small dots identify white matter interface of the MS sulcal cortex. Note very little uptake of 2DG on the banks of MS sulcus and note diminished uptake, compared to homologous equivalents in the opposite hemisphere in superficial and deep layers of areas 18 and 19; through the cortical thickness of fundal area 19; and within areas SVA, CVA and pCG. 2DG uptake at other sites such as in LGN, layer 4 in areas 17 and 18 and auditory cortex (Aud) are equal in both hemispheres. In (B) note diminished 2DG uptake in the right pulvinar and lateral posterior nuclei compared to high levels in equivalent region on left. The lowered 2DG uptake in these two nuclei is separated by an obliquely oriented band of higher 2DG uptake (asterisk). No significant asymmetries in uptake in LGN or ventrobasal nucleus (VB) are evident. Scale bar represents 4 mm. Abbreviations: see legend to Fig. 1.

is transferred from the  $-80^{\circ}\text{C}$  freezer to the cryostat for 45 min to acclimate to the new temperature.

Frozen sections are cut at a thickness of  $26\ \mu\text{m}$ , and every fifth section is collected on coverslips previously coated with 0.5% chrome alum/gelatin mixture, and placed on a  $50^{\circ}\text{C}$  warming plate to first thaw and then rapidly evaporate any water. The coverslips, with sections outermost, are attached with rubber cement in a  $5 \times 5$  grid on a  $28 \times 23\ \text{cm}^2$  white card. Completed cards are stored in the  $-80^{\circ}\text{C}$  freezer. Caution is exercised at all steps to avoid moisture condensing on the brain or sections, because condensate permits the movement of 2DG and introduces artifacts into the radiograms. Additional sections for routine histological procedures are collected as necessary. The latter sections are decontaminated under running water, and they are stained, dehydrated, cleared and the coverslip-with-section attached to a slide with DePeX (Gurr). Sections stained for cytochrome oxidase (Payne and

Lomber, 1996) are particularly useful for verifying the structural and functional integrity of cortex in contact with the cryoloop (Lomber et al., 1999).

## 2.6. Autoradiograms

### 2.6.1. Film exposure

The white-board cards with sections are transferred sequentially from the  $-80^{\circ}\text{C}$  freezer through  $-40^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$  freezers,  $4^{\circ}\text{C}$  refrigerator to room temperature. This graded sequence prevents water condensing on the cassettes and films. Under red-light illumination ( $24 \times 30\ \text{cm}^2$  X-ray film cassettes (Amersham Life Science Inc., Hypercassette, # RPN 1643) are opened, and white board cards placed in the cassettes with sections upper most. For single-label 2DG Amersham  $^{14}\text{C}$  microscale standards (# RPA504L) are placed on one or more coverslips, and the Agfa-Gevaert Structurix ( $24 \times 30\ \text{cm}^2$  film is applied. For double-label 2DG the

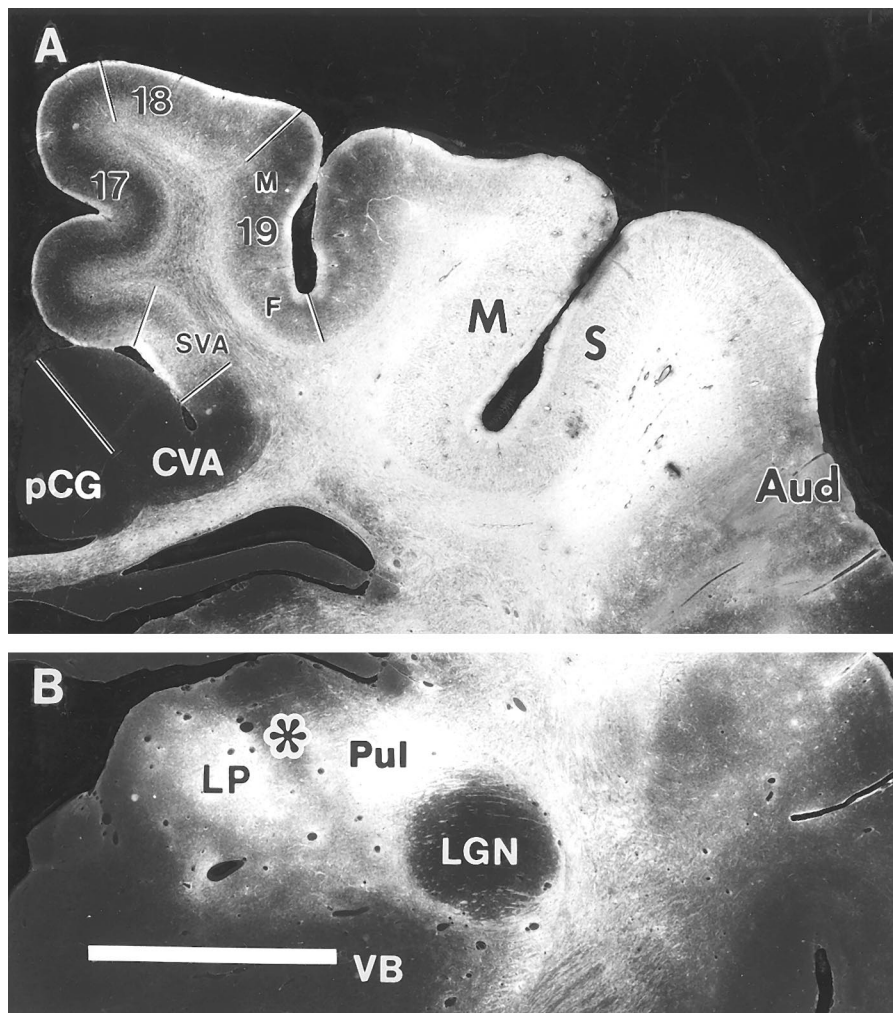


Fig. 4. Distribution of a  $^3\text{H}$ -proline and  $^3\text{H}$ -leucine mixture at the injection site in cortex lining the middle suprasylvian (MS) sulcus and after transport to cortical (A) and thalamic (B) targets. Darkfield illumination. In A, note uniform and high density of label within the uptake region of MS cortex in region extending from A3 to A13. Also note high density of label within superficial and deep layers of area 18, within medial area 19, and across the full thickness of fundal area 19 and splenial visual area (SVA), and very high density on layer 1 of the visual areas. Density of label in upper and lower layers of area 17 is lower. Label is slight in CVA and pCG, and is not visible in a low power darkfield photomicrograph. In B, note very high density label in pulvinar and lateral posterior nuclei. The labeled region is separated by an oblique band of lower density label (asterisk). Scale bar = 4 mm. Figure reproduced with permission from Vanduffel et al. (1997b).

sections are apposed, with Amersham  $^3\text{H}$  (# RPA507) and  $^{14}\text{C}$  microscales, to tritium Hyperfilm and to Polaroid 891 film. For pathway tracers, Amersham  $^3\text{H}$  microscales are applied to the coverslips. These procedures are repeated until the cassette is full or all cards are loaded. The cassette is locked closed, wrapped in two layers of aluminum foil and photographic tape, labeled and placed in the  $-80^\circ\text{C}$  freezer for storage and exposure of films. In a companion series of cassettes sections from several brains are prepared on single cards. These sections are the 'test sections' for evaluating the progress of the exposures. Films exposed to  $^3\text{H}$  are developed after 25–50 days and films exposed to  $^{14}\text{C}$  are developed after a period of 3–5 months. These periods produce strong labeling and clear differentiation between weakly and strongly labeled struc-

tures. The actual length of the exposure period is based upon test films developed and assessed during the temporal window.

#### 2.6.2. Film developing

The cassettes are transferred sequentially through increasing temperatures and permitted to adjust to room temperature for 1 h. Kodak<sup>®</sup> D-19B developer (15 min), Kodak<sup>®</sup> stop bath (2 min), water wash (1 min) and Kodak<sup>®</sup> Rapid fixer (8 min) are used at regular strength and the developer is maintained at  $5^\circ\text{C}$  by placing the developer tray in a larger tray containing crushed ice. Developed films are developed one at a time. Films are rinsed thoroughly for 10 min in running water, hung to dry, and placed in labeled plastic protective covers.

## 2.7. Identification of cortical areas and subcortical structures

Cortical areas are identified using standard criteria, and when necessary, regional unbiased quantitative analyses from computer based images are used. Identification can be based, with comparisons among nearby regions, upon cyto-, myelo-, and cytochrome oxidase-architecture; and on the supragranular: infragranular ratios of SMI32 immunoreactive neurons (Hof and Morrison, 1995). The boundaries of subcortical structures can be determined in sections stained for the same markers or reacted for the presence of acetylcholinesterase.

## 2.8. Data analysis: FINs and ACSs

### 2.8.1. Single label $^3\text{H}$ and $^{14}\text{C}$ images

Optical densities of  $^3\text{H}$  and  $^{14}\text{C}$  radiograms are measured using a modern computer-based imaging system (e.g. Imaging Research Inc. MCID™ or AIS™ systems) and optical densities are represented as colors with black representing highest 2DG density and blue the lowest (Fig. 3). Individual pixels cover an area of  $20 \times 20 \mu\text{m}^2$  but can be grouped into larger areas if some form of averaging is required. The optical density of both the background and a blank, processed film from the same batch is stored for subsequent subtraction. This step compensates for any potential inhomogeneities of illumination camera sensitivity, and background exposure of films. We have used sample areas are  $200 \times 200 \mu\text{m}^2$  and separated by  $\sim 150 \mu\text{m}$ , and extending through a structure but ignoring boundary regions.

Areal, laminar and nuclear boundaries derived from histologically processed sections (above) are applied to the image, and at least 100 measurements are made independently in the upper, middle and lower thirds of the cortical thickness (cerebral cortex) or zones (thalamus). Mean uptake (2DG) and mean transport (amino acid) values are determined. Reference measures are taken in pulvinar, which receives dense projections from all visual areas studied, and contralateral auditory cortex, which receives no fibers from any area studied. Pulvinar is used to define a maximum point and auditory cortex is used to define a baseline optical density (zero-point). These regions can be varied as appropriate if non-visual regions of cortex are deactivated. Measures of FINs and ACSs are calculated for each region against values for the homologous region in the contralateral hemisphere. Differences are tested for significance ( $P < 0.05$ ) with Student's  $t$ -test, and comparisons among animals or areas/nuclei within the same hemisphere are made using analysis of variance (Spjotvoll and Stolline honest significance test; Vanduffel et al., 1995).

### 2.8.2. Double label $^3\text{H}$ and $^{14}\text{C}$ images

The digitized pairs of  $^3\text{H}$  and  $^{14}\text{C}$  images, after subtraction of background, are aligned using an iterative algorithm which matches cross-correlations between selected contours in pairs of images generated from the same histological section. Both images are converted to radioactivity levels via piecewise linear interpolation based on  $^3\text{H}$  and  $^{14}\text{C}$  standards coexposed with each film (Friedman et al., 1987; Gonzalez-Lima, 1992). Finally, the small ( $< 5\%$ ) contaminating  $^{14}\text{C}$  label on the Hyperfilm image is subtracted, pixel by pixel, from the  $^3\text{H}/^{14}\text{C}$  composite Hyperfilm image (Friedman et al., 1987; Redies et al., 1990). This procedure reveals a quantitatively correct image of  $^3\text{H}$ -2DG uptake. The reverse of this procedure does not need to be carried out on the Polaroid film because (1) virtually all  $^3\text{H}$  is trapped by phosphorylation within cells prior to administration of  $^{14}\text{C}$  (Redies et al., 1990); and (2) the Polaroid film is insensitive to  $^3\text{H}$  signals. Thus, three types of images are obtained for each tissue section: (1) a direct visualization of  $^{14}\text{C}$ -2DG uptake; (2) a direct visualization of  $^3\text{H}/^{14}\text{C}$ -2DG uptake; and (3) a computed veridical image of  $^3\text{H}$ -2DG uptake. The  $^{14}\text{C}$  and veridical  $^3\text{H}$ -2DG images are then equalized by linear interpolation using 2DG uptake in several control regions (any region connected to system under study and white matter). Finally, for ease of viewing 2DG densities, pixels are converted to spectral colors with blue representing lowest 2DG uptake, through green, yellow, orange, red and black representing the highest 2DG uptake concentrations ( $\text{nCi g}^{-1}$ ).

The veridical  $^3\text{H}$ -2DG image is subtracted from the  $^{14}\text{C}$ -2DG to identify regions in the  $^{14}\text{C}$  image that differ from those in the veridical  $^3\text{H}$  image and to generate the FINs. This subtracted image, with black showing the largest FINs and blue the smallest, is then used to guide detailed comparisons of FINs.

## 3. Local and distant effects of cooling on 2DG uptake

In this section we provide examples and interpretations of local and distant cooling induced modulations on 2DG uptake. We tested the cooling/2DG approach in cat cerebral cortex and have shown the approach is pragmatic for assessing the functional impact of connections (Vanduffel et al., 1997b). We have shown for a cooling loop inserted into the middle suprasylvian sulcus that the local impact zone of negligible 2DG uptake is readily identifiable with respect to higher 2DG uptake in both adjacent cortices and in the homologous region of the opposite hemisphere. The depressed 2DG uptake is essentially confined to the two banks of the sulcus (Figs. 1 and 2). However, the spread of the cooling deactivation is slightly greater medially than laterally, and reflects the overall flow of blood from

lateral to medial in the terminal branches of the middle cerebral artery. The deactivated zone extends from coronal level A3 to level A13.

The extent of the lowered 2DG uptake is in accord with both the silencing of neuronal activity and lowering of temperatures below 20°C (Lomber et al., 1999). For a probe cooled to 1°C, the 20°C thermocline forms an envelope with boundaries 1–3 mm distant from the probe. On the basis of  $Q_{10}$  values of biological reactions, the temperatures recorded indicate metabolic activities of  $\ll 1/8$  to  $\ll 1/4$  of activity in brain at normal temperatures. Under the same conditions, cortices on banks of the lateral sulcus (area 19) and lateral ectosylvian gyrus (auditory cortex) were at 35–38°C, which has negligible effects on either  $Q_{10}$  values or neuronal activity (Lomber et al., 1999).

In addition, there are numerous and widespread distant impact zones, which can be identified by carefully comparing 2DG uptake in the cooled hemisphere against 2DG uptake at homologous sites in the contralateral hemisphere (Fig. 1). These regions of reduced uptake include: lateral-posterior and pulvinar nuclei in thalamus, and deep area 18, medial and fundal area 19, splenial visual area (SVA), cingulate visual area (CVA) and posterior cingulate gyrus (pCG), and they are immediately obvious in color-coded images of 2DG densities (Fig. 3). Note absence of cooling-induced reductions in 2DG uptake in LGN and middle layers of areas 17 and 18, the earliest stages of visual processing, and in auditory cortex (Aud) only about 2 mm lateral from the local cooling impact zone.

The distant modulations are the result of neither passive spread nor active export by the arterial system of cold from the cooled MS sulcal cortex. Passive spread is excluded because there is no systematic linkage between distant modulations in 2DG uptake and distance from the cooling probe ( $r = -0.23$ ,  $P = 0.19$ ; Vanduffel et al., 1997b). Active export of cold for considerable distances by the arterial system is an unlikely mechanism to account for the distant modulations in 2DG uptake. We make this suggestion because cooling of the loop in the MS sulcus cools blood in the distal branches of the middle cerebral artery, which does not supply any of the regions showing distant 2DG modulations. All distant structures receive blood via independent arterial systems: areas 18, 19 and SVA receive blood from occipital and occipitoparietal branches of the posterior cerebral artery; CVA and pCG receive blood via the posterior and anterior cerebral arterial anastomoses; and the posterior thalamus, geniculate nuclei receive blood from branches of the posterior cerebral artery. Branches of the posterior cerebral artery also supply the superior colliculus, parahippocampal gyrus and occipitotemporal regions.

In the absence of other mechanisms to account for the distant modulations in 2DG uptake, we conclude

that the distant modulations in 2DG uptake are induced by deactivation of nerve fiber projection systems originating in the deactivated cortex. This conclusion is supported by the close concordance between structures showing modulated 2DG uptake and structures in receipt of direct projections from cooled cortex (Fig. 4). This concordance permits a comparison of functional impact numbers (FINs) and anatomical connective strengths (ACSs) for each of the projections.

### 3.1. Comparisons of FINs and ACSs and interpretation

The concordance between patterns and signs of functional linkages revealed by 2DG (Fig. 3) and pathway terminations identified with  $^3\text{H}$ -amino acids is considerable. However, there is much less concordance for the magnitudes of the FINs and ACSs.

The good concordance between the spatial location of distant diminished 2DG uptake and presence of direct inputs revealed by pathway tracers is exemplified by several regions distant from MS cortex (cf Figs. 1 and 4). The most obvious example is the pair of zones in dorsal thalamus separated by a zone of higher uptake which spatially mirrors almost exactly the pair of regions receiving massive projections from MS cortex and separated by a zone of lower input (Fig. 3B and 4B). This high degree of concordance at the distant site depends critically upon congruence between the deactivated region and the region exposed to tracers.

The sign of the distant 2DG modulation is also in accord with the existence of long-distance excitatory projections between cortical regions (White, 1989). In every instance, the presence of projections is matched by lowered 2DG uptake, which indicates that cooling blocks transmission of excitatory signals. Even so, there is substantial variation in the magnitude of distant cooling-induced effects, and they cannot be predicted in any systematic way from anatomical connective strengths (ACSs;  $r = 0.48$ ,  $P = 0.003$ ; Vanduffel et al., 1997b). Importantly, the decreased 2DG uptake in area 19 cannot be accounted for by direct cooling effects because, as noted earlier, temperature is in the normal range. Thus, all distant decreases in 2DG uptake result from blockade of efferent signals that are normally transmitted from MS cortex, but which are silenced during cooling.

The apparent lack of concordance between the magnitudes of the functional and anatomical measures is more straightforward to comprehend when the data are presented in graphical form, and the functional contribution of each projection system to network operations is known. With reference to a standard line linking concordance between very low FINs and ACSs and very high FINs and ACSs (Fig. 5), regions in receipt of forward projections are usually located above or on the standard line (triangles); regions in receipt of lateral

projections tend to be grouped on or close to the standard line (filled squares), whereas regions in receipt of backward projections are grouped below the standard line (diamonds). Interestingly, subcortical targets in receipt of descending projections either overlap lateral projections or are clustered between lateral and backward projections (stippled squares). Overall, the functional impact of forward projections is greater than predicted from the anatomy, the impact of lateral connections is substantial and is in close accord with anatomical strength, and backward projections are weaker than predicted from connectional strength, with descending projections between the former two.

We might also expect to identify modulations of 2DG uptake in the absence of direct anatomical connections. In this instance, it is likely that modulations are in regions that receive substantial secondary projections from a region in receipt of strong primary projections from the deactivated cortex. In such a system, it is likely that functional coupling in the multisynaptic pathway is high. This pattern of connectivity may contribute to significant 2DG modulations in CVA and pCG regions, which receive only minor direct inputs from MS cortex, but both regions receive additional signals, relayed via the strong primary projections from

MS cortex to SVA cortex (Rosenquist, 1985; Maekawa and Ohtsuka, 1993)

At present, absence of topographic registration has not been identified as a significant factor in analyses carried out because the size and shape of our probes cover a substantial fraction of each cat extrastriate area examined. However, topographic registration may become a significant factor when the technique is applied to larger primary areas or to some extrastriate monkey areas that are larger than their counterparts in the cat and possess well ordered visual field representations. In these instances, visuotopic correspondence between deactivated loci and distant loci selected for 2DG measures should be ensured.

#### 4. Future applications and avenues of investigation

In this section we comment on future applications and future avenues of investigation. It is straightforward to conceive of several additional strategies to gather useful data on the functional impact of connections. In addition to the ‘passive’ viewing in awake preparations with which we have already worked, we will also consider the usefulness and drawbacks of using the ‘active’ viewing preparation, anesthetized

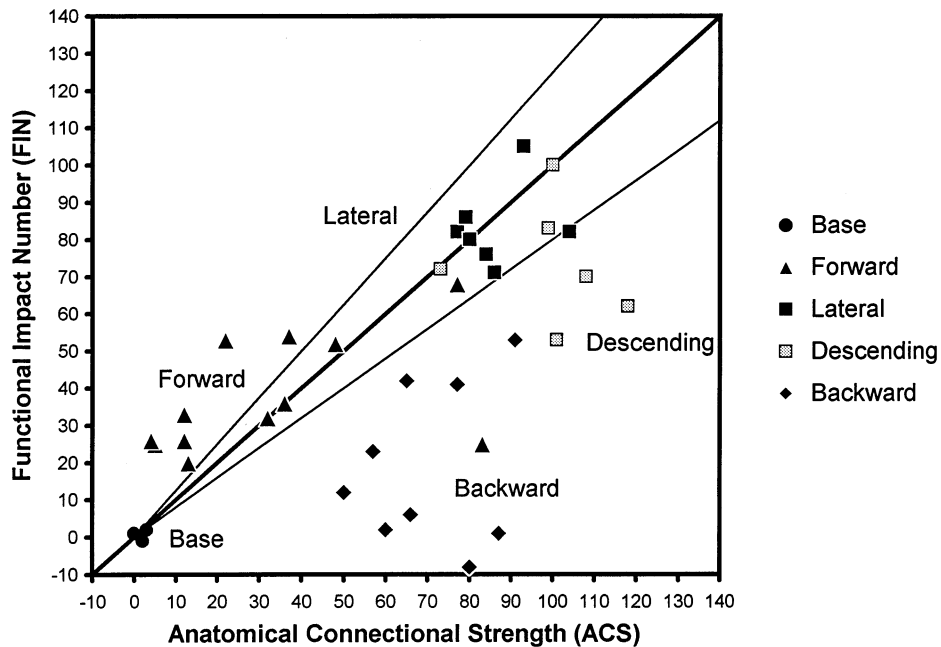


Fig. 5. Graph of anatomical connectional strength (ACS; normalized pathway tracer density) versus functional impact number (FIN; normalized cooling effect on 2DG concentration) for a variety of cortical and subcortical structures. Normalization based on equating tracer density and decrease in 2DG uptake in contralateral auditory cortex to 0% and in the pulvinar nucleus to 100%. Standard, diagonal line represents slope of unity: tracer density and effects on 2DG uptake are equal. Thinner lines on either side represent a deviation of  $\pm 20\%$  from unity. Triangles, forward projections (to CVA, pCG, caudate nucleus, claustrum); filled squares, lateral projections (to area 19, SVA, putamen); stippled squares, descending projections (to lateral posterior nucleus, pulvinar nucleus, superior colliculus); diamonds, backward projections (to area 17, area 18, LGN); circles, baseline data (auditory cortex, no projection from MS cortex and no modulation of 2DG uptake). Measures are taken in upper, middle and deep cortical layers; upper and lateral parts of basal ganglia; layers A, A<sub>1</sub> and C of LGN; and top four layers of superior colliculus. Figure adapted from Vanduffel et al. (1997b).

preparations, and brains with discrete lesions. However, the largest and most comprehensive gains in knowledge are likely to be reached by applying all approaches to produce the broadest and most accurate interpretations of the meaning of functional impacts of cerebral connections.

#### 4.1. Awake preparations

The use of the awake preparation is highly desirable, and likely most advantageous, because 2DG uptake levels are higher and there is greater differentiation between active and less active brain regions (Löwel et al., 1987; Vanduffel et al., 1997a; Tootell et al., 1988a,b). The awake preparation can be used in either a passive condition where the animal is not specifically engaged with its environment or in an active condition where brain and behavior are both actively interacting with surroundings.

*Passive condition:* We have already successfully applied the cooling/2DG technique to cats in the passive viewing condition and shown the usefulness of the technique to probe the functional impacts of cerebral connections (Vanduffel et al., 1997b, 1998). In this condition deactivation of one region in the mid-level of the cerebral hierarchy has repercussions on earlier, similar and higher levels. In broad terms, the relative strengths of the functional impacts reflect the anatomically defined hierarchy of connections. In this instance, inputs from earlier levels of visual processing have a greater influence on LGN and areas 17 and 18 that largely overshadow the backward projections from MS cortex, which have a modest or undetectable impact. At mid-levels of visual processing, the lateral projections from MS cortex to area 19, SVA and the lateral-posterior and pulvinar nuclei have a substantial impact that parallels the strengths of anatomically-defined linkages. At CVA and pCG levels in the hierarchy, which are higher than MS cortex, rather weak direct anatomical projections have significant functional impacts that exceed the magnitude predicted from the anatomical data. These results suggest that there may be substantial differences in the form, number, size and strength of synapses in backward, lateral and forward projection systems. In the passive viewing condition, the FINs obtained may be considered a baseline set of measures against which active viewing measures may be gauged.

As pointed out by Scannell (personal communication) even in the passive viewing condition, there may be unanticipated effects of the deactivation on 2DG uptake which are not mediated via direct or indirect anatomical pathways but via feedback from reduced behavioral interaction with the environment. This may be especially important if the direct effects of the deactivation has significant attentional or mnemonic components.

*Active condition:* With active engagement of the environment the functional state of parts of the brain may deviate significantly from functional levels revealed in the passive mode, as attentional and mnemonic influences originating from higher processing levels modify activity at lower levels of signal processing. With active engagement of surroundings and the induction of ‘top-down’ influences, the functional impacts revealed in cooling/2DG studies on feedback systems may be demonstrably stronger. In this regard the active preparation benefits considerably from the use of two differently tagged 2DG markers to provide ‘active’ experimental and ‘passive’ baseline measures in the same animal with exact registration of anatomical assay sites in the two sets of radiograms. Moreover, comparisons made with two differently tagged 2DG labels made during two active behavioral states have potential to permit linkages of differences between functional impacts to differences in behavioral task. In either case, the gains in knowledge on the dynamic aspects of cerebral networks are likely to be considerable, and outweigh the high economic cost of the two 2DG labels identified earlier.

#### 4.2. Anesthetized preparations

It may also be highly desirable to assess functional impacts of cerebral projections in anesthetized preparations to eliminate all attentional and mnemonic influences and the secondary, behavioral repercussions on the system under investigation. As recognized by Scannell (personal communication) modulations of 2DG uptake at sites distant from the cooling deactivation could be more easily attributable to effects mediated by direct and indirect anatomical pathways. Moreover, there could be greater control over the exposure to sensory stimulation of the animal. However, these potential benefits are mitigated by lower overall levels of 2DG uptake and lesser overall scale of activity levels. These drawbacks may not be a major problem for connections where activity and functional impacts are large, but they may be significant when activity and functional impact levels are low. In the latter, differences may merge and be obscured. Even so, the use of the anesthetized preparation is likely to produce interesting results, and provide valuable measures against which values from the awake preparation can be compared and interpreted.

#### 4.3. Lesions

Lesions also have their place for assessing the functional influence of one cerebral region on other brain regions. However, their value in the direct sense is limited. In the initial post-lesion period distant orthograde and retrograde degenerations are active and they

compromise interpretation of data on functional impacts of cerebral connections. In the long-term, the degenerations reach completion and result in modified 2DG uptake. Of greater biological interest are the functional compensations that emerge in cerebral circuits to ameliorate lesion-induced deficits. Functional compensations are increasingly attributed to modifications in occipital, parietal and temporal circuits (Yamasaki and Wurtz, 1991; Milner and Goodale, 1996; Payne et al., 1996a, 1997), and they are likely to be features of all parts of the cerebral mantle. While these compensations may obscure the identification of accurate functional impact numbers, measures of functional strengths of compensations can be obtained by comparing cooling and 2DG measures in intact animals and animals with lesions. Such comparisons are likely to provide valuable information on functional plasticity within the cerebral network and on sites of lesion-induced compensations. A similar strategy has been adopted in humans and animals to assess the repercussions of lesions (Soh et al., 1978; Kuhl et al., 1980; Martin and Raichle, 1983; Deuel and Collins, 1984; Kosslyn et al., 1993).

#### 4.4. *Optical imaging*

One final important direction to mention is the substitution of the optical imaging method (Blasdel and Salama, 1986; Bonhoeffer and Grinvald, 1996) for 2DG to assess the repercussions of cooling deactivation on distant neural activity. The optical imaging method has the potential to make maximum use of the complete reversibility of the cooling technique and for differences between active and deactivated conditions to be assessed in real-time without the imposed lags necessary for histological and radiographic processing of 2DG containing tissue. Moreover, it permits multiple stimulus paradigms to be implemented and the multiple measures of neural activity to be made with the network circuitry functioning normally and in the absence of one or more distant sources of inputs. The combination of these two techniques has substantial potential to reveal many transcortically mediated network interactions which were heretofore unattainable at the population level of analysis.

#### 4.5. *Electrophysiology*

The combined cooling/2DG approach also shows the value of carrying out a preliminary study that combines a deactivation technique and modified 2DG to identify neuron populations under a substantial influence by distant neuron populations. The data collected can then be used to focus single-neuron

electrophysiological studies employing reversible deactivation technique on specific neuron populations. The strategy reduces the needle-in-a-haystack problem for the single-neuron electrophysiologist examining neuronal interactions to a more tractable level, and likely increase efficiency and yield of neurons showing significant effects. Such investigations, when coupled with cooling to investigate behaviors, are likely to advance our understanding of neural networks considerably and link them to perceptual and cognitive aspects of brain functions.

### 5. **Epilogue**

Measures of anatomical connectional strengths (ACSSs) between network nodes and functional impact numbers (FINs) of the influence of each node on others in the network are essential prerequisites for the formulation of biologically realistic models of perceptual, cognitive and sensorimotor networks. While the extant nominal data on patterns of connectivity are comprehensive, measures of both anatomical strength and functional impact of connections are virtually non-existent. We have shown the practicality of acquiring essential data on anatomical strength and functional impacts.

The development of biologically realistic neural network models describing the structural and functional organization of cerebral systems is an essential endeavor for improving knowledge of cerebral network operations. So far, only a limited number of attempts at systems-level modeling have been carried out (e.g. Goldman-Rakic, 1988; Gonzalez-Lima, 1992; McIntosh and Gonzalez-Lima, 1992; Young, 1992, 1993; McIntosh et al., 1994; Young et al., 1994, 1995; Scannell et al., 1995, 1999; Sommer and Kötter, 1997; Tagamets and Horwitz, 1997; Vanduffel et al., 1997a; Jouvé et al., 1998), and future models will likely benefit considerably by incorporating measures of functional impacts of cerebral connections. These models will likely cut through the jungle of large and small anatomically identified transcortical and subcortical pathways to reveal either dominant or tuned functional connections. Identification of these connections is important because they will greatly simplify the complex neural webs currently being generated by anatomists and modelers. Moreover, they will elevate current models of static cerebral networks to a functional description that recognizes network component functions and interactions. The importance of these models lies in the conceptual framework they provide for integrating and comprehending experimental results, and for identifying new avenues of investigation.

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