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Giant pyramidal neurons of the primary motor cortex express vasoactive intestinal polypeptide (VIP), a known marker of cortical interneurons

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Vasoactive intestinal polypeptide (VIP) is known to be present in a subclass of cortical interneurons. Here, using three different antibodies, we demonstrate that VIP is also present in the giant layer 5 pyramidal (Betz) neurons which are characteristic of the limb and axial representations of the marmoset primary motor cortex (cytoarchitectural area 4ab). No VIP staining was observed in smaller layer 5 pyramidal cells present in the primary motor facial representation (cytoarchitectural area 4c), or in the premotor cortex (e.g. the caudal subdivision of the dorsal premotor cortex, A6DC), indicating the selective expression of VIP in Betz cells. VIP in Betz cells was colocalized with neuronal specific marker (NeuN) and a calcium-binding protein parvalbumin (PV). PV also intensely labelled axon terminals surrounding Betz cell somata. VIP-positive interneurons were more abundant in the superficial cortical layers and constituted about 5–7% of total cortical neurons, with the highest density observed in area 4c. Our results demonstrate the expression of VIP in the largest excitatory neurons of the primate cortex, which may offer new functional insights into the role of VIP in the brain, and provide opportunities for genetic manipulation of Betz cells.

Keywords Vasoactive intestinal polypeptide, Layer 5 pyramidal cells, Betz cells, Primary motor cortex, Inhibitory neurons, Marmoset

Similar to other primates^{1–3}, in the marmoset monkey, the primary motor cortex (M1, Brodmann Area 4) is formed by distinct cytoarchitectural subdivisions. According to the contemporary view, the representations of the limb and body axial musculatures are encompassed within cytoarchitectural area 4ab, located medially, whereas the representations of the head musculature (including facial and oral) are located laterally, in cytoarchitectural area 4c^{4,5}. In particular, A4ab contains the unique “gigantopyramidal” neurons, known as Betz cells in primates^{6–8}. In contrast, in A4c, layer 5 is populated by smaller pyramidal neurons⁴. Betz cells are characterised by their pyramidal shape, large size, and prominent apical dendrites that are oriented along a vertical axis. Although more sparsely distributed, they can be up to 20 times larger in volume when compared to other pyramidal cells in humans⁹.

Betz cells have specialised molecular and physiological characteristics⁶. Given their distinctive action-potential properties^{10,11} and direct connectivity onto the ventral horn of the spinal cord¹², they are well placed for initiation and modulation of fine movement. Betz cells can be identified using markers of pyramidal neurons, such as antibodies against non-phosphorylated neurofilament (e.g. SMI-32)^{13,14}, as well as general markers of neuronal nuclei (e.g. NeuN)¹⁴. In addition, some of these cells specifically express nitric oxide synthase (NOS)¹⁵ and/ or the calcium-binding protein parvalbumin (PV)¹⁴. However, no single molecular criterion has been shown yet that distinguishes adult Betz cells from other layer 5 extratelencephalic projection neurons¹⁶.

There is a large body of evidence to indicate that the local vasoactive intestinal polypeptide-positive (VIP⁺) interneurons exert inhibition onto excitatory pyramidal cells, either directly or indirectly, to shape their outputs^{17–22}. Here, we present evidence that staining M1 with antibodies against VIP reveals not only a subset

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of GABAergic interneurons, as expected from previous studies^{23–27}, but also Betz cells. The spatial and laminar distributions of VIP⁺ interneurons and Betz cells are compared.

Materials and methods

Animals

The study is reported in accordance with ARRIVE guidelines. Materials were sourced from nine healthy adult marmosets (*Callithrix jacchus*) as detailed in Table 1. The experiments were conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All experimental protocols were approved by the Monash University Animal Ethics Committee. The animals had no veterinary record of serious acute or chronic health conditions. Some of the animals were also used in an anatomical tracing experiment unrelated to the present study; for details of the tracer experiments, see^{28,29}.

Tissue perfusion and processing

The marmosets were euthanized with an overdose of sodium pentobarbitone (100 mg/kg, i.v.) and transcardially perfused with 0.1 M heparinized phosphate buffer saline (PBS; pH 7.2), followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. The brains were dissected and post-fixed in 4% PFA for one hour prior to immersion in PBS, with increasing concentrations of sucrose (10%, 20%, and 30%), over several days at 4 °C. The one-hour post-fixation was a critical step for VIP visualisation. Longer post-fixation times were not successful unless heat-induced antigen retrieval steps were used which produced variable degrees of success. For consistent staining across cases, we exclusively used one-hour post-fixation for data presented in this study. Using a cryostat, frozen 40 µm coronal sections were obtained. Five sequential series were collected, with the first two being stained for myelin³⁰ (modified version³¹), and for cell bodies (Nissl stain). These series were used to delineate boundaries of the motor cortex⁴. The other series were used for immunostaining, as detailed in Table 1. In cases also used for anatomical tracing, the sections used for the present analyses were sourced from the hemisphere contralateral to the tracer injections.

Immunostainings were conducted by incubating sections in blocking solution (10% normal horse serum and 0.3% Triton X-100 in 0.1 M PBS) for 1 h at room temperature before undergoing incubation in primary antibody at 4 °C for 42–46 h (Table 2). For immunohistochemistry using DAB (IHC), a biotinylated horse anti-mouse IgG secondary antibody incubation was applied for 30 min, followed by treatment with Avidin–Biotin Complex (ABC) reagent and DAB (3,3'-Diaminobenzidine) substrate working solution. Alexa Fluor secondary antibodies were used for immunofluorescence staining (IF) (Table 2).

To test for the specificity of anti-VIP primary antibodies, we ran control immunohistochemistry experiments in which either the primary antibodies were removed (Supplementary Fig. S1) or pre-absorbed with the VIP peptide (absorption test, Supplementary Fig. S2). Pre-absorbed VIP primary antibody was made through incubation with the VIP peptide (Cat# ABIN2958145) in the blocking solution for 24 h at 4 °C (molar ratio of peptide to antibody; 100: 1 for Cat# L108/92 and 400:1 for Cat# HPA017324). Sections were incubated in the pre-absorbed antibody solution for 42–46 h, followed by the application of secondary antibodies as described above (Supplementary Fig. S2).

Subsequently, Aperio Scanscope AT Turbo (Leica Biosystems) was used to scan myelin, Nissl, and IHC stained sections. Fluorescent images from the motor cortex were captured using a Nikon C1 laser scanning confocal microscope.

Quantification of VIP+ neurons and statistical analysis

The three distinct regions of the primary motor cortex, namely areas 4a, 4b (encompassing representations of the axial and limb musculatures), and 4c (the representation of the head musculature), along with the caudal subdivisions of the dorsal premotor area (A6DC) were delineated using previously defined criteria^{4,32}.

Four VIP-stained sections were selected from each subdivision of the primary motor cortex from three cases that underwent IHC (CJ226, CJ227, and CJ240). Using standard features of the Aperio Image Scope software, square counting frames (150 × 150 µm) were aligned across the cortical thickness. The uppermost frame was

Subject (sex)	Neuronal marker	Staining method	Age at perfusion (months)
CJ195 (M)	PV	IHC	43
CJ200 (F)	PV, CB, CR, NeuN	IHC	43
CJ205 (M)	CB, CR	IHC	43
CJ226 (F)	VIP, NeuN	IHC/IF	24
CJ227 (M)	VIP, NeuN	IHC/IF	37
CJ232 (F)	VIP, PV	IF	30
CJ240 (M)	VIP, NeuN	IHC/IF	36
CJ249 (F)	VIP, GABA	IF	36
F1741 (F)	CB, CR, NeuN	IHC/IF	40

Table 1. Details of sex, age, and staining. PV parvalbumin, CB calbindin-D28k, CR calretinin, VIP vasoactive intestinal polypeptide, NeuN neuronal nuclei, GABA gamma-aminobutyric acid, IHC immunohistochemistry using DAB, IF immunofluorescence.

Staining method	Primary antibody	Source and identifier	Secondary antibody	Source and identifier
Parvalbumin (PV)-IHC	Mouse monoclonal (1:8000)	Swant Cat# 235; PRID: AB_10000343	Secondary antibody (Mouse IgG, 1:200) and Vectastain elite ABC HRP kit; DAB substrate kit	Vector laboratories Cat# PK-6102; RRID: AB_2336821; vector laboratories Cat# SK-4100; RRID: AB_2336382
Calbindin-D28k (CB)-IHC	Mouse monoclonal (1:8000)	Swant Cat# 300; RRID: AB_10000347	Same as above	Same as above
Calretinin (CR)-IHC	Mouse monoclonal (1:8000)	Swant Cat# 6B3; RRID: AB_10000320	Same as above	Same as above
Neuronal marker (NeuN)-IHC	Mouse monoclonal (1:800)	Millipore Cat# MAB377; Clone A60; RRID: AB_2298772	Same as above	Same as above
Vasoactive intestinal polypeptide (VIP)-IHC	Mouse monoclonal (1:500)	Abcam Cat# ab30680; RRID: AB_778830	Same as above	Same as above
VIP-IF	Rabbit polyclonal (1:200)	Atlas antibodies Cat# HPA017324; RRID: AB_1858754	Donkey Anti-Rabbit IgG H&L (Alexa Fluor* 594) preadsorbed (1:600)	Abcam Cat# ab150064; RRID: AB_2734146
VIP-IF	Mouse monoclonal (1:500)	NeuroMab Cat# L108/92; PRID: AB_2651163	Donkey Anti-Mouse IgG H&L (Alexa Fluor* 488) preadsorbed (1:600)	Abcam Cat# ab150109 RRID: AB_2571721
GABA-IF	Rabbit polyclonal (1:500)	Sigma-Aldrich Cat# A2052; RRID: AB_477652	Donkey Anti-Rabbit IgG H&L (Alexa Fluor* 594) preadsorbed (1:600)	Abcam Cat# ab150064; RRID: AB_2734146
PV-IF	Mouse monoclonal (1:500)	Swant Cat# 235; PRID: AB_10000343	Donkey Anti-Mouse IgG H&L (Alexa Fluor* 488) preadsorbed (1:600)	Abcam Cat# ab150109 RRID: AB_2571721
NeuN-IF	Rabbit polyclonal (1:700)	Millipore Cat# ABN78; RRID: AB_10807945	Donkey Anti-Rabbit IgG H&L (Alexa Fluor* 594) preadsorbed (1:600)	Abcam Cat# ab150064; RRID: AB_2734146

Table 2. List of primary and secondary antibodies used for immunostaining.

aligned at the border of layers 1 and 2. The frames were equidistant (10 μm apart) and covered all cortical layers up to the white matter³³. The number of counting frames varied depending on the thickness of the cortex in each section. Each counting frame was bounded by 2 exclusion lines (left and bottom) and 2 inclusion lines (right and top), as previously described³³. A cell was counted if it was completely within the counting frame or if the cell body contacted the inclusion lines.

The number of counted cells per frame was averaged in each section and used to calculate the neuronal density. The section thickness (40 μm) and a shrinkage factor of 0.801 obtained in a previous study³⁴ were considered when calculating the number of cells per volume unit. For the analysis presented in Fig. 5e, we measured the VIP fluorescence intensity of 8 neurons from each type (Betz cell or interneuron) in one section as a relative quantity, using built-in functions of ImageJ software. We further calculated the corrected total fluorescence [CTF = integrated density – (area of selected cell $\times$ mean fluorescence background)] (Fig. 5f), to best assess the amount of VIP expression in these two cell types. Integrated density is the sum of the values of the pixels in the selected part of the image, i.e. cell soma. Mean fluorescent background was obtained by averaging the value of fluorescence intensity across random points in the area between cells.

Statistical analysis was conducted using GraphPad Prism v9.2.0 (Graph-Pad Software, La Jolla, CA, USA) and involved the use of unpaired t-test or one-way ANOVA followed by post hoc Tukey's tests. Statistically significant differences were those with a p-value < 0.05. Figures were created using Adobe Illustrator.

Results

Immunostaining against VIP in the motor cortex led to the visualisation of giant Betz cells of layer 5, as well as a subset of putative inhibitory neurons (Fig. 1). While the expression of VIP in interneurons has been demonstrated in other species^{22–25,35}, our data also indicates weak VIP expression in Betz cells, which was confirmed using three different antibodies and validated by control experiments involving the removal of the primary antibody (Table 2 and Supplementary Fig. S1) and absorption test (Supplementary Fig. S2). The VIP antibodies used in different analyses are given in the corresponding figure legends.

Betz cells

VIP staining intensity was strongest in the largest layer 5 pyramidal cells of A4ab, which are located more medially in this area, as defined by Nissl and myelin staining (Fig. 1a–c)⁵. This region corresponds to the representations of the leg and trunk musculatures⁴. The locations of stained Betz cells are shown by black circles in Fig. 1d. VIP staining intensity in Betz cells diminishes toward the more lateral parts of A4b (Fig. 1e–j). The cell body and apical dendrites of Betz cells were clearly visible in the VIP staining, similar to the observations with Nissl staining, with a more pronounced delineation of the apical dendrites (Fig. 1e–j). There was no VIP staining in layer 5 pyramidal cells of cytoarchitectural areas A4c and 6DC (Fig. 2), which lack the gigantopyramidal characteristics^{4,32}.

We confirmed the weak presence of VIP staining for Betz cells by colocalization with the neuronal marker (NeuN), and the calcium-binding protein PV (Fig. 3). PV-positive (PV⁺) axonal endings, which are known to

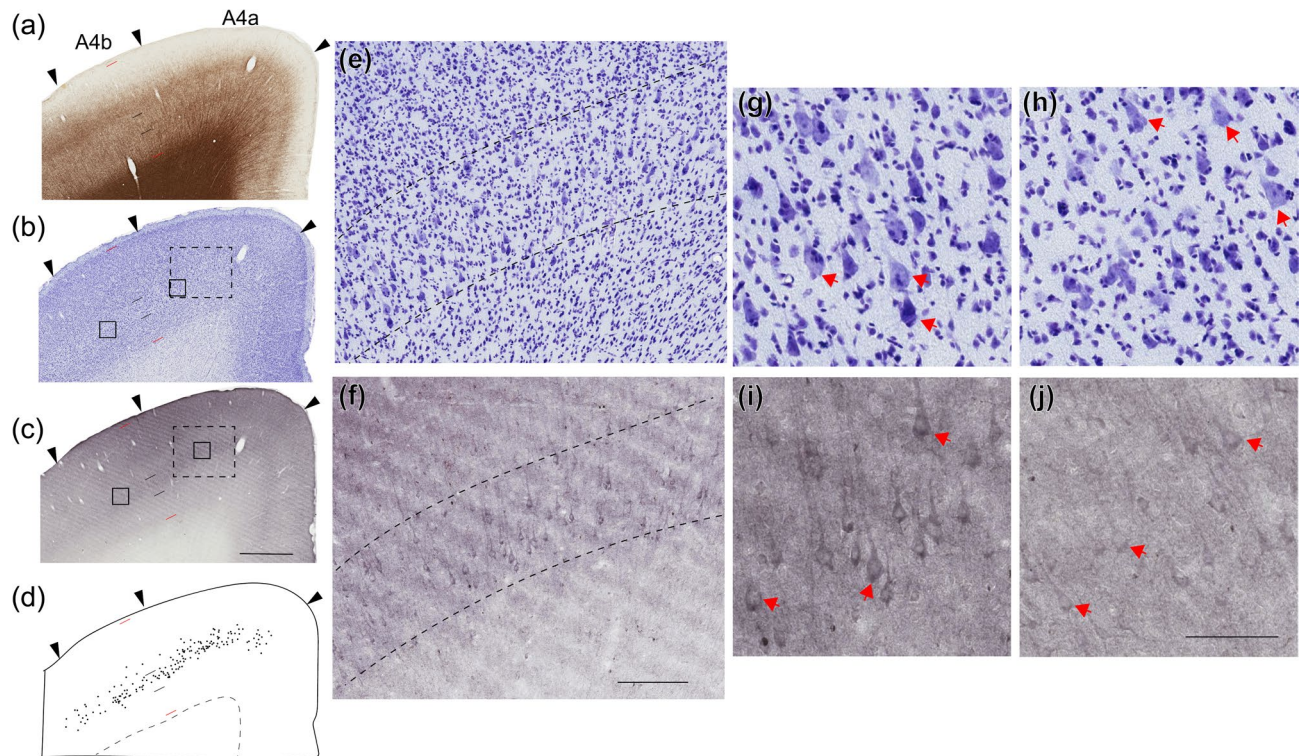


Fig. 1. Vasoactive intestinal polypeptide (VIP) is expressed in giant pyramidal (Betz) cells of primary motor cortex. Representative images of primary motor cortex (M1, Area 4a and Area 4b) are shown using myelin (a), Nissl (b) and VIP staining (c) in CJ226. Arrowheads point to the areal boundaries⁵. Red lines identify layers 2 to 6, while layer 5 is indicated by small black lines in (a–d). Schematic of stained layer 5 Betz cells is indicated in (d). Dashed rectangles in (b,c) are shown at higher magnification in (e,f) in which Betz cells are clearly visible within layer 5 (between the two dashed lines). Solid rectangles in (b,c) are magnified in (g,i) representing A4a and in (h,j) representing A4b. Red arrows point to Betz neurons. Scale 1 mm for (a–d), 200 μ m for (e,f) and 100 μ m for (g–j). VIP antibody used in production of images is from Abcam (Cat# ab30680).

heavily innervate Betz cells¹⁴, were very evident in our material, forming perisomal networks that delineated the Betz cell bodies (Figs. 3 and 4). We also found that PV is present in the soma of a subset of Betz cells in marmoset (Fig. 4), as reported previously in the human brain¹⁴. The PV signal was far less intense in Betz cell bodies, compared to interneurons or PV⁺ terminal nets delineating soma boundaries, but was clearly distinguishable from the background, and particularly evident by comparison with PV negative (PV⁻) Betz cells. Examples of PV⁺ and PV⁻ Betz cells alongside interneurons are shown in Fig. 4. The two other calcium-binding proteins tested, calbindin D28-K (CB) and calretinin (CR), were not expressed within Betz cells (Fig. 4). This is despite the known expression of CB in some populations of pyramidal cells in primates, including in the marmoset^{36,37}. The largest of Betz cells reached up to 25 μ m in soma diameter in the marmoset brain (Figs. 3 and 4).

VIP⁺ inhibitory neurons

VIP staining revealed a subset of inhibitory neurons in the marmoset motor cortex (Fig. 5a–c). VIP⁺ interneurons had different morphologies likely including bipolar cells, double bouquet cells, and bitufted cells³⁸ (Fig. 5a). Co-staining for VIP and GABA confirmed the inhibitory nature of VIP⁺ interneurons (Fig. 5b,c). The arbitrary fluorescence intensity was much higher in the VIP⁺ interneurons than in VIP⁺ Betz cells (Fig. 5d,e, unpaired t-test; 3165 ± 281 vs. 1333 ± 125 , $p < 0.0001$). However, considering the somatic area, the corrected total fluorescence within the cell bodies was similar (Fig. 5f, unpaired t-test, $18,347 \pm 2473$ vs. $14,404 \pm 258$, $p = 0.16$).

In all subfields of the primary motor cortex, VIP⁺ inhibitory neurons were mostly concentrated in the supragranular layers and their density gradually decreased toward layer 6 (Fig. 6a–c). The mean density of VIP⁺ interneurons was significantly higher in A4c compared to the other subdivisions of primary motor cortex [Fig. 6d, one-way ANOVA: $F(2, 33) = 21.26$, $p < 0.0001$; Tukey's test; A4c ($4745 \pm 146/\text{mm}^3$) vs. A4a ($3065 \pm 224/\text{mm}^3$) or A4b ($3410 \pm 199/\text{mm}^3$), $p < 0.0001$]. VIP⁺ interneurons corresponded to 4.5, 5.2, and 6.6% of the total neuronal population in cytoarchitectural subdivisions A4a, A4b, and A4c, respectively (Fig. 6e).

Discussion

The primary motor cortex is not only involved in motor control, but also in sensory integration, behavioural strategising, working memory, and decision-making³⁹. Its susceptibility in several diseases, including neurodegenerative conditions and ageing^{40–44}, warrants further understanding of its neurochemistry. Here, we demonstrate that VIP, a peptide with widespread distribution in many organs, is expressed in the largest excitatory pyramidal

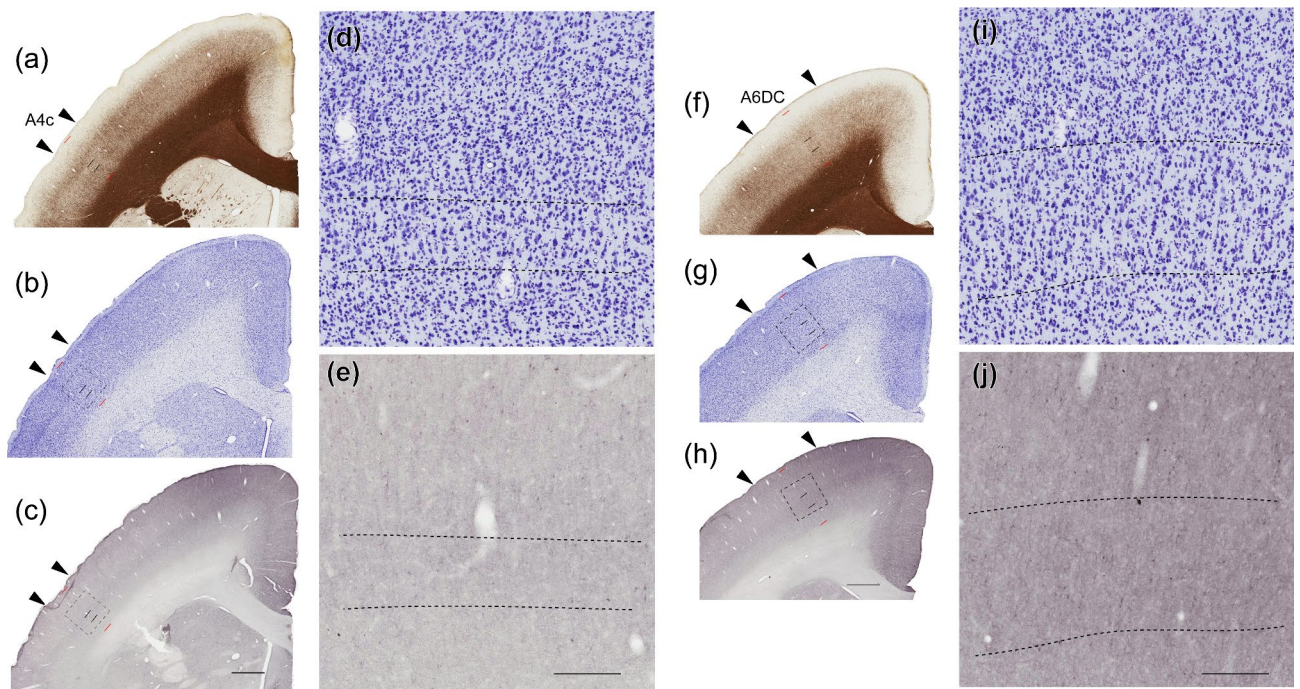


Fig. 2. Expression of vasoactive intestinal polypeptide (VIP) is limited to the largest layer 5 pyramidal cells. Representative images of Area 4c (**a–e**) and A6DC (caudal subdivisions of the dorsal premotor area, **f–j**) are shown using myelin (**a,f**), Nissl (**b** and **g**) and VIP staining (**c** and **h**) in Cj226. Arrowheads point to the areal boundaries⁵. Red lines in (**a–c** and **f–h**) identify layers 2–6, while layer 5 is indicated by small black lines. Dashed rectangles in (**b,c,g,h**) are shown at higher magnification in (**d,e,i,j**), respectively. Large pyramidal cells are visible within layer 5 (between the two dashed lines) only in Nissl staining (**d,i**). Scale 1 mm for (**a–c**) and (**f–h**), 200 μ m for (**d–e**) and (**i–j**). VIP antibody used in production of images is from Abcam (Cat# ab30680).

neurons of the primate neocortex, adding to the intricacy of distinctive gene expression and physiological features of the giant layer 5 Betz cells⁶. This finding extends VIP expression to a new class of cortical neurons, raising new questions about the physiological role of this peptide in excitatory neurons of the motor cortex.

The specificity of VIP staining was confirmed using three different primary antibodies. Control experiments, in which these antibodies were omitted, along with an absorption test supported the specificity (see Supplementary Figs. S1 and S2), ruling out alternative explanations such as non-specific background staining. Our findings are supported by transcriptomic data in humans, suggesting the weak presence of VIP mRNA in cortical pyramidal cells⁴⁵, including layer 5 extratelencephalic projecting glutamatergic cortical neurons of motor cortex (<https://cellxgene.cziscience.com/gene-expression>).

VIP expression in Betz cells

VIP is widely distributed in the nervous system as well as in many other peripheral organs, acting both as a neuromodulator and neuroendocrine releasing factor^{46–48}. In the brain, VIP regulates the secretion and expression of neurotrophic factors^{49,50}, has neuroprotective and anti-inflammatory effects^{46,48}, and is up-regulated in neurons and immune cells after injury and/or inflammation^{48,51}.

Owing to VIP's versatile and widespread effects, it is currently difficult to pinpoint its possible role in Betz cells without additional functional assessments. However, the particularly large size of Betz cells may imply unique metabolic/homeostatic demands, for which VIP may contribute by having neuroprotective effects.

Betz cells, with their large body size and long myelinated axons, are amongst the most vulnerable neurons⁵², which are affected in ageing and motor neuron disease^{40,42,44}. High energy requirement and reliance on long distance axonal transport, along with a large cell surface area, increases exposure to undesirable factors⁵³, likely rendering Betz cells in need of extra regulatory protection. In line with this hypothesis, it has been shown that VIP mRNA is induced in facial motor neurons of mice following the application of an inflammatory stimulus directly to the nerve⁵¹. Moreover, the expression of PV in Betz cells^{14,54} may add another layer of protection through calcium regulation. Consistently, the proportion of PV-containing Betz cells decreases with age in humans⁵⁵, making them more susceptible to degeneration⁴².

The presence of VIP in the largest pyramidal cells may be related to the high demands of these cells for energy substrate. VIP induces concentration-dependent glycogenolysis in mouse cortical slices, which may indicate a regulatory mechanism for the local control of energy metabolism⁵⁶. Such a hypothesis is consistent with the maximal VIP staining in the largest pyramidal cells, which are located more medially in A4ab. Overall, the expression of VIP in Betz cells was weak and whether they actually produce VIP and use it as a neurotransmitter or neuromodulator needs to be clarified by further studies.

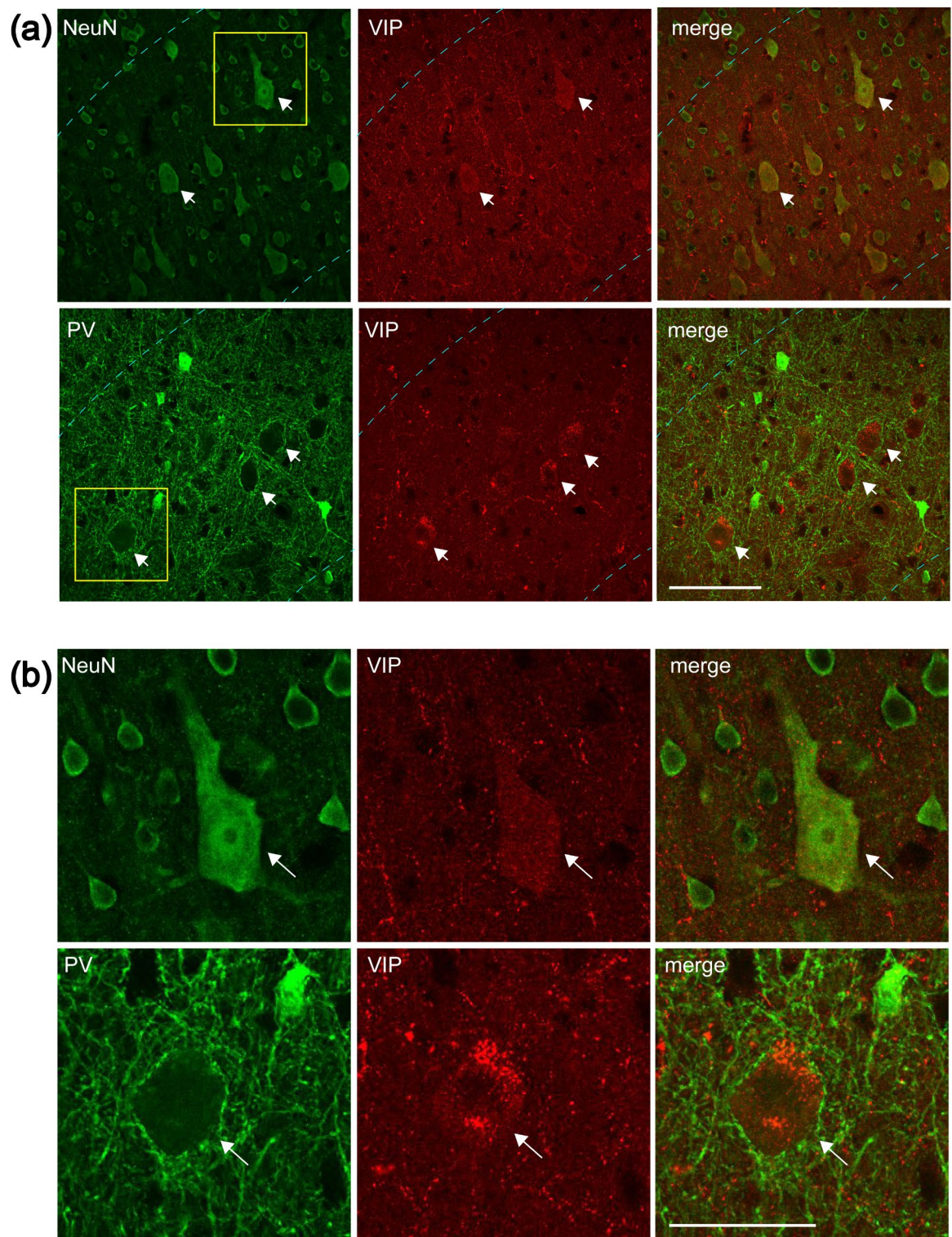


Fig. 3. Co-localisation of vasoactive intestinal polypeptide (VIP) with a neuronal marker (NeuN) and parvalbumin (PV) in the Betz cells. **(a)** Confocal images of cortical layer 5 showing immunoreactivity to VIP either with neuronal marker NeuN (top) or PV (bottom). The two cyan dashed lines identify layer 5. **(b)** Insets at higher magnification. White arrows point to the Betz cells in all images. Scale 100 μm **(a)** and 50 μm **(b)**. VIP antibody used in production of images is from Atlas Antibodies (Cat# HPA017324).

VIP⁺ interneurons in the motor cortex

VIP⁺ interneurons were primarily located in the supragranular layers of the cortex in the areas examined in this

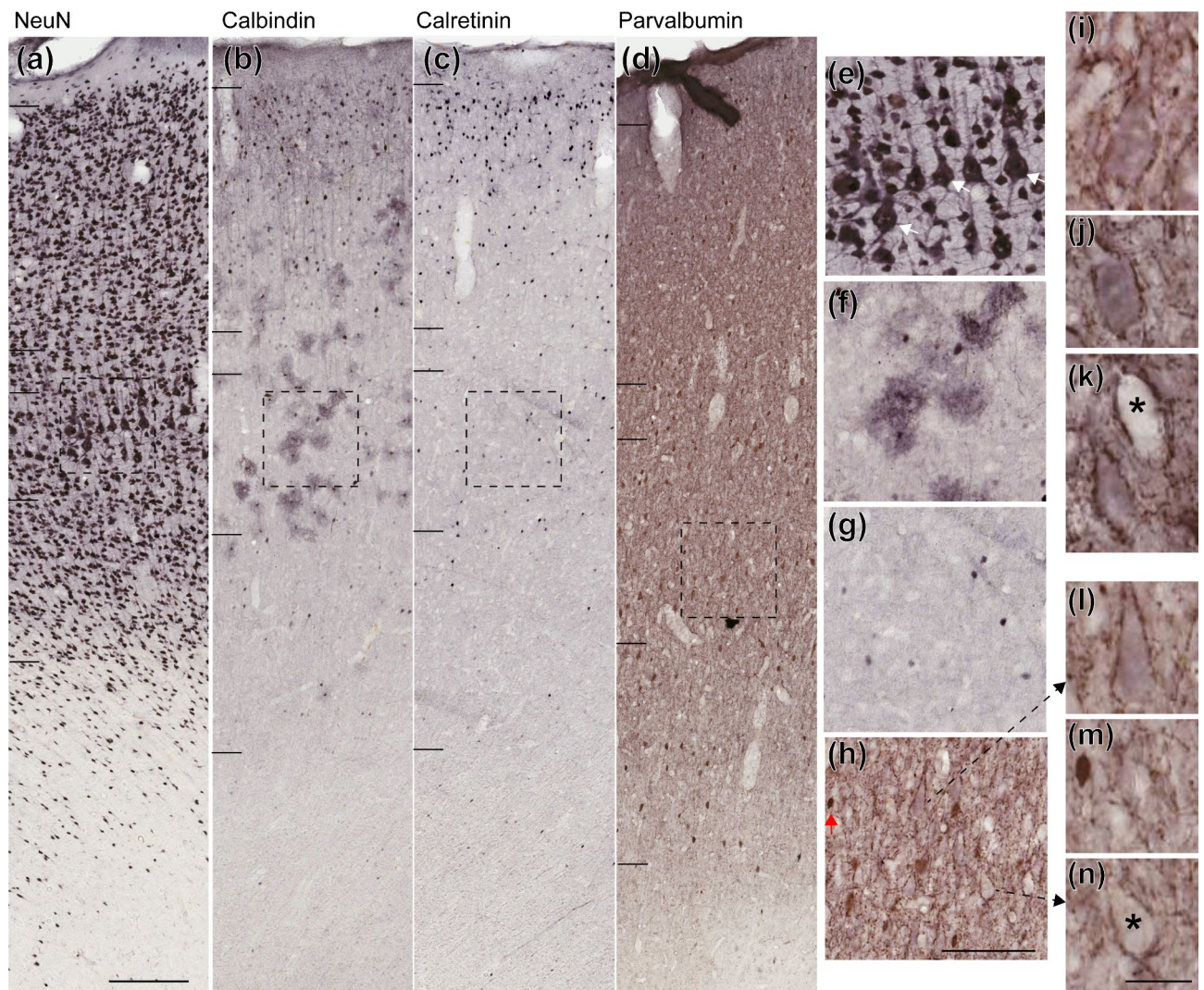


Fig. 4. Giant pyramidal cells of the primary motor cortex are identified by parvalbumin (PV) but no other calcium binding proteins. Examples of NeuN (**a**), calbindin-D28k (**b**), calretinin (**c**) and parvalbumin (**d**) staining of area 4A. The dashed squares in (**a–d**) are shown in higher magnification in (**e–h**), respectively. White arrows in (**e**) point to Betz cells. Betz cells in (**i–k**) are taken from sections not shown here and those in (**l,n**) are magnified from panel **h**. Asterisks (in panels **k** and **n**) point to Betz cells that do not contain PV in their soma. PV content is also compared to an interneuron visible in (**m**), highlighted by a red arrow in panel (**h**). Scale 200 μm for (**a–d**), 100 μm for (**e–h**) and 25 μm for (**i–n**).

study, similar to observations in other species^{23–26,35}. VIP⁺ interneuron density across cortical layers showed a sharp decline toward the white matter, with far fewer VIP⁺ interneurons present in the infragranular layers. Supra- and infragranular VIP⁺ interneurons may have different functional roles as the two populations are transcriptionally distinct in mice⁵⁷. Although the drop in the density of VIP⁺ interneurons in the infragranular layers was consistent in the three examined areas, the mean density of VIP⁺ interneurons and their relative proportion to the total neuronal density were higher in A4c compared to the areas 4a and 4b. This may reflect regional differences in network organisation for facial versus limb and body movements. Together with the differences in expression of VIP in Betz cells, this result further highlights the fact that functionally defined cortical areas are not necessarily homogeneous, a principle which has been demonstrated in terms of cellular structure⁵⁸ (including present results), connections^{59,60} and physiological properties^{61,62}.

Overall, we report a 5–7% contribution of VIP⁺ interneurons to the neuronal population in the motor cortex in the marmoset monkeys. Our density estimates for VIP⁺ interneurons in the motor cortex (3065–4745/mm³) are 50–100% higher than those in the mouse motor cortex²⁶. Based on the 23% prevalence of GABAergic interneurons in marmoset's motor cortex⁶ and total neuronal density³³, we estimate that VIP⁺ interneurons of the motor cortex constitute around 20–29% of the total interneuron population. These estimates are higher than those reported in several cortical areas of mice, which are around 11–15% of total GABAergic neurons^{22,25,63,64}. Analysis of single-nucleus RNA expression in marmosets suggests that although the relative percentage of VIP⁺ neurons is much smaller in A4ab compared to prefrontal or somatosensory cortices, they constitute the second

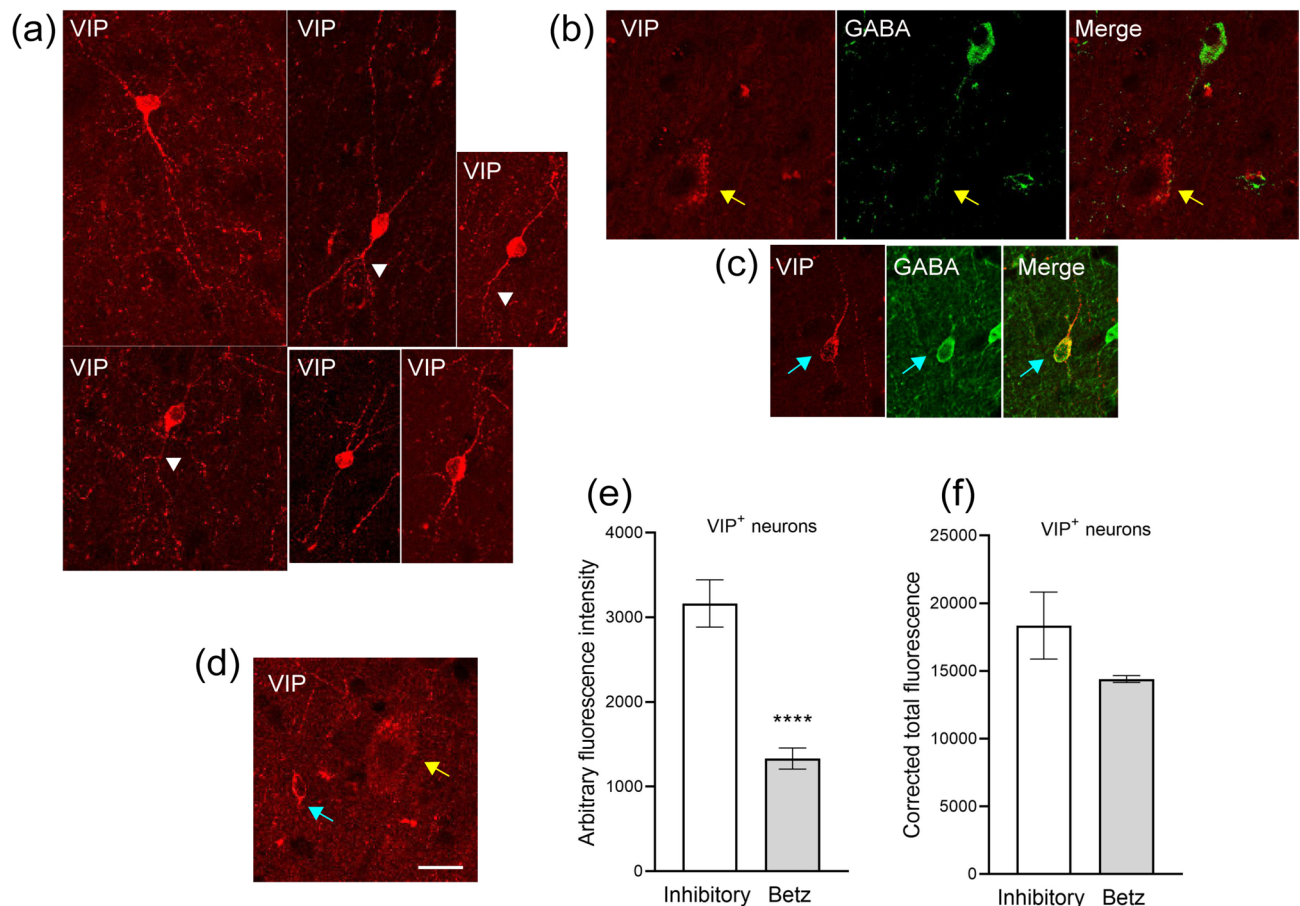


Fig. 5. Antibody against vasoactive intestinal polypeptide (VIP) stains inhibitory interneurons and Betz cells. Representative images of different morphologies of VIP-positive (VIP⁺) inhibitory neurons of the primary motor cortex (a). White arrowheads point to the dendritic bifurcations. Inhibitory nature of VIP⁺ interneurons is shown by co-localisation with gamma aminobutyric acid (GABA) that is missing in Betz cells (b,c). Fluorescence intensity comparison for a VIP⁺ inhibitory neuron and Betz cell (d). Cyan and yellow arrows point to VIP⁺ inhibitory neurons and Betz cells, respectively in b–d. Mean $\pm$ SEM density of arbitrary fluorescence intensity obtained from variable somatic locations (e) and corrected total fluorescence for somatic area (f) for VIP⁺ neurons. Scale bar in (d, 20 μ m) applies to all fluorescence images. VIP antibodies used in production of images are from Atlas Antibodies (Cat# HPA017324, a,d) and Neuro Mab (Cat# L108/92, b,c).

highest population of interneurons in A4ab²⁷. The observed morphologies of VIP⁺ interneurons in the marmoset motor cortex were similar to those reported in other species^{23,38,65}.

In analyses that take into consideration the soma size of neurons, the arbitrary fluorescence intensities in the VIP⁺ interneurons were similar to the Betz cells, indicating a comparatively similar expression of VIP. While the modes of connectivity of VIP⁺ interneurons with Betz cells remain to be elucidated, current literature suggests that VIP⁺ interneurons provide inhibitory inputs to pyramidal neurons mainly through forming disinhibitory circuits^{17,19,64}. However, direct inhibition of pyramidal neurons by this population has also been reported^{18,20,21}.

The abundance of studies on VIP⁺ interneurons in rodents has shed light on the specific roles of this neuronal population, which include motor integration^{17,66,67} and gain control¹⁹. However, we are far from attaining a similar understanding of their function in primates, where only limited studies have been made available^{23,27,68,69}, possibly due to the technical difficulty of successful staining for VIP, including visualisation of interneurons. Here, we have determined that reliable VIP staining in the marmoset can only be achieved with short periods of post-fixation, an observation that may facilitate further studies.

Conclusion

The distribution of VIP⁺ neurons in the marmoset motor cortex demonstrates that this neuropeptide is expressed both in interneurons and in large pyramidal cells with putative projections to the spinal cord (Betz cells). The specific presence of VIP in Betz cells hints at a different set of functions for this neuropeptide, as well as differences in neuronal circuitry involved in generating and controlling different types of body movements.

Data availability

All data generated or analysed during this study are included in this published article.

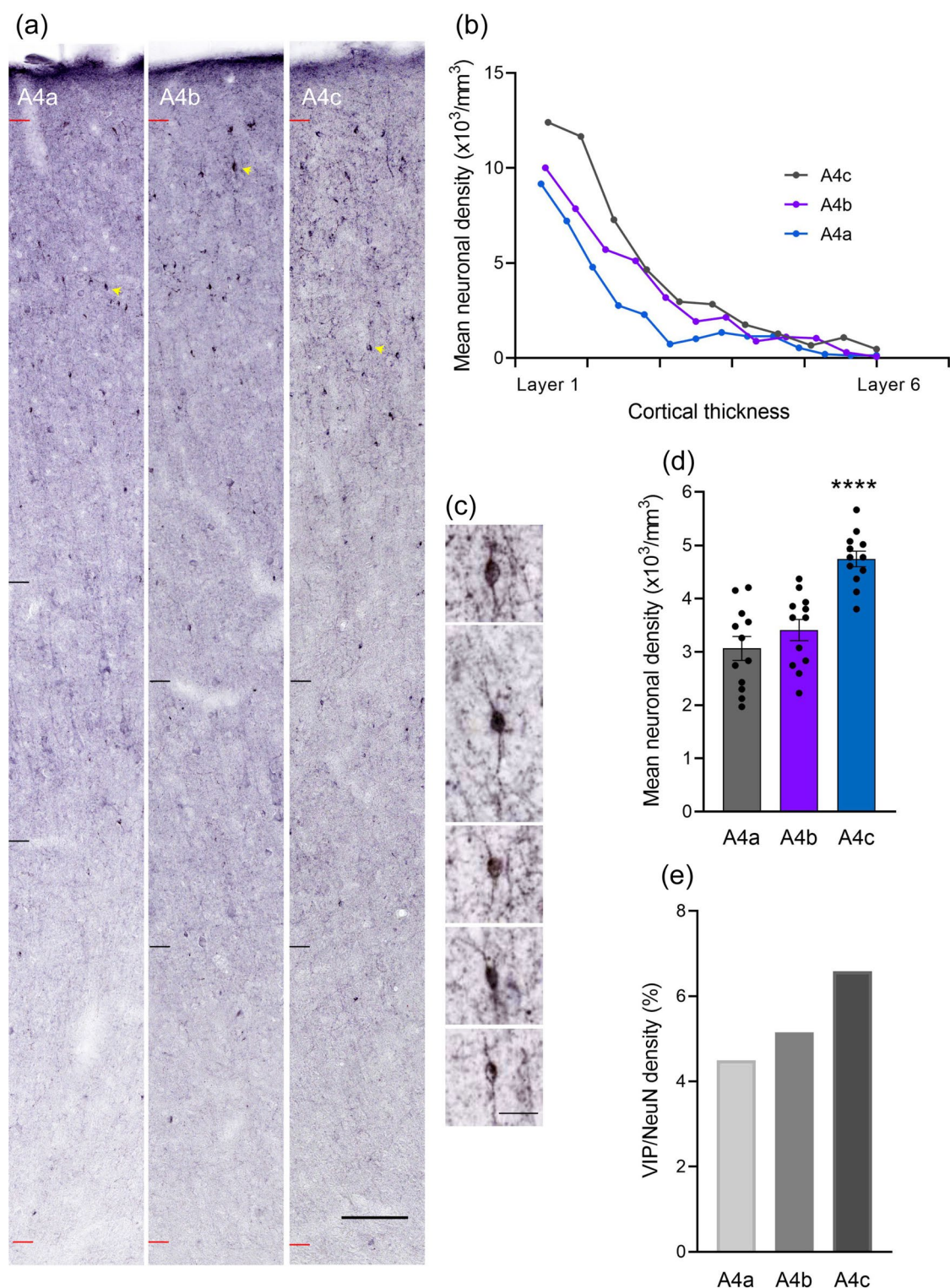


Fig. 6. Layer profile of vasoactive intestinal polypeptide-positive (VIP⁺) interneurons of the primary motor cortex. Representative VIP-stained strips from different parts of the primary motor cortex including areas A4a, A4b and A4c (a). Individual cells are indicated by yellow arrows. Small red lines identify the border with layer 2 and white matter. Limits of layer 5 are shown by small black lines. Quantification of VIP⁺ interneuron density across cortical depth (b). Some VIP⁺ interneurons are shown at higher magnifications in (c). Scale 100 μm in (a) and 20 μm in (c). Mean $\pm$ SEM density of VIP⁺ interneurons (d) and its percentage, as ratio of total neurons stained by NeuN in our previous study³³ (e). Black circles in (d) represent values obtained from individual sections. VIP antibody used in production of images is from Abcam (Cat# ab30680).

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Author contributions

N.A. and M.G.P.R. designed the experiments. S.T., K.H.W. and N.A. performed the experiments. N.A. and S.T. analysed the data and N.A., M.G.P.R. and S.T. wrote the manuscript. All listed authors have approved the final manuscript before submission.

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Competing interests

The authors declare no competing interests.

Ethics approval

The experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All procedures were overseen by the Animal Ethics Experimentation Committee at Monash University.

Consent to participate

All listed authors agreed to participate in this study.

Additional information

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