Cellular Diversity in Mouse Neocortex Revealed by Multispectral Analysis of Amino Acid Immunoreactivity

Cortical cells were classified using an unsupervised cluster analysis based upon their quantitative and combinatorial immunoreactivity for glutamate, γ-aminobutyric acid (GABA), aspartate, glutamine and taurine. Overall, cell class-specific amino acid signatures were found for 12 cellular types; seven GABA-immunoreactive (GABA-IR) populations (GABA1–7), three classes containing high glutamate levels (GLUT1–3) and two putative glial (GLIA1, 2) cell types. From their large somata, associated vertical processes and high glutamate content, the GLUT classes most probably correspond to pyramidal neurons. Two of the GLUT classes demonstrated complementary distributions in different cortical layers, suggesting spatial separation of cells differing in amino acid immunoreactivity. Of the seven GABA classes, two comprised cells with large somata and displayed medium to low glutamate levels. On the basis of size, these somata populations may correspond to large basket cell interneurons. Glial populations could be divided into two classes: GLIA1 cells were more frequently associated with blood vessels and GLIA2 cells were more commonly seen in the lower cortical layers. This work demonstrates that signature recognition based upon amino acid content can be used to separate cortical cells into different categories and reveal further subclasses within these categories. This approach is complementary to other methods using physiological and molecular tools and ultimately will enhance our understanding of neuronal heterogeneity.

Introduction
Cortical neurons can be classified broadly into two groups based on morphological and electrophysiological characteristics: the pyramidal and nonpyramidal neurons. Pyramidal neurons, which constitute the majority, have a pyriform morphology and use glutamate as their excitatory neurotransmitter. These cells are the projection neurons of the neocortex and have different connectivity characteristics depending on their anatomical location within the cortical depth (pyramidal neurons located in the supragranular cortex predominantly make corticocortical projections, whereas neurons projecting to extracortical targets are located infragranularly). However, nonpyramidal neurons exhibit a diverse range of morphologies. These cells have local connections and (with the exception of layer IV spiny stellate cells) use the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Fairén et al., 1984; Nieuwenhuys, 1994).

Several studies have tried to further classify cortical neuronal populations using anatomical and immunocytochemical means (Hendry et al., 1984, 1989; Conti et al., 1987b; Lewis and Lund, 1990; Gupta et al., 2000). Morphological analysis has uncovered at least four subtypes of pyramidal neurons based on axo-dendritic morphology and the laminar location of cell somas (Peters and Jones, 1984; de Lima et al., 1990). For nonpyramidal neurons, five distinct cell classes are distinguishable based on cell morphology and soma size (Gupta et al., 2000; Hendry and Jones, 1981; Jones, 1987). In addition, nonpyramidal neurons may be further subclassified using electrophysiological techniques coupled with single-cell gene expression studies for cell-specific markers such as vasointestinal peptide (VIP), neuropeptide Y (NPY), somatostatin (SS) and calcium-binding proteins. For example, the VIP-immunoreactive (VIP-IR) subpopulation of fusiform nonpyramidal neurons may be subclassified as either regular-spiking or irregular spiking (Cauli et al., 2000).

In addition to a multitude of peptides, cortical neurons can be labelled for amino acids, which serve important roles in neurotransmission and/or cellular metabolism. Different amino acids can be selectively found in different neurons, but additionally, distinct patterns of amino acid overlap may subclassify various groups of neurons. We have previously shown that glutamate is present in almost all cortical cells, and that there is a wide range of variation in cellular amino acid levels which is consistent across cortical areas. By superimposing images of amino acid distributions it was also shown that GABA-immunoreactivity in cortical neurons might coexist with variable levels of amino acid precursors such as glutamate, glutamine and aspartate (Hill et al., 2000). These findings raise the possibility that cortical neurons may be further partitioned on the basis of differential amino acid content (qualitative and quantitative) in individual neurons. If successful, such an analysis may potentially reveal amino acid signatures for individual neurons, something not possible prior to the use of classification techniques combined with quantitative immunocytochemistry (Kalloniatis et al., 1996; Marc et al., 1990, 1995, 1998).

The aim of this study was to identify amino acid signatures in the adult mouse neocortex. Because amino acids are important in basic cellular functions such as metabolism and neurotransmission, subclasses distinguished by different signatures may identify cells with real functional differences. In the retina, similar analysis has shown correlation of distinct cellular neurochemical signatures with independently verified cell types (Marc et al., 1990, 1995, 1998; Kalloniatis et al., 1996). Remarkably, up to eight different categories of amacrine cells may be partitioned on the basis of signature analysis (Kalloniatis et al., 1996; Zhang et al., 1996). The task ahead is to ask whether neurons in the neocortex, a less anatomically defined structure, also possess definable signature classes as a basis for describing further cellular heterogeneity. Our results confirm this to be the case, with statistically verifiable separation of three classes of pyramidal neurons, seven classes of nonpyramidal neurons and two glial classes.

Materials and Methods
We have previously carried out amino acid immunocytochemistry in adult mouse neocortical tissue (cortical areas 5 and 6) and established that there are no significant differences in labelling distributions between cortical areas, or between different cortical samples (Hill et al., 2000). For the present study, the aim was to examine in detail the signature of every cell present in a representative sample of cortical space (area 6). Multispectral

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image analysis was conducted for three cortical samples obtained from adult mice (C57BL/6; 6 weeks of age). Tissue processing was carried out as previously described (Hill et al., 2000). To enable individual cortical neurons to be simultaneously labelled by antibodies directed against different amino acid antigens, serial sections (0.25 µm) were cut using a Reichert-Jung Ultracut S, and each section incubated with a different primary antibody.

**Immunocytochemistry**

To ensure uniform treatment, all sections were incubated simultaneously with reagents under identical conditions (with the exception of primary antibodies) as previously documented (Hill et al., 2000). Serial sections were incubated on Teflon-coated slides (CelLine, NJ, Fig. 1A) for post-embedding immunocytochemistry (Kalloniatis and Fletcher, 1995; Kalloniatis et al., 1994, Marc et al., 1995). Rabbit polyclonal antibodies (kindly donated by Dr Robert Marc, University of Utah) were diluted in 1% goat serum in buffered saline at the following working concentrations: anti-glutamate (1:4500), anti-GABA (1:4500), anti-glutamine (1:800), anti-aspartate (1:100) and anti-taurine (1:600). Sections were rinsed in 1% buffered saline for 5 min before addition of goat anti-rabbit secondary antibody (coated with 1 nm gold; Immunodiagnostics, Australia) at a dilution of 1:100. Following this, silver intensification was carried out using a solution of 200 mM silver nitrate containing 38 mM hydroquinone in 200 mM citrate buffer for ~8 min (Moeremans et al., 1984).

**Image Analysis**

Images of immunolabelled tissue were captured using a Zeiss Axiohot light microscope with a Kodak Megaplus 1.4 camera and NIH Image software (Bethesda, MD; Fig. 1B). To obtain relative estimates of neuronal amino acid content, data were collected from a single immunocytochemical run with constant light settings and fixed camera gain. Non-overlapping images of the equivalent tissue region in each of the five serial sections were captured at 40× magnification (using an oil immersion lens) in sequence from the pial surface to the white matter. Images from a strip of tissue (measuring ~500 µm in width) were captured as two adjacent columns, each column containing five consecutive frames spanning the cortical depth.

Images were low-pass filtered (Image-Pro Plus software, Media Cybernetics, MD) to reduce registration error and subsequently overlaid using conventional image registration methods (GCWorks software from PCI Remote Sensing, Richmond Hill, Canada). Anatomical landmarks such as blood vessels and cell somas were used as reference points, as previously described (Kalloniatis et al., 1996; Marc et al., 1995, 1998). Pixel intensity data representing labelling intensity within a maximum range of 0–255 was also collected from blood vessel lumina to provide control readings for background immunoreactivity.

Amino acid labelling patterns were qualitatively assessed by viewing the registered images in rgb format (Fig. 1B). An rgb image is comprised of up to three separate images (i.e. a triplet image representing the distribution of three amino acids), which have been overlaid and digitally coloured to produce red, green and blue hues. Images can also be viewed in pairs (i.e. as a doublet) to examine differences between distribution patterns (Fig. 2F,G). PV data from each of the five serial images were classified using an unsupervised K-means cluster analysis procedure (KCLUS, XPACE, PCI Remote Sensing).

We generated a map consisting of cell bodies, processes and blood vessels (serving as landmarks) present in an area of cortical tissue (Fig. 3). This procedure involved defining a mask area, to enable only areas of interest within the entire cortical image to be selected for analysis (Fig. 1B; ImageWorks, PCI Remote Sensing). The use of a mask in the analysis was advantageous because it enabled classification of the neuropil to be avoided. This was desirable because misregistration of cellular processes within the neuropil area yielded nonsensical classifications (unpublished observations).

From the masked area, each signature or cell class corresponding to cortical cell populations or blood vessels is denoted by a specific hue within the map (Fig. 1B). A signature or cell class was identified as statistically separable from other populations by calculating transformed divergence values (TD; KCLUS algorithm, PCI Remote Sensing). All pseudocolours in the map refer to unique cellular areas with distinct amino acid distributions (blood vessel lumina are shown in white). The TD separability measure yields real values between 0 and 2, where 0 indicates complete overlap between the signatures of two classes, and 2 indicates a complete separation between the two classes. We used a criterion of TD > 1.9 as significant separability, which translates to <1% error of misclassification. All cell classes analysed in this study had a separability of TD > 1.9. Following generation of the map, the SIEVE program was run (STHRESH = 100 pixels, XPACE, PCI Remote Sensing) to merge small areas of cell classes with the adjacent cell class. This procedure serves to remove ‘specks’ of alternate pseudocolours from single signatures, thus improving the clarity of the map for presentation without altering original PV data.

**Data Presentation**

We used established single letters to denote the different amino acids (E = glutamate, γ = GABA, D = aspartate, Q = glutamine and T = taurine). To depict individual cell classes separable by signature differences, separate mask areas corresponding to the various classes were generated. Univariate histograms were subsequently plotted for each cell class (Jandel SigmaPlot; Fig. 1C panel 1) with PV depicted on the x-axis, and number of pixels (normalized) shown on the y-axis. For example, three different GABA cell classes may be distinguished from each other, and from the GLUT3 class. The collective PV data may also be represented in two other ways to highlight biochemical diversity between different cell populations. Firstly, while a signature is the result of the multiparametric analysis (of five amino acids), each cell class can also be uniquely depicted by considering bivariate data (or ellipses) for subsets of amino acids. An ellipse provides a bivariate description of PV values (±2SD) for a pair of amino acids; the first stated amino acid is depicted on the x-axis and the second on the y-axis. A composite overlay made up by different ellipses (Fig. 1C panel 2) would provide a ‘fingerprint’ for that cell class. In the example shown (Fig. 1C panel 2), the GLUT3 class was defined using five different amino acids considered in six paired-combinations. For example, glutamate content in this class was separately considered against GABA and taurine. The red ellipse depicts low GABA content (x-axis) in the presence of high glutamate (y-axis), whereas the pink ellipse depicts universally high taurine and glutamate in this class. Taken together, the positions of the six ellipses, and their overlap, represent the ‘fingerprint’ pattern for the GLUT3 class.

Bivariate PV data for a set pair of amino acids can also be used to separate different cell classes (Fig. 1C panel 5). The power of bivariate analysis is demonstrated in the given example where single-dimensional analysis of GABA-IR cells would have obscured differences in aspartate distribution because of similar taurine levels in the three GABA cell classes (Fig. 1C panel 3). Using this method of data presentation (Fig. 1C), cell populations can be clearly separated in two-dimensional comparisons (e.g. the GABA4 and GABA2 populations are completely separated, while GABA1 has overlaps with the other two populations). However, the GABA1 and GABA2 overlap may disappear as separation power is increased by considering more parameters (Marc et al., 1995), in our case up to five different amino acids.

**Results**

In the present study, serial section quantitative immunocytochemistry for amino acids and computer image analysis were used to classify different neuronal subtypes based upon their neurochemical content. By superimposing various combinations of immunocytochemical distribution patterns, information regarding amino acid cellular colocalization and relative abundance was obtained using rgb images (Fig. 2F-J). Using various amino acid doublet and triplet combinations, it is possible to demonstrate cellular differences in amino acid levels prior to cell classification.

**Greyscale Images**

Figure 2A–E shows greyscale images of individual amino acid distribution patterns (glutamate, GABA, aspartate, glutamine and taurine) from consecutive sections of layer VI. Because these
images have been inverted (using a digital NOT function, Image-Pro Plus), strongly immunoreactive neurons (with high PV) appear lighter and weakly labelled cells (and unlabelled vascular lumina) appear dark (and display low PV). To demonstrate the variability in PV for cells labelled for different antibodies, nine cells are depicted by numerals and adjacent arrows in Figure 2A–E. Accordingly, areas of increased labelling for one amino acid do not necessarily correspond for other antisera (e.g. cell 1 in panels C and D shows low aspartate and high glutamine labelling). As expected (Hill et al., 2000),
glutamate immunoreactivity was relatively high in most of the cortical neurons visualized (Fig. 2A). GABA levels were consistently lower than glutamate for the population shown, with a few cells exhibiting similar levels for both amino acids (Fig. 2B; A; cells 5 and 6). Aspartate levels for the same cell population were generally lower in comparison with glutamate (Fig. 2C). Similarly, glutamine levels were predominantly low, with the exception of a few cells containing significant levels of labelling (Fig. 2D; cell 1). Of the four amino acids shown, taurine immunoreactivity was the highest in the neuropil (Fig. 2E). However, several cells that demonstrated low levels of glutamate also contained low taurine labelling (i.e. cells 5 and 6; Fig. 2A,E).

rgb Images
Of the five amino acids under study, glutamate and taurine showed the highest general immunoreactivity, while glutamine, for example, showed lower levels. This can be demonstrated in doublet images in which these amino acids are depicted as red–green (rg) images (Fig. 2F,G). In the rg → glutamate–taurine pair (rg = Et), both glutamate and taurine showed intense labelling, although the dominance of the green hue reflects a higher presence of taurine (Fig. 2F). Some small cells were bright green (cells 1–4; Fig. 2F), indicating substantial levels of taurine over lesser amounts of glutamate. These cells were either located at the periphery of blood vessels (cells 3 and 4; arrows) or found adjacent to large cell bodies presumed to be neurons (cells 1 and 2). When the same cells were viewed as a glutamate–glutamine (rg = EQ) doublet (Fig. 2G), some of these cells (cells 1 and 2) showed substantial glutamine levels, while the vascular-associated cells appeared to be less immunoreactive for glutamine. Aside from these cells, the remaining cellular population and neuropil also showed strong overall glutamate immunoreactivity, evidenced by the overall red hue (Fig. 2G).

Taken together, these results demonstrate biochemical heterogeneity for the small-sized cells under consideration. While the cells with high taurine, low glutamine (cells 1 and 2; green in both Fig. 2F,G) appeared to be associated with neurons; cells with high taurine, low glutamine (cells 3 and 4) were associated with blood vessels. This combination of biochemical criteria (low glutamate, high taurine), with physical proximity (to vasculature) would suggest these smaller-sized cells to be glia (Marc et al., 1995, 1998; Kalloniatis et al., 1996).

rgb Images
A greater range of cellular diversity was apparent when the immunoreactivity of the same cellular population was considered as rgb images (in amino acid triplets). When taurine–glutamate–GABA immunoreactivities were viewed together (rgb = tEG, Fig. 2H), the putative glial cells considered above continued to reflect high taurine levels (shown here in the red channel) even when GABA labelling is taken into consideration. In addition, GABA-IR cells had a blue–purple hue (cells 5 and 6; Fig. 2H), demonstrating GABA immunoreactivity in the presence of glutamate. The green channel depicts glutamate and many cell bodies with high glutamate content are evident (e.g. cell 7; Fig. 2H). In the upper layers, high levels of glutamate were also found in fibers resembling apical dendrites (data not shown).

When the tissue was viewed as an rgb image comprising of glutamine–glutamate–GABA (rgb = QET; Fig. 2F), the resulting image was predominantly green, indicating high levels of glutamate in comparison with glutamine and GABA. However, GABA-positive cells were variable for glutamine content (e.g. the hue for cell 5 appears to contain a greater component of red, indicating more glutamine, compared to cell 6; Fig. 2F). The combination of glutamine–glutamate–aspartate (rgb = QED, Fig. 2F) revealed predominantly blue–green hues, indicating generally higher levels of aspartate.

These combinations of amino acid profiles enabled several cells to be distinguished by variable taurine, glutamate, aspartate and GABA levels (Fig. 2H–J). In particular, the cells indicated by the red arrows contained contrasting hues in Figure 2H (demonstrating glutamine–glutamate–GABA/aspartate levels), but appeared to be similar when viewed in Figure 2H (taurine–glutamate–GABA triplet).

Cellular Classes
In order to identify cellular populations differing in amino acid immunoreactivity, PV data from a mask area of the five overlaid images were classified using K-means cluster analysis. The 12 classes resulting from the multispectral analysis were depicted in 12 different pseudocolours on a cortical map. The map represented a strip of cortical space spanning the cortical thickness within which every cell (and blood vessels) was accounted for (Fig. 3). The analysis revealed statistically verifiable cell classes corresponding to diversity in amino acid immunoreactivity. For example, three different classes of glutamatergic neurons (GLUT1–3) and seven classes of GABA-ergic neurons (GABA1–7) were distinguishable (Fig. 3, key). Glial cell classes fell into two subclasses (GLIA1 and 2) with GLIA 1 frequently encountered adjacent to blood vessel lumina (white pseudocolour map area). The number of cell classes and distributions were similar for each of the three cortical samples analysed.

Three classes of GLUT neurons were identified by their high glutamate content, large somata and superficial layer cell processes. Coupled with their relative abundance across different cortical layers, we infer them to be pyramidal neurons. In one map (Fig. 3A), two classes of GLUT neurons are seen with large somata and represented here as blue (GLUT1) and yellow (GLUT2) areas. GLUT2 cell class was prominently associated with cell processes with an identical amino acid signature (depicted by the same pseudocolour) in vertical orientation and seen in layers II/III. The same appears to be true for GLUT3 cells (Fig. 3C). In contrast, cellular processes for GLUT1 neurons were more difficult to define from neighbouring neuropil, suggesting similar amino acid signatures between the processes and neuropil. With respect to layer distribution, each of the GLUT classes appear to have distinctive features. GLUT1 cells (blue) were found at a higher density in layer V compared with other layers, but were exceptionally scarce in layer IV (Fig. 3A). The GLUT2 class (yellow) was also found throughout the cortical depth; however, in contrast to GLUT1 cells, few GLUT2 (yellow) cell bodies were seen in the upper layers – instead GLUT2 cells were mostly observed in layer VI. These two cell classes (GLUT1

**Figure 2.** Visual depiction of PV data in greyscale or rgb format. (A–E) Inverted images (blood vessel lumina in black, strongly labelled cells in white) from consecutive sections labelled for different amino acids (E, y; D, O, t). In this example, nine cells with different labelling characteristics may be seen (arrows and numerals). (F–J) Overlaid images from (A–E) viewed as doublet or triplet combinations reveal differences in amino acid composition between cells (e.g. cells 1 and 6 in F appear similar but are clearly different in J). Doublet images can distinguish cells that may appear similar (cells 1 and 3; F) but are clearly different when viewed through a different amino acid combination shown in (G). Similarly, cells indicated in boxes and arrows demonstrate that rgb images enable cells to be distinguished from each other by hue. Scale bar = 20 μm.
Figure 3. Cortical map showing 12 different cell classes and blood vessels derived from multispectral analysis. (A–C) Twelve statistically separable cellular groups (GLUT1–3, GABA1–7 and GLIA1 and 2) were allocated different pseudocolours (see Key) and represented on a map of the cortical space spanning from layer I to the white matter (WM). (A) Two cell populations containing high levels of glutamate (GLUT1 and GLUT2) demonstrate complementary distribution patterns with GLUT1 cells (blue) more prevalent in layer V, while GLUT2 (yellow) is more strongly represented in layer VI. (B) Cells containing GABA (GABA1–7) and those resembling glia (GLIA1 and 2) are sparsely distributed. GLIA1 cells (red) are frequently found adjacent to blood vessels, whereas the GLIA2 population (purple) is more densely distributed in the lower cortical layers. (C) The third GLUT class (GLUT3, beige) is evenly distributed throughout the cortical layers. (D) GABA cell class (1–7) distribution in various layers was determined by binning the layer location of GABA cells found in a cortical area. GABA2 (orange), containing the higher levels of GABA, are mainly found in the upper layers. Roman numerals indicate cortical layers. Blood vessel lumina, serving as landmarks, are shown in white. Dotted line indicates position of white matter boundary.
and GLUT2) demonstrated complementary distributions with regard to cortical layers, i.e. few GLUT2 somas were present in layer V where GLUT1 cells were abundant, and vice versa in layer VI (Fig. 3A). The third type of glutamatergic neuron (GLUT3, beige) may be observed in the composite map of all cell classes (Fig. 3C). GLUT3 neurons were evenly distributed throughout the cortical depth (Fig. 3C).

For GABAergic cells, seven unique classes of GABA-IR neurons were identified (Fig. 3, key). These are depicted as maroon (GABA1), orange (GABA2), green (GABA3), khaki
Some nonpyramidal populations contained significant levels of content, medium-high aspartate levels for the GABA6 class. Pyramidal subpopulations (e.g. high glutamine content and aspartate and glutamine were not correlated within the non-pyramidal classes of GLUT neurons, high glutamine consistently varied considerably across the total neuronal population. Within the three classes of GLUT neurons, high glutamine was present at the same level in both GLUT2 and GLUT3. Consistent with the pyramidal phenotype, levels of GLUT1-immunoreactivity were minimal for the three GLUT classes (i.e. approaching control values expressed as background immunoreactivity in blood vessel lumina; Fig. 4, bottom row). The relative levels of amino acid immunoreactivity across the three classes were also consistent; i.e. GABA < glutamine < aspartate < glutamate/taurine.

The pyramidal populations (GLUT1–3) also displayed differences in amino acid profiles. GLUT1 showed higher levels across all five amino acids compared to GLUT2, with GLUT 3 showing intermediate levels. The exception was taurine, which was present at the same level in both GLUT2 and GLUT3. Consistent with the pyramidal phenotype, levels of GABA-immunoreactivity were minimal for the three GLUT classes (i.e. approaching control values expressed as background immunoreactivity in blood vessel lumina; Fig. 4, bottom row). The relative levels of amino acid immunoreactivity across the three classes were also consistent; i.e. GABA < glutamine < aspartate < glutamate/taurine.

Biochemical Signatures: Univariate Histograms

The 12 amino acid signatures revealed by the cluster analysis allow the extraction of individual (univariate) amino acid distributions. Univariate histograms for each cellular population (GABA1–7, GLUT1–3, GLIA1–2) were plotted for each of the five amino acids (Fig. 4). In addition, data obtained from blood vessel areas (BVs) were plotted as a background concentration reference (Fig. 4, bottom row). This method of data presentation allows comparison of individual amino acid levels across all cellular populations.

GABA and GLUT classes showed consistent differences in amino acid composition. Nonpyramidal neurons consistently contained higher GABA levels compared with the GLUT subclasses. In contrast, GLUT neurons contained higher taurine levels than nonpyramidal cells. However, the levels of the two major glutamate precursor amino acids, glutamine and aspartate, varied considerably across the total neuronal population. Within the three classes of GLUT neurons, high glutamate consistently coincided with high aspartate levels. In contrast, the levels of aspartate and glutamine were not correlated within the non-pyramidal subpopulations (e.g. high glutamine content and medium aspartate levels for GABA5 cells, or low glutamine content, medium-high aspartate levels for the GABA6 class). Some nonpyramidal populations contained significant levels of glutamate, e.g. the mean for the GABA3 class was higher than for GLUT1 cells. However, mean glutamate content for the GLUT1–3 populations was generally higher than that for the nonpyramidal populations.

Within the seven GABA4–IR cell classes, much variation in amino acid content was evident. The GABA1 population contained medium-low levels of glutamate in comparison with the GABA2–7 populations. In addition, this cell class contained a level of GABA clearly above that of the pyramidal populations (Fig. 4). Of the seven nonpyramidal cell types analysed, GABA2 and GABA7 populations contained the highest levels of GABA.

The GABA3 distribution profile showed higher levels of glutamate than the GABA1–6 and GLUT1 classes. GABA3 cells also contained the highest level of aspartate immunoreactivity for the nonpyramidal group, and the highest mean glutamine of all cell types analysed. The GABA4 class contained the lowest GABA levels of the nonpyramidal group (however, these cells were labelled more strongly for GABA than the GLUT cells). The glutamine profile for some of the GABA5 cell showed the highest level of glutamine, whereas GABA6 cells contained the lowest glutamine content, and the highest mean taurine value of all nonpyramidal classes (Fig. 4).

Bivariate Distributions (2SD ellipses)

The data in univariate histograms may be presented in another way to highlight signature differences between cell classes (Fig. 5). Recall that a cell class is a statistically defined entity, which represents all cells with amino acid distribution ranges falling within the limits of a histogram row shown in Figure 4. Two amino acids coexisting in a given cell class may then be graphically depicted as a bivariate plot of their PV values. The resulting ellipse describes the quantitative range for the two amino acids in that class (Fig. 1C). Immunoreactivity data for five different amino acids is capable of generating 10 different bivariate ellipses (i.e. $N/[(x(N - x)^2)]$; where $N$ = the number of samples, and $x$ = the number in each group) although for the

Figure 5. Characteristic fingerprints define each of the 12 cell classes. Data indicating labelling intensities for six representative pairs of amino acids (see legend) were plotted in ellipse format (mean $P = 2SD$) for each cell class. The first-mentioned amino acid is represented on the $x$-axis, the second on the $y$-axis. A–C The GLUT1–3 populations are clearly distinguishable from the GABA1–7 (D–F) and GLIA1 and 2 (G–I) populations. Within the three major cell groups there are also subtle differences in amino acid distribution, e.g. the blue and green ellipses are separate for GLIA1 but slightly overlapping for GLIA2 (J–K). M When data for a single amino acid pair is plotted on a bivariate axis, cell classes may be separated on the basis of differences in amino acid content, whereas when viewed on a univariate axis the data for both classes may overlap substantially. Both the GABA4 and GABA2 populations overlap in their GABA content (y-axis) but are clearly separable when viewed for glutamate-IR (M).
purpose of the study, we chose the six most informative pairs for highlighting cell class characteristics. In this manner, the spatial positions and overlaps of ellipses would provide a ‘fingerprint’ for that cell class. This method of analysis, using a subset of the data used for signature classification, provides further validation of cell class separation.

The ‘fingerprints’ for all three GLUT classes showed greater separation of ellipses, compared to the GABA classes. Within the GLUT classes, GLUT2 was clearly distinct, with greater ellipse diameters and degrees of overlap in the blue (Q versus D), green (Q versus τ), yellow (τ versus D) and white (D versus τ) ellipses (Fig. 5A–C). Although GLUT1 and GLUT3 had similar fingerprints, they clearly differed in the degree of overlap of green (Q versus τ), blue (Q versus D) and white (D versus τ) ellipses. For the GABA classes, the tighter clustering of ellipses suggests a constant relationship between amino acid distribution ranges. However, the seven GABA classes were separable by their fingerprints by a number of criteria. For example, the yellow (τ versus D) and white (D versus τ) rings had grossly different spatial relationships between classes. Whereas GABA7 show almost complete overlap of yellow and white ellipses (Fig. 5F), they were fully unlinked in GABA3 (Fig. 5J).

For a single pair of amino acids, the coordinates of the ellipse may differ depending upon the cell class. For example, the position of the red ellipse (γ versus E) clearly distinguished GABA2 from GABA4 (Fig. 5C; see also Fig. 1C, panel 3 for aspartate/taurine). Taking GABA immunoreactivity alone, GABA2 and GABA4 had similar quantitative ranges, but their differing glutamate levels permit clear separation of the two classes. In a similar vein, for the GLIA classes, the blue (Q versus D) and green (Q versus τ) ellipses had characteristic fingerprint differences (Fig. 5K).

Hierarchical Cluster Analysis Dendrogram

To view the relatedness or otherwise between individual members of all 12 classes, a dendrogram was plotted by comparing the mean amino acid values from each class (obtained from regression analysis of univariate histograms using a three-parameter Gaussian curve; Fig. 6). A total of 60 mean values were fed into a spreadsheet as sets of five (representing five means for each class) for hierarchical cluster analysis (SPSS 9.0 for Windows). As expected, GLUT classes as a whole were more related to one another, compared to GABA or GLIA classes. Within either GLUT or GABA classes, minor subgroups demonstrating greater degrees of similarity were also found (e.g. GLUT1/3, GABA3/4, GABA 1/6). In contrast, the dendrogram highlighted the singular appearance of GABA2 from all other GABA classes. GLIA classes were also set apart from all putative neuronal classes. The data was subject to repeated analysis using a variety of other parameters to seek out other possible dendrograms. The results showed a high degree of convergence validity, i.e. groupings similar to the above were obtained.

Discussion

In a previous study (Hill et al., 2000), we proposed that quantification of multiple amino acids in individual neurons of the cortex may be meaningfully used to uncover cellular heterogeneity. We suggested that biochemical diversity in individual neurons might reflect a cell’s identity in the way amino acids are utilized for metabolic and neurotransmitter functions. In the present study, we have used combinatorial data (with unsupervised clustering) for five amino acids to assign amino acid signatures to all the cells of all cortices in a given cortical space. The analysis yielded at least 12 different neurochemical signatures, which fell into three broad groups (denoted as the GLUT, GABA and GLIA classes). In a previous study (Hill et al., 2000), we raised the concept that quantification of multiple amino acids in individual neurons of the cortex may be meaningfully used to uncover cellular heterogeneity. We suggested that biochemical diversity in individual neurons might reflect a cell’s identity in the way amino acids are utilized for metabolic and neurotransmitter functions. In the present study, we have used combinatorial data (with unsupervised clustering) for five amino acids to assign amino acid signatures to all the cells and blood vessels in a given cortical space. The analysis yielded at least 12 different neurochemical signatures, which fell into three broad groups (denoted as the GLUT, GABA and GLIA classes).

The first group (GLUT1, GLUT2, GLUT3) encompasses cells rich in glutamate and taurine, but low in GABA. The three classes may be differentiated by their characteristic signatures but the high glutamate content suggests correspondence to pyramidal neurons. Although stereology was not performed, a visual analysis suggests that taken as a whole, GLUT cell classes outnumber either the GABA or GLIA classes. This asymmetry is confirmed by known cellular ratios for the different cell types (Beaulieu, 1993).

Cortical pyramidal neurons have not previously been classified using quantitative immunocytochemistry for neuronal markers or metabolites. Using a qualitative approach, three pyramidal subtypes (glutamate only, aspartate only, glutamate plus aspartate) with overlapping distribution patterns have been mapped (Conti et al., 1987a). Our analysis refines this classification by providing quantitative evidence of high aspartate/high glutamate for GLUT2 class, while GLUT1 and GLUT3 classes appeared to be low in aspartate. Additionally, we found further evidence for separating these cell classes, with higher glutamine in GLUT2 compared to GLUT1 and GLUT 3. The distribution of GLUT2 cells in cortical space suggests that this class is predominantly situated in layers V and VI, and their corresponding signature patterns in apical cellular processes provide strong intimation of large projection neurons. Elevated levels of glutamate and aspartate content may indicate high rates.
of neurotransmitter usage, or at least high levels of accumulation and possibly a high synthesis rate in these cells.

Classically, pyramidal neurons can be subgrouped based upon distinguishing criteria such as morphology and projection patterns, layer location and receptor profiles (Feldman, 1984; Jones, 1984; Munoz et al., 1999; Cauli et al., 2000). Not surprisingly, the number of definable classes is heavily influenced by the criteria upon which the separation is based. For example, morphological examination of primate cortex has described up to eight subgroups of pyramidal neurons (de Lima et al., 1990).

By signature analysis, the present study has uncovered at least three classes of pyramidal neurons, but whether or not they correspond to any known morphological or physiological subtypes remains undetermined.

An unexpected finding was low levels of GABA-IR in all three GLUT cell classes. The level of GABA was lower compared to neurons identified in the GABA classes but clearly above control immunoreactivity in blood vessel space. Perhaps the greater sensitivity of the post-embedding method is able to uncover trace levels of GABA in neurons rich in glutamate, which we presume to be pyramidal in character. On the other hand, this phenomenon is not new: the presence of GAD67, a GABA-synthesizing enzyme, has been reported in glutamatergic neurons of the rodent and primate hippocampal granule cells (Sloviter et al., 1996). Furthermore, GAT-1, a GABA-transporter for GABA uptake from extracellular space, has been localized in granule and pyramidal neurons of rat hippocampus (Frahm et al., 2000). Therefore, it is possible that trace GABA levels in GLUT cell classes may have been imported as part of the process of GABA clearance from extracellular space. Following uptake or synthesis, these cells may utilize GABA in energy production, as GABA metabolism reportedly contributes to a large proportion (approximately one-third) of overall cerebral TCA cycle activity in the mouse (Hassel et al., 1998).

Signature analysis provided at least seven classes of GABA cells, based upon their high GABA content and associated fingerprints. GABA1 and GABA6 were most related to one another by their fingerprints (Fig. 5D.f) and by their larger soma sizes with preferential distribution layers IV and V1 (Fig. 3B). Morphologically, large soma GABA cells have been associated with basket cells, one of the nonpyramidal neuron descriptors (Hendry and Jones, 1981). Interestingly, both GABA1 and GABA6 also exhibit a similar trend of low glutamine immunoreactivity. In the retina, this class signature has also been described for starburst amacrine neurons (Kalloniatis et al., 1996). On morphological and physiological grounds, at least five types of GABAergic neurons have been observed in the neocortex (Gupta et al., 2000). Whether or not the signature classes depicted in this study will ultimately correspond to morphological groups remains unclear, but there is precedence for the idea, first proposed as the ‘signature hypothesis’ (Lam et al., 1985), that morphology has unifying themes with neurochemical signatures in neurons of the central nervous system. For example, vasointestinal peptide (VIP) and calretinin immunoreactivity is correlated to certain populations of bipolar interneurons in the neocortex (Cauli et al., 1997). In the enteric nervous system, at least 14 different kinds of myenteric neurons can be reliably distinguished from one another by their neurochemical coding (Furness, 2000).

Central to cell class separation of GABA cells were the wide-ranging differences in aspartate and glutamine content between all seven GABA classes (GABA1–7). This may possibly reflect variation in glutamate production, the immediate precursor to GABA. Some GABAergic cells may preferentially use aspartate as a precursor to glutamate production (via transaminase reactions), whereas other cells may synthesize glutamate via glutaminase reactions, from glutamine (Erecińska and Silver, 1990). In contrast, there was less differentiation between the three GLUT classes for glutamine and aspartate, possibly reflecting similar usage of these precursors in metabolic and neurotransmitter functions. GABA5 is noteworthy for its high glutamate content, in this instance, exceeding even the GLUT1 class. A similar pattern emerged from retinal studies where known glutamatergic neurons display lower glutamate levels compared with known GABAergic or glycinergic neurons (Kalloniatis et al., 1996; Marc et al., 1998). This is counter-intuitive to what is currently known about the use of glutamate and GABA in excitatory and inhibitory neurons. It emphasizes the fact that glutamate level alone is not a reliable indicator of cell type. High glutamate levels in GABA cells may reflect either increased steady-state glutamate during synthesis or bona fide usage of glutamate as a co-neurotransmitter in GABA cells (Ehinger, 1989).

Signatures presumed to be GLIA classes were clearly different compared to neuronal classes (see Fig. 6) in terms of their low glutamate/high taurine levels. A similar signature for Müller glia has been described in the retina (Kalloniatis et al., 1996; Marc et al., 1998). Glial cells are essential to glutamate recycling whereby extracellular (transmitter) glutamate is taken up and recycled to form glutamine via the glial-specific glutamine synthetase enzyme (Norenberg, 1979; Moscona, 1983). The high efficiency of the glutamate–glutamine conversion is thought to be responsible for the lack of glutamate in this cell type (Erecińska and Silver, 1990).

The two glial classes disclosed by the cluster analysis also showed different anatomical distributions. Cells of the GLIA1 population were commonly located in close vicinity of blood vessels and hence considered strong candidates for perivascular astrocytes. The GLIA2 was less numerous and more frequently seen in lower cortical layers. In this regard, GLIA2 share similar spatial locations with oligodendrocytes which are known to predominate in lower layers (Cammer and Tansey, 1988).

In conclusion, multispectral analysis of amino acids in adult neocortical tissue provides an important means of partitioning cellular classes. As illustrated by the dendrogram (Fig. 6), signature classes fall into broad categories that fit into known categories of glial or neuronal cells. In the neuronal category, further subdivisions into putative excitatory or inhibitory classes may be seen. This biochemical classification awaits physiological and functional validation and it would be of interest if molecular properties are seen to coincide with electrical properties.

Notes

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References


References


