Selective Neurofilament (SMI-32, FNP-7 and N200) Expression in Subpopulations of Layer V Pyramidal Neurons In Vivo and In Vitro

There are two main types of layer V pyramidal neurons in rat cortex. Type I neurons have tufted apical dendrites extending into layer I, produce bursts of action potentials and project to subcortical targets (spinal cord, superior colliculus and pontine nuclei). Type II neurons have apical dendrites, which arborize in layers II–IV, do not produce bursts of action potentials and project to ipsilateral and contralateral cortex. The specific expression of different genes and proteins in these two distinct layer V neurons is unknown. To distinguish between distinct subpopulations, fluorescent microspheres were injected into subcortical targets (labeling type I neurons) or primary somatosensory cortex (labeling type II neurons) of adult rats. After transport, cortical sections were processed for immunohistochemistry using various antibodies. This study demonstrated that antigens recognized by SMI-32, N200 and FNP-7 antibodies were only expressed in subcortical (type I) — but not in contralateral (type II) — projecting neurons. NR1, NR2a/b, PLCβ1, BDNF, NGF and TrkB antigens were highly expressed in all neuronal subpopulations examined. Organotypic culture experiments demonstrated that the development of neurofilament expression and laminar specificity does not depend on the presence of the subcortical targets. This study suggests specific markers for the subcortical projecting layer V neuron subpopulations.

Keywords: cell differentiation, corpus callosum, intracortical and intercortical connections, spinal cord, superior colliculus

Introduction

The cerebral cortex is comprised of many different neuronal types (Peters and Jones, 1985), which can be classified according to distinct morphology, connectivity, and neurochemical and electrophysiological characteristics; these characteristics can be related to the laminar location of the cell body. Although there is basic structural similarity across the neocortex, specific functions are clearly localized to distinct areas, which are characterized by microcircuity, input and output connectivity, local cytoarchitecture and proportions of cell types (Brodmann, 1909). The subtle variations in cytoarchitecture reflect important differences in the computational role of numerous cortical areas (Kaas, 1993). Although much research has been conducted on brain development, fundamental questions remain about how specific neuronal subpopulations differentiate and form functional circuits.

Layer V pyramidal neurons provide an excellent model for addressing these important questions. In adult rodent cortex, there are two major subpopulations of layer V pyramidal neurons that have distinct projection targets, somatodendritic morphologies and electrophysiological properties (Larkman and Mason, 1990; Koester and O’Leary, 1992; Kasper et al., 1994). Type I subcortical projecting neurons (projecting to the spinal cord, superior colliculus and pontine nuclei) have tufted apical dendrites terminating in layer I and fire bursts of action potentials in response to depolarizing current. In contrast, type II contralateral cortex projecting neurons have, non-tufted apical dendrites, which arborize in layers II–IV and never fire bursts of action potentials (Kasper et al., 1994). Our main question is, are there molecules that characterize the different somatodendritic morphologies of these two neuronal subpopulations?

The aim of this study was to identify proteins that are differentially expressed in layer V pyramidal neuron subpopulations in the adult rat. Several proteins have been reported to be strongly expressed in layer V, including: (i) neurotransmitter receptors — N-methyl-D-aspartate receptor subunit 1 (NR1; Aoki et al., 1994) and NR2a/b (Conti et al., 1999); (ii) neurotrophin receptors — tyrosine kinase receptor type B (TrkB; Tongiorgi et al., 1999; Miller, 2000); (iii) neurotrophins — nerve growth factor (NGF; Miller, 2000) and brain-derived neurotrophic factor (BDNF; Murer et al., 1999); and (iv) neurofilaments — Sternerberger monoclonal incorporated antibody 32 (SMI-32; Hof et al., 1995; Gabernet et al., 1999), medium-sized neurofilament clone (FNP-7; Hornung and Riederer, 1999) and neurofilament 200 (N200; Sasaki and Maruyama, 1994). However, these studies did not investigate the protein expression in layer V pyramidal neuron subpopulations based on axonal projection. Therefore, for most of these proteins, it is not clear if all — or only a subset of pyramidal cells — express these molecules. The medium-sized neurofilament proteins (SMI-32, N200 and FNP-7) were of particular interest because previous studies in primate (monkey and human) have shown that these proteins are expressed in ∼30% of cortical pyramidal neurons (Campbell et al., 1991; Hof and Morrison, 1995; Hof et al., 1996b; Bussiére et al., 2003a). These studies have described the overall regional and laminar distributions of cells containing these neurofilaments, but have only examined corticocortical neurons. This paper further characterizes the neuronal pyramidal populations that express these neurofilament proteins by examining not only the corticocortical — but also the subcortical — projecting neurons in layer V. The only layer V pyramidal cell subpopulation marker identified is the transcription factor Otx1, which is specific for subcortical projecting neurons (Weimann et al., 1999). In this study, layer V pyramidal neurons were divided into subpopulations based on their axonal projection site, which were identified by retrograde labeling. Immunohistochemistry was then used to screen the identified subpopulations using a panel of antibodies.
against proteins expressed in layer V. Three proteins were identified that are selectively expressed in subcortical (superior colliculus and spinal cord projecting type I neurons) – but not contralateral cortex (type II neurons) – projecting layer V pyramidal neurons in adult rats. Their specific and selective expression pattern was maintained in early postnatal cortical slice cultures, suggesting that the development and maintenance of these neurofilament protein expression patterns are not dependent on their target.

Materials and Methods

Animals
Nine young adult wistar rats (120–180 g) were used for this study (n = 5, cortical injections; n = 5, spinal cord injections; n = 5, superior colliculus injections). Embryonic day 16 (E16) rats (n = 11) and postnatal day 4 (P4) rats (n = 12) were used for the organotypic cultures. All experimental protocols were approved by and in accordance with the regulations and guidelines of the University of Oxford (UK), the Home Office (UK) and the University of Lausanne (Switzerland).

Retrograde Tracing with Fluorescent Latex Microspheres
Green fluorescent latex microspheres (Lumafuor, Naples, FL) were used to identify subpopulations of cortical layer V pyramidal cells based on their axonal targets.

Adult rats were anesthetized with 2.7 mg/kg Hypnovel (Roche, Basel, Switzerland), Hypnorm (Janssen, Titusville, NJ) and distilled H2O (1:1:2 volume ratio), which was delivered i.p. and placed in a stereotaxic frame. After the skin was disinfected and incised, a micro-drill was used to perform a craniotomy. Glass micropipettes (Clark Electromedical Instruments, Reading, UK) and a binocular stereomicroscope (Zeiss, Germany) were used to inject 0.3–1.0 µl of microspheres into one of three pyramidal cell targets: primary somatosensory cortex (n = 5; 3.0 mm posterior to bregma, 3.5 mm lateral of sagittal suture, 1 mm deep; Welker et al., 1996), superior colliculus (n = 5; 6.5 mm posterior to bregma, 1.5 mm lateral of sagittal suture, 3.5 mm deep; Paxinos et al., 1985) or spinal cord (n = 5; between thoracic 1 (T1) and (T2)). Each target received two or three injections –100 µm from each other. The micropipette was kept in place for 1–2 min before retraction. During the postoperative period, animals were kept under a heating lamp before returning them to their cages. All animals recovered quickly and resumed normal behavior following the procedure. Animals were allowed to survive for 24–48 h to permit adequate retrograde transport of the microspheres to the pyramidal soma (Fig. 1A). For the organotypic culture experiments, P5 rats were anesthetized by hypothermia and injection into either the somatosensoory cortex (n = 6; 2 mm posterior to bregma, 2–3 mm lateral of sagittal suture, approximately 150–200 µm deep) or the center of the superior colliculus (n = 6; under direct visual guidance since it is not yet covered by the occipital pole of the cortex) were made. The rest of the retrograde-labeling procedure for P5 animals followed the adult protocol above.

Immunohistochemistry
After 1–2 days, animals were anesthetized with sodium pentobarbitone (25 mg/kg) delivered i.p. and perfused transcardially with cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were removed and post-fixed for 6 h to overnight in 4% paraformaldehyde in 0.1 M PB (pH 7.4) at 4°C. Brains were sectioned at 60 µm in the coronal plane using a vibrorlicer (VT1000S; Leica, Heidelberg, Germany) and serial sections through the primary somatosensory and motor cortices were collected. Free-floating sections were washed in 0.05 M tris buffer containing 0.9% NaCl (TBS; pH 7.4) and incubated for 2 h in TBS containing 10% normal goat serum (NGS) and 0.1% Triton X-100 to mask non-specific binding sites. Sections were then incubated overnight in the primary antibody diluted in TBS containing 1% NGS and 0.1% Triton X-100 to mask non-specific binding sites. Sections were then incubated overnight in the primary antibody diluted in TBS containing 1% NGS and 0.1% Triton X-100 (see Table 1 for dilutions). After rinsing in TBS, sections incubated with polyclonal primary antibodies were further incubated in cyanine (Cy3)-conjugated goat anti-rabbit (Jackson Immunoresearch Labora-
tories, West Grove, PA) in TBS with 1% NGS for 2 h (see Table 1 for dilutions). Sections incubated with monoclonal primary antibodies (Table 1) were initially incubated in biotinylated-conjugated goat anti-mouse antibody (Vector Laboratories, Burlingame, CA) in TBS with 1% NGS (all diluted 1:100) for 2 h, rinsed in TBS and incubated in CY3-conjugated to streptavidin (Jackson Immunoresearch Laboratories, West Grove, PA) for 2 h (all diluted 1:500). After a final rinse in TBS, sections were counterstained with bisbenzimide Hoechst trihydro-chloride (2.5 µg/ml PBS; Sigma-Aldrich, St Louis, MO), mounted on gelatin-coated slides, air-dried and cover slipped with PBS. Slides were stored in 4°C and protected from light. The immunohistochemistry controls were negative (data not shown).

Organotypic Cortical Cultures
To determine if targets of layer V pyramidal neurons are responsible for the maintenance and the selective expression of neurofilament antigens, two types of organotypic culture experiments were performed (Fig. 1B,C).

Cortical slices were prepared from the parietal cortex of E16 rats (n = 11) and maintained for 2 weeks in culture conditions according to the methods described in Molnár and Blakemore (1991, 1999; see also Fig. 1D). A tissue chopper was used to cut 350 µm thick cortical slices from E16 rats. The tissue blocks were transferred to a Petri dish containing Hanks balanced salt solution (Sigma) supplemented with glucose to a final concentration of 6.5 mg/ml. Explants were then placed on Transwell-COL culture chamber membranes (pore size 0.4 µm, 25 x 5 mm diameter inserts; Costar, Cambridge, MA). Cultured explants were maintained in N2 medium [1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12, supplemented with insulin (5 µg/ml), transferrin (100 µg/ml), progesterone (20 nM), putrescine (100 mM) and selenium (50 nM, as Na2SeO3)]. These constituents were made up into stock solutions, filter-sterilized (pore size 0.2 µm) and then added to the medium individually. In most cases, we used N2 stock solution (Gibco BRL, Grand Island, NY). The
cultures were maintained in Transwell-COL culture chambers (Costar) under standard culturing conditions with continuous flow of humidified carbogen (5% CO₂ and 95% air, 100% humidity, 40°C) in a modular incubator chamber (Flow Laboratories). After culturing, the slices were fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.5) and the entire slice (without resectioning) was immunostained using SMI-32 and FNP-7 antibodies (for details, see above).

Organotypic culture experiments with cortical slices containing pre-labeled pyramidal cells were used to examine whether the subpopulations of layer V neurons maintain their antigenicity in the absence of the target tissue (Fig. 1C). At P3, after the corticofugal projections have reached the superior colliculus and the contralateral cortex, fluorescent latex microspheres were injected using a Leica DC 500 digital camera (Leica, Bensheim, Germany). The presentation study is based on the examination of 6501 layer V pyramidal neurons. An average of 240 cells per layer V subpopulation was identified relative to the total number of cells counted (fluorescent-labeled only plus double-labeled) was calculated for each antibody using a student's t-test, with P < 0.05 being considered statistically significant.

**Confocal Microscopy**

Confocal scanning confocal microscopy (Leica TCS NT, Germany) validated the quantification conducted under the fluorescence microscope. Excitation was obtained with an argon–krypton laser, with lines set at 488 nm for fluorescein isothiocyanate (FITC) and 568 nm for tetramethyl rhodamine isothiocyanate (TRITC). Between six and eight optical sections (z distance between each: 0.2–0.5 μm) were scanned through a single pyramidal cell. Images were taken in a 1024 × 1024 pixel format using a 100×/1.4 N.A. oil immersion objective. Individual optical sections and the Z-axis reconstructions were examined before images were compiled into a single image. The single images were then processed using Adobe Photoshop 6.0.
Results

Distribution of Labeled Cells from the Fluorescent Microsphere Injections into Different Pyramidal Cell Targets

Spinal Cord Projecting Neurons

Spinal cord injections (Fig. 2A) revealed retrogradely labeled cells in the somatosensory and motor cortices (Fig. 2B,C). Cells were more numerous in the dorso-medial part of the frontal poles. The labeled neurons were limited to layer V (Fig. 2B,D). The soma size of the spinal cord projecting neurons appeared similar to that of the superior colliculus projecting neurons, both of which were larger than those projecting to the contralateral hemisphere (Fig. 2C,G,K).

Superior Colliculus Projecting Neurons

All cells labeled after superior colliculus injections (Fig. 2E) were located exclusively in layer V (Fig. 2F,H). The majority of back-labeled cells were found in the upper portion of layer V. A minority of labeled cells were scattered in the lower portion of layer V as well; however, no sharp boundary between the upper and lower layer V cells was observed. The band of labeled cells extended throughout the primary somatosensory cortex, while more posterior regions (i.e. occipital cortex) contained a slightly greater number of labeled neurons.

Contralateral Cortex Projecting Neurons in the Primary Somatosensory Area

Although it is known that primary cortical areas have fewer callosal connections (Akers and Killackey, 1978), numerous contralateral projecting neurons were found in the primary somatosensory cortex after cortical injections (Fig. 2I). The labeled cells were distributed in all cortex layers, with the exception of layer I (Fig. 2J,L). Layers II, III and V had the greatest number of labeled cells, whereas layer IV contained relatively few labeled cells. The callosal projecting neurons were smaller than the spinal cord or superior colliculus projecting neurons (Fig. 2K).

Immunohistochemical Analysis of Layer V Projection Neuron Subpopulations

Markers Ubiquitously Expressed in all Three Subpopulations Examined

Most neurons in the neocortex were stained with the antibody against the glutamate receptor, NR-1. However, layers II, III, V and VI contained especially large numbers of heavily labeled cells bodies. The apical dendrites, as well as some parts of the terminal tufts, exhibited strong immunoreactivity. NR2a/b was also expressed in all cortical layers, with cell bodies most strongly labeled in layers II–V. Similar to NR-1 staining, NR2a/b also stained the proximal dendrites. The isozyme, phospholipase Cβ₁ (PLCβ₁) was diffusely expressed in layers II, III, IV, V and VI on neuronal apical dendrites. Staining for the neurotrophin BDNF showed expression in cortical layers II, III, V and VI throughout the cortex with labeling of the soma, proximal dendrites and axons of pyramidal cells. NGF was expressed in almost every cell in the cortex with a particularly high density in layers II, III and V. Pyramidal cell bodies and dendrites were most strongly immunolabeled. The BDNF receptor, TrkB was expressed in large numbers of neurons in all cortical layers. Layers V and VI contained the greatest number of immunoreactive cells. Only the initial segment of the layer V neuronal apical dendrites and primary dendrites were labeled. The apical dendrites and the apical tufts were not immunoreactive for TrkB.

Markers Specific for Subcortical Projecting Pyramidal Neurons (SMI-32, FNP-7 and N200)

SMI-32, which reacts with non-phosphorylated epitopes in neurofilament-M (150 kDa) and -H (200 kDa; Lee et al., 1988), was intensely expressed in the cortical white matter. Layers II, III and V cells were strongly stained in the medial somatosensory cortex; however, the immunoreactivity in layers II and III decreased when progressing laterally, while layer V staining remained the same (Fig. 3A,B). SMI-32 was expressed in the soma and the dendrites, but only some thick axons of pyramidal neurons (Fig. 3C,D). FNP-7, which reacts exclusively with non-phosphorylated NF-M, is most prominently expressed in layers III, V and VI (Fig. 3E,F). FNP-7 also stained the somata and apical and basal dendrites, as well as the proximal axon extending into the white matter (Fig. 3G,H). N200, which reacts with both the phosphorylated and non-phosphorylated NF-H, most strongly stained layers III, V and VI (Fig. 3I,J). Like SMI-32 and FNP-7, N200 stained apical and basal dendrites more strongly than the soma, whereas the soma was also stained by N200.
dendrites as well as a few thick axons (Fig. 3K, L). Immunostaining from all three neurofilament markers indicated that the majority of stained fibers that exited the cortex entered the cortical white matter and turned laterally toward the internal capsule (not toward the corpus callosum). Nevertheless, strongly immunoreactive fibers were observed within the corpus callosum itself and layers II–III consistently showed a very small percentage of contralateral cortex projecting cells which contained both green microspheres and stained with one of the three neurofilament antibodies (average ± SEM; SMI-32 = 2.31 ± 0.58; N200 = 8.89 ± 2.27; FNP-7 = 1.74 ± 0.39). Other fiber tracts — including the cerebral peduncle and the optic tract — as well as the thalamic reticular nucleus were also heavily stained. A sizable percentage of corticocortical neurons projecting to ipsilateral cortical areas showed neurofilament reactivity (SMI-32 = 21.8% and FNP-7 = 61%). This finding suggests that a large proportion of the cells that establish intracortical connections also express SMI-32 and FNP-7; therefore, these markers are not restricted to the longer-range intercortical and subcortical projection neurons.

### Quantification of Immunoreactive Layer V Pyramidal Cells Based on their Projection Sites

**Markers Ubiquitously Expressed in all Three Subpopulations Examined**

Layer V pyramidal cells containing fluorescent microspheres were quantified based on their immunoreactivity. Figure 4 shows confocal images of the three cell populations (contralateral cortex, superior colliculus and spinal cord projecting) stained with NR1, NR2a/b, PLCβ1, BDNF, NGF and TrkB antibodies. The panels illustrate that all six antibodies co-localized with the pyramidal cells that contained fluorescent microspheres labeled from the spinal cord, superior colliculus and contralateral cortex. The percentage of double-labeled cells compared to the total number of cells counted (fluorescent-labeled only plus double-labeled) was calculated. Regardless of their projection site, a very high percentage of back-labeled layer V pyramidal cells expressed NR-1, NR2a/b, PLCβ1, BDNF, NGF and TrkB (Table 2). There was no significant difference between the expression of these six proteins in the three pyramidal cell populations.
We performed two experiments to determine the likelihood that the neurofilament immunoreactivity in layer V projection neurons is elicited and maintained by their subcortical targets. Therefore do not project to the contralateral cortex. Scale bar = 20 µm.

Table 2

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Spinal cord</th>
<th>Superior colliculus</th>
<th>Contralateral cortex</th>
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<tbody>
<tr>
<td>NRI</td>
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<tr>
<td>NRI2b</td>
<td>197</td>
<td>198</td>
<td>99.73 ± 0.27</td>
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<tr>
<td>PLC1</td>
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<td>168</td>
<td>98.65 ± 1.53</td>
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<tr>
<td>BDNF</td>
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<td>207</td>
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<tr>
<td>NGF</td>
<td>179</td>
<td>180</td>
<td>99.57 ± 0.42</td>
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<tr>
<td>TrkB</td>
<td>165</td>
<td>165</td>
<td>100.00 ± 0.00</td>
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<tr>
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<td>N200</td>
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<td>275</td>
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<tr>
<td>FNP-7</td>
<td>205</td>
<td>209</td>
<td>98.27 ± 0.46</td>
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Av. %DLN ± SEM

NR1     | 208         | 212                 | 98.16 ± 1.11         |
NR2a/b  | 261         | 266                 | 97.87 ± 0.70         |
PLC1    | 248         | 255                 | 97.37 ± 1.78         |
BDNF    | 354         | 357                 | 99.31 ± 0.38         |
NGF     | 372         | 374                 | 99.64 ± 0.36         |
TrkB    | 248         | 250                 | 98.66 ± 0.60         |
SMI-32  | 285         | 290                 | 98.27 ± 0.01         |
N200    | 190         | 195                 | 98.24 ± 1.00         |
FNP-7   | 244         | 246                 | 97.99 ± 1.01         |

Av. %DLN ± SEM

Contralateral cortex projecting neurons expressed NR1, NR2a/b, PLC1, BDNF, NGF and TrkA. The difference between the three populations of cells was not statistically significant for these antibodies. However, the immunoreactivity for the neurofilament markers (SMI-32, N200 and FNP-7) was selectively expressed in subcortical projecting cells (i.e. projecting to the spinal cord and superior colliculus). Virtually none of the contralateral cortex projecting neurons expressed these three neurofilament markers. The difference between the subcortical and callosal projecting cells was statistically significant. SMI-32 SpC versus CC (P = 0.00038); SMI-32 SC versus CC (P = 0.00014); N200 SpC versus CC (P = 0.00025); N200 SC versus CC (P = 0.00034); FNP-7 SpC versus CC (P = 0.000024); FNP-7 SC versus CC (P = 0.00016). Abbreviations: Av. %DL, average percentage of double-labeled layer V neurons quantified; CC, contralateral cortex projecting neurons; DLN, double-labeled layer V neuron; SEM, standard error of the mean; SpC, spinal cord projecting neurons.

Markers Specific for Subcortically Projecting Pyramidal Neurons (SMI-32, FNP-7 and N200)

Figure 5 shows confocal images of the three cell populations (contralateral cortex, superior colliculus and spinal cord projecting) stained with three antibodies (SMI-32, N200 and FNP-7) that are specific for different neurofilament epitopes. The panels demonstrate that SMI-32, N200 and FNP-7 were expressed in neurons projecting to the spinal cord and to the superior colliculus, but these three antigens were not expressed in the neurons projecting through the corpus callosum to the contralateral hemisphere. Sections stained with these three antibodies were analyzed using the same quantification technique described above. Most of the subcortical projecting pyramidal cells expressed SMI-32 (mean ± SEM; spinal cord = 97.35 ± 1.33%; superior colliculus = 98.27 ± 0.0077%; N200 (spinal cord = 98.08 ± 1.04%; superior colliculus = 98.24 ± 1.00%) and FNP-7 (spinal cord = 98.27 ± 0.46%; superior colliculus = 97.99 ± 1.01%; Table 2). There was no significant difference between the percentage of cells expressing these three neurofilament markers in the subcortical projecting neurons. However, the contralateral cortex projecting cells barely expressed a quantifiable level of these neurofilament markers (SMI-32 = 1.06 ± 0.54%; N200 = 2.10 ± 1.56%; FNP-7 = 0.33 ± 0.33%; Table 2). The percentage difference between neurofilament expression in callosal – compared to subcortical – projecting neurons was statistically significant (Table 2).

Neurofilament Protein Expression in Organotypic Culture

Figure 5. Confocal microscopic images of layer V pyramidal neurons showing that the three neurofilament antibodies (red) specifically stained neurons that project subcortically, but they did not stain cells with projections to the contralateral cortex (all populations contain green microspheres). SMI-32 (A, B), N200 (D, E) and FNP-7 (G, H) were specifically located in the spinal cord (A, D, G) and superior colliculus (B, E, H) projecting layer V neurons (arrows indicate examples of double-labeling), (C, F, I) contralateral cortical projecting neurons (green microspheres indicated by arrowheads), did not express SMI-32 (C), N200 (F) or FNP-7 (I). The open arrow heads indicate neurons expressing the neurofilaments, but do not contain green microspheres and thus do not project to the contralateral cortex. Scale bar = 20 µm.
without contact with their target tissues. The slices derived from the embryonic cortex did, in fact, express SMI-32 (Fig. 6A) and FNP-7 (Fig. 6B). In high power images, layer V pyramidal neurons were clearly identified by strong SMI-32 (Fig. 6C) and FNP-7 (Fig. 6D) immunoreactivity. Layers II and III did not develop as well as the other layers, which is a typical observation in embryonic explants grown in culture (Molnár and Blakemore, 1999).

To further investigate whether or not subpopulations of layer V neurons maintain their SMI-32 and FNP-7 immunoreactivity specificity in the absence of contact with their target tissue, pyramidal cells were pre-labeled and cortical slices were cultured. At P3, after the corticofugal projections had reached the superior colliculus and the contralateral cortex, fluorescent latex microspheres were injected into one of these targets. At P4, cortical slice cultures were prepared from regions containing labeled cells. These slices were cultured in isolation for two weeks, then fixed and immunostained for FNP-7 and SMI-32 without resectioning (Fig. 7). In 40 cultures, individual microsphere-labeled layer V neurons (n = 1163) were examined for immunohistochemistry. Of the 320 layer V cells that were labeled from the contralateral hemisphere (Fig. 7), only a small percentage (mean ± SEM; 1.69 ± 1.40) expressed SMI-32. In contrast, of the 196 layer V cells that were labeled from the superior colliculus (Fig. 7), a much higher percentage (27.11 ± 4.67) of labeled cells were SMI-32 immunoreactive. Of the 286 layer V neurons that were labeled from the contralateral cortex (Fig. 7), only 4.2% were FNP-7 immunoreactive, whereas 44.6% of layer V neurons (n = 361) that were labeled from the superior colliculus were immunoreactive (Fig. 7). Laminar specificity of neurofilament immunoreactivity was also maintained with stronger staining in layers II, III, V and VI (Fig. 7). Although the absolute number of double-labeled neurons in the cultures was decreased, the overall pattern of neurofilament expression was consistent with the in vivo observations.

### Discussion

**Differential Protein Expression in Layer V Pyramidal Cell Subpopulations**

In this study, a combination of retrograde labeling and immunostaining techniques were used to determine whether or not subpopulations of layer V pyramidal cells projecting to distinct targets express specific neurofilament proteins in the absence of their target tissues. SMI-32 and FNP-7 were used as markers for these neurofilaments. The results showed that layer V pyramidal neurons express these proteins in the absence of their target tissues, suggesting that the expression of these proteins is not dependent on the presence of their target tissues.

**Figure 6.** Organotypic cultures of E16 rat cortices express SMI-32 and FNP-7 immunoreactivity in their layer V pyramidal neurons after 2 weeks in vitro. The presence of SMI-32 and FNP-7 immunoreactive layer V neurons suggests that the expression of these neurofilaments does not depend on the presence of the subcortical target tissues. (A) SMI-32 was expressed in layer V pyramidal neurons in vitro. (C) Higher magnification of neurons taken from A. (B) FNP-7 was expressed in layer V pyramidal neurons in vitro. (D) Higher magnification of neurons taken from B. Scale bars = 1 mm (A, B); 100 µm (C, D).

**Figure 7.** The subpopulation-specific expression of SMI-32 and FNP-7 immunoreactivity is maintained in organotypic cortical cultures from postnatal rats after 2 weeks in vitro. Layer V pyramidal neurons were back-labeled from the contralateral cortex (see inserts in A, C) or from the superior colliculus (see inserts in E, G) at P3 (both populations contain green microspheres). At P4, the somatosensory cortex of both groups was cultured for 2 weeks. SMI-32 and FNP-7 maintained a laminar (A, C, E, G) pattern of expression similar to that found in vivo. This suggests that the presence of specific targets is not necessary for the maintenance of the particular layer V populations after P4. (B, D) Confocal images of pyramidal cells taken from A and C respectively. Both SMI-32 (B) and FNP-7 (D) failed to co-localize with contralateral cortex projecting neurons (green microspheres indicated by the arrowheads). (F, H) Confocal images of pyramidal cells taken from E and G respectively. A large percentage of both SMI-32 (F) and FNP-7 (G) co-localized with superior colliculus projecting neurons (indicated by the arrow). P, postnatal day. Scale bars = 1 mm (A, C, E, G); 20 µm (B, D, F, H).
targets show differential expression of antigens known to be located in layer V of adult rats. These antigens include the following: NR1, NR2a/b, PLCγ1, BDNF, NGF, TrkB, SMI-32, FNP-7 and N200. Staining patterns found in this study confirm previous observations with the same antibodies: NR1 (Aoki et al., 1994), NR2a/b (Conti et al., 1999), TrkB (Tongiorgi et al., 1999; Miller, 2000), NGF (Miller, 2000), BDNF (Murer et al. 1999), SMI-32 (Hof and Morrison, 1995; Gabetter et al., 1999), FNP-7 (Hornung and Riederer, 1999) and N200 (Sasaki and Maruyama, 1994). However, these experiments show for the first time a striking difference in neurofilament expression (SMI-32, FNP-7 and N200) between subcortical (superior colliculus and spinal cord projecting type I neurons) and contralateral cortex (type II neurons) projecting neurons. While a large proportion of spinal cord and superior colliculus projecting neurons were heavily immunoreactive for the neurofilament antigens (SMI-32, FNP-7 and N200), a visual assessment indicated that none of the callosal projecting layer V cells had significantly detectable immunoreactivity. In contrast, all of the other proteins tested (NR1, NR2a/b, PLCγ1, BDNF, NGF and TrkB) were expressed equally in the layer V pyramidal subpopulations examined.

**Differential Neurofilament Expression Layer V Pyramidal Neuron Subpopulations**

Neurofilaments (NF), which are part of the intermediate filament family, are one of the earliest recognizable features of the developing central nervous system (Ulfig et al., 1998). Neurofilaments are heteropolymers consisting of three subunit proteins: NF-L (68 kDa), NF-M (150 kDa) and NF-H (200 kDa) (Hoffman and Lasek, 1975; Nixon and Sigh, 1991; Nixon, 1998). These subunits combine to form filaments (~10 nm diameter). All triplets consist of two structural domains: an α-helical domain, which is the functional backbone and a carboxy end. The carboxy terminals vary in their amount of phosphorylation, which is responsible for the different molecular weights (Shaw, 1991). Decreases in neurofilament expression or abnormal neurofilament phosphorylation have been implicated in normal aging (Vickers et al., 1992; Budinger et al., 2000) as well as in neurological diseases (Bickford et al., 1998) such as: Alzheimer’s disease (Morrison et al., 1987; Hof et al., 1990; Hof and Morrison, 1990; Trojanowski et al., 1994; Vickers et al., 1994; Bussière et al., 2003a,b), Parkinson’s disease (Goldman et al., 1983), Pick’s disease (Perry et al., 1987), amyotrophic lateral sclerosis (Manetto et al., 1988; Munoz et al., 1988; Mizusawa et al., 1989; Tsang et al., 2000), traumatic brain injury (Saatman et al., 1998) and multiple sclerosis (Trapp et al., 1998).

Neurofilaments have a very specific laminar distribution pattern in the cortex that reflects the functional and anatomical brain divisions. Therefore, neurofilament staining is a powerful tool for confirming and extending previous architectural observations (Hof et al., 1995; Preuss et al., 1997; Geyer et al., 2000). SMI-32 recognizes the non-phosphorylated NF-H form, N200 recognizes the non-phosphorylated and phosphorylated NF-H forms and FNP-7 recognizes the non-phosphorylated NF-M form. These protein epitopes initially appear at P7, which interestingly coincides with the divergence of the layer V pyramidal neuronal subpopulations with respect to their somatodendritic morphologies (Riederer and Matus, 1985; Riederer, 1995; Riederer et al., 1995; Kogan et al., 2000).

It is important to relate the onset of neurofilament expression to other aspects of layer V pyramidal cell development. Neurons from layer V subpopulations are born around E15–18 and migrate to layer V by E19–20 (Miller, 1988). Morphologically, they initially appear indistinguishable: all have stout apical dendrites with terminal tufts in layer I and they do not fire action potential bursts in response to depolarizing current (Kasper et al., 1994). Shortly after reaching layer V, they begin extending their axons toward different targets. The callosal axons start to cross the corpus callosum at E16–18, whilst the corticofugal axons enter the internal capsule at E17 and enter the cerebral peduncle at E19. The axons reach the basal pons at E19 and the superior colliculus and spinal cord shortly after birth (De Carlos and O’Leary, 1992). Axonal target invasion occurs postnatally. It is only after axonal outgrowth that the dendritic morphologies begin to diverge (P5; Koester and O’Leary, 1992). The different electrophysiological properties are first detected much later (P14; Kasper et al., 1994). These findings raise the question: how do seemingly similar cell populations differentiate into a heterogeneous group with different targets?

It is not clear what causes these morphological changes, but it has been shown that neurofilaments participate in the structural organization and stabilization of dendrites (Riederer and Matus, 1985; Riederer et al., 1995; Kogan et al., 2000). Therefore, the neurofilament quantity may be important in establishing and maintaining different morphologies of layer V neuronal subpopulations; therefore, playing a role in connections reaching their developmental endpoint (Liu et al., 1994; Kogan et al., 2000).

Previous studies in macaque monkeys have shown that SMI-32 is differentially expressed in certain subpopulations of pyramidal cells. These studies have exclusively examined corticocortical – not subcortical – projecting neurons in layers II, III, V and VI. The corticocortical neuron populations that express low levels of SMI-32 include cells that project from the anterior cingulate to prefrontal cortex; from prefrontal to prefrontal cortex; and from V1, V2 or V3 to V4 (Campbell et al., 1991; Hof et al., 1995, 1996b). Corticocortical neuron populations that express high levels of SMI-32 include cells that project from polysensory association cortices (superior temporal sulcus) to prefrontal cortices and from V1, V2, V3 to MT (Campbell et al., 1991; Hof et al., 1995, 1996b). Equal numbers of corticocortical neurons that send their projections from high level visual association cortex (intraparietal sulcus) to prefrontal cortex express SMI-32 (Campbell et al., 1991). Our results confirm previous findings in rats, which show that somatotopic corticocortical pyramidal neurons in layers III and V projecting contralaterally express very low levels of SMI-32 (Kirkcaldie et al., 2002). One must, therefore, take into account that SMI-32 specificity may vary between primate and non-primate species.

Several studies have correlated the neurofilament amount or type with specific cellular populations in order to better understand the functional significance of each cell type. It is known that the amount of neurofilament increases with the increase of the cell size (Campbell and Morrison, 1989; Tsang et al., 2000); therefore, layer V projection neurons would be expected to have high neurofilament levels compared to other cortical layers. Campbell and Morrison (1989) first showed that non-phosphorylated neurofilaments are found in neurons with long axonal projections extending to cortical and subcortical layers.
areas. This finding is supported by studies that show high neurofilament levels in: long distance ipsilateral and contralateral cortex projections (Campbell et al., 1991), long ipsilateral association connections of the visual system (Hof et al., 1996a), large retinal ganglion cells (Stranzicky et al., 1992) and long corticofugal projections (Hornung and Riederer, 1999). Few cells having short corticocortical, callosal or limbic projections express neurofilament epitopes (Hof et al., 1995, 1996a; Hornung and Riederer, 1999). Our observations demonstrate that layer V pyramidal neurons with callosal (type II) projections do not express these selected neurofilament epitopes, in spite of their long axons. However, it has been suggested that the neurofilament amount might not be associated with axonal length. Instead, the neurofilament quantity might be associated with the amount of axonal myelination (Kirkcaldie et al., 2002). The degree of myelination corresponds with the projection target and would explain the contradictions between the different neurofilaments in ipsilateral connections and callosal connections. Cortical projections extending to subcortical targets and projections from primary sensory cortex extending to association areas (heterotopic) have heavily myelinated axons and are integral for important cognitive and motor processing. Goldstein et al. (1987) found that NF-H protects against proteolysis, thus increasing structural stability, which is critical in large, fast-conducting neurons. In addition to microtubules, neurofilaments have been implicated in regulating the nutrient transport rate, which is especially important in maintaining the structural integrity of these large neurons (Lasek, 1988).

We investigated whether these two distinct classes of layer V cells preserve their neurofilament specificity in two different organotypic slice culture paradigms, in which the projection neurons have been axotomized and lack contact with their targets. When E16 cortical slices were cultured for 2 weeks, SMI-32 and FNP-7 immunoreactive cells were still present, suggesting that the subpopulation phenotype is not dependent upon the target and is an inherent property of neurons. Obviously, before the connections reach their target, the back-labelling of selective layer V subpopulations is not possible; therefore, the issue of subpopulation specificity cannot be addressed with the techniques used in the current study at this embryonic stage. However, maintenance of subpopulation neurofilament specificity in the absence of target tissue contact can be addressed at later developmental stages. In this study, we demonstrated that after 2 weeks of culturing P4 slices, the overall pattern of SMI-32 and FNP-7 immunoreactivity was consistent with the \textit{in vivo} observations. The pyramidal neurons labeled from the superior colliculus at P3 maintained specific expression of SMI-32 and FNP-7, but neurons labeled from the contralateral cortex hemisphere did not express these epitopes. These findings suggest that whilst the specific subpopulation phenotype development normally occurs once the axons have innervated their targets, this process is not dependent upon those targets. Therefore, it stands to reason that by P4, the two layer V neuronal subpopulations might already be committed to different differentiation programs which continue in the absence of their target tissue contact.

In summary, this study demonstrates that layer V neuronal subpopulations not only have specific projection targets and somatodendritic morphologies, but also express different structural protein levels recognized by SMI-32, NFP-7 and N200 antibodies. The finding that neurofilament proteins are expressed in subcortical (superior colliculus and spinal cord type I) projecting neurons (but not in type II, homotopic callosal projecting cells) that have large dendritic tufts supports theories that neurofilaments are associated with dendritic arborizations, long projecting axons and axons with a high degree of myelination. The characterization of molecular and cellular differences in adult rat layer V neuronal subpopulations sheds light on the mechanisms involved in functional circuit differentiation in mammalian cerebral cortex development. The specific neurofilament expression in certain neuronal populations, even in the absence of long projections and maintenance after distant target removal through slice culturing, suggests that early in corticogenesis, neuronal subpopulations are already committed to distinct protein expression patterns.

Notes

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Address correspondence to Dr Zoltán Molnár, Department of Human Anatomy and Genetics, University of Oxford, South Parks Road, Oxford, OX1 3QX, UK. Email: zoltan.molnar@anat.ox.ac.uk.

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