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# **Projections, Partaken Circuits and Axon Initial Segments of Cortical Principal Neurons**

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Additional information is available at the end of the chapter

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## **1. Introduction**

The axon initial segment (AIS) is the portion of the neuron immediately distal to the axon hillock. The AIS has a specialized membrane that works for a manifold function. The first portion of the AIS membrane possess a collection of ion channels that allows for the modulation of the membrane potential of the parent cell whilst blocking the back propagation of the axon potential. The last portion of the AIS membrane possesses in addition voltage-dependent ion channels that are responsible for the ultimate display of the cell, that is, the generation of the axon potential. Much has been investigated recently on the ion channels that are embedded in the AIS membrane of nerve cells. Yet basic parameters such as the length and diameter of the AIS and, of no lesser importance, the number and distribution of boutons synapsing the AIS membrane remains largely unknown for distinct subpopulations of principal cells of cerebral cortex. Principal cells are heterogeneous in many anatomical, molecular and functional aspects but, in agreement with their distinctive possession of combinations of these aspects, they can be classified in different subpopulations. Taking as core features for this grouping the cell laminar address and the pattern of axon projection, this paper reviews subtypes of cerebral cortical principal cells and their AIS features. In doing so, this paper also presents an account of our past and present research on the AIS of principal cells in visual and other areas of cerebral cortex. Yet, aiming to furnish a background to the function of the AIS of cerebral cortical principal cells, we shall begin by reviewing the cellular types and circuits in cortex, and the axon projections arising from the cortex.

## **2. Cerebral cortical neurons**

Spiny neurons are of an excitatory nature and most have extrinsic axons, that is, axons that project outside the cortical area where their somata lie. These cells employ glutamate or

predominantly aspartate as a neurotransmitter (for percentages of aspartergic and glutamatergic cells out of projection cell subpopulations to several telencephalic and extratelencephalic targets, see [1]). By contrast, aspiny neurons are inhibitory, and most of their axons are intrinsic, i.e. they use gamma-amino-butyric acid (GABA) as a neurotransmitter and their axons remain within the cortical area in which the parent cell soma lies. They are usually named interneurons.

## 2.1. Interneurons

Interneurons are present in all cortical areas and layers and represent approximately 10%–20% of cortical neurons in rats [2] or 15%–30% of the total population in other species [3]. Interestingly, while in the occipital, parietal and frontal cortex of the rat the same proportion of GABAergic neurons among all neurons was found (15%, in [2]), the numerical density of all neurons in the frontal cortex (34,000 per cubic millimetre) was significantly lower than those in the occipital and parietal regions (52,000 per cubic millimetre and 48,000 per cubic millimetre, respectively) [2]. The fixed proportion of interneurons, irrespective of the number of neurons, is in keeping with the idea of the uniformity of cortical circuits. However, this does not exclude the possibility that the proportion of the different types of interneurons might vary in each cortical area, resulting in regional specialization of inhibitory circuits.

Interneurons show great morphological, biochemical and physiological diversity. However, interneurons with the same morphology may have different biochemical characteristics and connectivity [3]. Taken into account this consideration certain interneurons can be recognized by their unique morphological characteristics or they can be more generally divided in subgroups on the bases of their pattern of axonal arborization, synaptic connections (both with pyramidal cells or between themselves) and physiological and biochemical characteristics. One most accepted classification of interneurons is based on the domain of pyramidal neurons their axons target combined with the immunoreactivity for different calcium binding proteins and neuropeptides [4,5].

### 2.1.1. Axosomatic targeting interneurons

The axosomatic targeting interneurons are chandelier cells and basket cells. Chandelier cells target on the AIS of principal cells and basket cells target the somata and proximal portions of dendrites of principal cells. Both interneurons are likely to exhibit a greater impact on the direct output of postsynaptic neurons. An important number of them are fast-spiking interneurons, most of which being immunoreactive for the calcium binding protein parvalbumin. In turn, most parvalbumin-immunoreactive cells are fast spiking large basket cells and chandelier cells. Parvalbumin-immunoreactive cells in rodents account for 40%–50% of GABAergic neurons [6]. A much higher percentage (74%) was found in macaque visual cortex [7].

*Chandelier cells.* These cells are the only interneuron that shows clearly recognizable terminal axonal specializations, which form short vertical rows of terminal buttons, resembling candlesticks. These cells only synapse with the AIS of principal cells [8–10]. For this reason they were named axo-axonic cells. Chandelier cells have been found not only in the

neocortex, but also in several areas of the mesocortex, paleocortex and archicortex, which include the piriform cortex, entorhinal cortex, subiculum, hippocampus, fascia dentata, cingulate cortex, claustrum and amygdala [5,11]. Chandelier cells are present in all cortical layers (except layer 1), most abundantly in layer 2/3 [5,12,13]. A single chandelier cell can innervate between 250 (in neocortex) and 1,200 (in hippocampus) principal cells, indicating the potential to synchronize many principal cells [5,14]. Thus, chandelier cells appear ideally suited to shut off entire groups of pyramidal cells, making them the ultimate cortical switches. Each chandelier-terminal innervates single AIS. Moreover, each AIS may be innervated by one or a few chandelier terminals (five or less), which may originate from the same or different chandelier cells (for a review, see [14]). Chandelier cells are parvalbumin-reactive and fast-spiking cells.

*Basket cells.* Basket cells are most difficult to classify for their somatodendritic variety and sizes. The term 'basket' comes from the basket-like appearance of their axonal terminals around pyramidal cell somata that results from convergent innervation by many basket cells. About 50% of all inhibitory interneurons are basket cells [12]. Basket cells target the somata (15%-30% of their synapses are on somata) and proximal dendrites of principal cells; yet basket cells also target somata and dendrites of interneurons, particularly other basket cells [5,15,16]. There are *small basket cells* with a dense local axonal arborization that targets more dendrites than somata of principal cells; these small basket cells predominate in infragranular layers. *Large basket cells* are the typical basket cells. They have an extensive horizontal axonal branching with few vertical types of collateral. They are therefore the primary source of lateral inhibition across the cortical columns. Large basket cell predominate in layer 2/3. There are intermediate sizes of basket cells too. Basket cells can be parvalbumin-immunoreactive and fast spiking cells [6,16,17]. Other subgroup of basket cells are regular spiking; large- and medium-size basket cells are cholecystokinin immunoreactive neurons; small basket cells are immunoreactive for other calcium binding protein, or the vasointestinal neuropeptide; all this depends on the species studied [6,12,17].

### 2.1.2. Dendritic targeting interneurons

The dendritic targeting interneurons are more suited to modify and gate incoming excitatory inputs. The dendritic targeting interneurons are the bipolar cells, Martinotti cells, neurogliaform cells and double-bouquet cells. The latter cells target dendrites and spines.

*Double bouquet cells.* The axon of a double bouquet cell forms a tight fascicular axonal cylinder that can extend across all layers, innervating distal dendrites and spines. The highly varicose collaterals that form these columnar bundles are unusually thicker than the axonal main stem. While the morphology and distribution of double bouquet cells are similar in the human and macaque neocortex, these cells are modified or less numerous in the neocortex of other species (e.g. the cat), and may even be absent (e.g. the mouse and rat). Thus, differences in the morphology, number and distribution of double bouquet cells may represent fundamental differences in cortical micro organization between primates and other species [3]. Double bouquet cells usually are calbindin-immunoreactive. Together with being calbindin-immunoreactive, they can be also calretinin-immunoreactive. Other double

bouquet cells are vasointestinal-peptide-immunoreactive and still others, cholecystokinin-immunoreactive [3,5,12].

*Martinotti cells* have a prominent axonal projection to layer 1. They have many fine unmyelinated ascending axon collaterals, which fan out as they ascend, bearing 'en passant' boutons in intermediate layers; some of these collaterals reach and branch as well in layer 1. Many other interneurons have ascending axon collaterals, but the prominent axonal arbour in layer 1 distinguishes Martinotti cells. Martinotti cells lie in particular in the deep cortical layers but also in layer 3 [18]. The majority of Martinotti cells are somatostatin-immunoreactive.

*Bipolar cells.* These are small cells with spindle or ovoid somata and narrow bipolar (most often) or bitufted dendrites that extend vertically. These cells can be found in all layers but predominate in layer 2/3 and layer 6. Bipolar neurons can be excitatory by releasing only vasointestinal-peptide, or inhibitory by releasing mainly GABA (though inhibitory bipolar cells also express vasointestinal-peptide). Bipolar cells commonly express calretinin too. Their axon forms a narrow band that crosses all layers leaving a little proportion of terminals, mainly on the basal dendrites of principal cells [12].

*Neurogliaform cells.* These are small-sized 'button-type' cells with many fine, radiating dendrites that are short and aspiny, finely beaded and rarely branched. They form a highly symmetrical and spherical dendritic field. The axon can arise from any part of the soma or from the base of a dendrite, and shortly after its origin, it breaks up into a dense, intertwined arborization of ultra-thin axons with as many as ten orders of branching. Fine boutons are distributed on the axonal collaterals to form GABA synapses onto the dendrites of target cells. The molecular characteristics of neurogliaform cells are still not well understood [3,12].

## 2.2. Spiny neurons

In turn, the spiny group of cortical cell consists of several subgroups. Pyramidal neurons with upright somatodendritic orientation are by far the largest subgroup within the group of spiny cells. Upright pyramidal neurons are projecting cells of all cortical layers other than layer 1. Upright pyramidal neurons can be further subdivided (see below). In addition to upright pyramidal neurons, in layers 5-6 there is another collection of projection cells, of spiny nature too, the polymorphic-cell subgroup [19-23]. The spiny stellate neurons of layer 4 are an exception to the spiny-cell/extrinsic-projection correlation, because they are implicated in the canonical thalamo-cortical reciprocal circuit. They are directly innervated by thalamic axons and almost exclusively establish synaptic contact with the neighbouring layer 3. In this study we consider as a cerebral cortical principal cell any spiny neuron that extends an axon branch outside the cortical area where its soma is located.

### 2.2.1. Principal cells of intratelencephalic projection (inclusive of the type I cell, of striatal projection)

Principal neurons have been sub classified by the laminar position of the cell body, somatodendritic morphology, electrophysiology and axonal target [24]. For a review, see [25].

Concerning the axonal target, there are associative neurons of intracortical projection in the same hemisphere and commissural neurons that project to homotopic and heterotopic sites of the opposite hemisphere by way of the corpus callosum and anterior commissure. Principal cells of intratelencephalic projection encompass also those that can extend their axon to the striatum, claustrum, amygdala and other sites that are not out of the telencephalon.

Associative and commissural neurons exist in all layers but with differences concerning projection and species. A limited number of associative and commissural axons arise from spiny stellate cells of layer 4 and low layer 3 [26]. Most associative and commissural axons emerge from pyramidal cells sited in layers 2/3 and 5-6, and also from spiny inverted pyramidal cells and other polymorphic cells of layers 5-6 [23,27,28]. The axons of all these cells extend forward to innervate the layer 4 of cortical areas of higher-order hierarchy [23,27-30]. This is the so-called associative forward projection [31]. In turn, the associative backward projection arise from cells sited in layers 2/3 and 5-6 of higher-order areas to innervate layers 1 and, to a lesser extent, 3, 5 and 6 of areas of lesser-order hierarchy [23,27,32]. This layer-segregation of forward and backward projections indicates that they target very different neuronal elements in recipient regions [18].

Spiny inverted pyramidal cells are morphologically and numerically conspicuous among the cells of layers 5-6 furnishing the associative backward projection in rabbits and cats; these cells also originate an important proportion of commissural, cortico-claustral and cortico-striatal projections; they negligably project to extratelencephalic centres such as the geniculate nuclei, colliculi and pons [23,27,33]. See Table 1 for a summary and [28] for a review; see also Section 4 in this Chapter for more data on AIS of these cells.

Commissural axons result from a broad and anatomically diverse population of principal cells that are located primarily in layers 2/3, 5 and 6 of restricted areas; there are interspecies differences in the laminar address of commissural neurons; in ferrets, rabbits and rats the commissural neurons predominate in infragranular layers [23,26,27,34,35]. Commissural cells can be further defined based on patterns of collateral projections to the ipsi- and contralateral striata and cortices, as well as by the expression of combinations of molecular markers [36].

Despite having common morphologies and similar laminar distributions, the commissural and associative neurons have been reported to differ from each other in the rat, cat, and monkey neocortex; there, at least in adult animals, they constitute two separate populations of neurons that rarely have dual projections [26,34]. The expression of the orphan nuclear receptor Nurr1 is associated to layer 6 neurons projecting to the ipsilateral cortex, but not to those cells projecting to contralateral cortical regions [37]. Recent studies in mice motor cortex show that 4% of the commissural cells of layer 2/3 and 34% of layer 5 extends dichotomous axons to ipsilateral prefrontal cortex and contralateral motor cortex [38]. Also, in the rat sensory-motor cortex, there are bifurcated projections to associative and contralateral areas from cells of layers 5-6 [39].

Moreover, certain principal cells of layer 5 of rodents project to the striatum in addition to the ipsilateral and contralateral cortex [40-44]. Cells sited in layer 5 can also project to the ipsi- and



contralateral claustra in rodents and rabbits [23,33,43]. These cortico-claustral cells may branch to the ipsilateral and contralateral cortical areas and striata, in addition to the claustra, but not to the thalamus [43]. By describing genes that identify molecularly distinct subpopulations of commissural neurons, a recent work has cast more light on the heterogeneity of these cells [45].

Projection type	Visual cortex	Auditory cortex	Retrosplenial cortex
Cortico-cortical intrahemispheric projection, total	25%	—	Not studied
Cortico-cortical intrahemispheric backward projection to primary cortex from columns across cortical layers of secondary cortex	26%	Not studied	Not studied
Cortico-cortical intrahemispheric backward projection to primary cortex from a horizontal, extended cell band along the layer 5/6 border of secondary and primary cortex*	82%	Not studied	Not studied
Cortico-cortical intrahemispheric forward projection to secondary cortex from columns across cortical layers of primary cortex	7.5%	42 %	Not studied
Cortico-cortical intrahemispheric lateral projection from columns across cortical layers of associative cortex	31%	30%	Not studied
Cortico-claustral projection from primary cortex	83%	Not studied	Not studied
Cortico-claustral projection from secondary cortex	23%	24%	Not studied
Cortico-claustral projection	—	—	10%
Cortico-striatal projection	< 20%	< 20%	Not studied
Cortico-thalamic projection to lateral and medial geniculate nuclei	Null	Null	Not studied
Cortico-collicular projection	Null	Null	Not studied
Cortico-pontine projection	< 1%	Not studied	Not studied

**Table 1.** Percentages of spiny inverted cells of layers 5-6 out of the total number of principal cells of layer 5-6 of identified projection in cerebral cortex of rabbits [23,27,33] (The commissural projection is not included in this Table, but see [23,27].) All but one\* of these percentages were estimated taking into account all infragranular neurons within clear-cut columns of retrograde labelling that extended along the radial dimension of the cortex. (\*) Spiny inverted neurons make up the majority of cells within a horizontal band of cells located at the border between layers 5 and 6 of primary and secondary visual cortex of projection to the ipsilateral primary visual cortex. This band of retrogradely labelled cells extends for millimetres in the secondary visual cortex from the sites of injection of retrograde tracer in the primary visual cortex. This band is particularly cell-populated in brains after multiple injections of tracer; with single injections, labelled cells lie scattered for millimetres in the border between layer 5 and layer 6 [27,28]. These findings have shown that there is a highly convergent yet diffuse projection from the layer 5/6-border principal cells of secondary visual cortex to discrete points of the primary visual cortex. Spiny inverted neurons are the principal source of this type of projection. This widespread projection is distinct from the backward cortico-cortical projection from secondary to primary visual cortex that originates in discrete columnar patches of cells.

All of these neurons of layer 2/3 and 5 that extend axons to telencephalic centres may be grouped in the type I principal cells upon the condition that they have branches to the striatum [44]. It should be noted, nevertheless, that this name (type I) had been chosen for the neurons of projection to extratelencephalic (or sub-cerebral centres) [1,46], which introduces confusion in the terminology.

### 2.2.2. *Principal cells of extratelencephalic projection*

The principal cells of extratelencephalic projection typically lie in layers 5-6. They include the projection cells to the thalamus and multiple sites in the brain stem and the spinal cord.

#### 2.2.2.1. *Principal cells of extratelencephalic projection sited in layer 5 (type II multiple target principal cells and cortico-thalamic cells)*

Cortico-striatal axons of layer 5 cells can be collaterals not only of the type I [44] projection but also of the type II, extratelencephalic projection to regions such as the spinal cord, brain stem nuclei, pontine nuclei, colliculi, substantia nigra, zona incerta and subthalamic nucleus [20,40-44,47]. The latter projection arises from type II cells that in their origin may belong to the same kind of principal neuron with extensive sub-cortical projections that during development loses particularly some of them depending on the cortical area in which the parent soma lies, as it is the instance for the visual cortex [1]. As well, the type II projection leaves driver collaterals to the core cells of *higher-order nuclei* of the thalamus, which are parvalbumin-positive cells and in turn project to the cortical layer 4 [41,44,48]. Such a branching pattern has been demonstrated for the axonal pathways of visual, somatosensory, pre-limbic and motor cortex of rats, cats and monkeys [41,42,47-51]. The presynaptic boutons of the axon of type II cells of layer 5 are usually of a big size, typically bigger than those leaved by axons of type I intratelencephalic cells [44,51].

Although principal cells share numerous common features within layer 5, they are heterogeneous in their somatodendritic morphology [1,25,52]. Type II cells of layer 5 have a thick apical dendrite extending into cortical layer 1 with a prominent terminal tuft; these cells produce distinctive initial bursts of tonic firing in response to current injection [46,53-55]. Depending on the cell-body position in layer 5, type I cells are characterized by having an apical dendrite that can tuft in cortical layer 1 or ascend to this layer without tufting; type I cells tend to fire phasically [44,46,54-57]. In addition, cells of layer 5b of short apical dendrite may project to the thalamus and superior colliculus but not to the striatum [18].

Thus, at least three subtypes of principal cells of layer 5 can be tentatively classified in agreement to their projection: the type I cell of intratelencephalic projection; the type II cell of sub-cerebral projection with collaterals to the striatum and thalamus; finally, the cortico-thalamic cell of layer 5b, which can project to the superior colliculus but not to the striatum.

#### 2.2.2.2. *Principal cells of extratelencephalic projection sited in layer 6*

It is well known that some cells of cortical layer 6 innervate the thalamus leaving collaterals to cortical layer 4. This cortico-thalamic projection is originated in pyramidal cells but not in spiny inverted or other polymorphic cells of layer 6 (Table 1) [23,28]. This projection is



specific of the cortico-thalamo-cortical loop [5,51,58]. Remarkably, the cortico-thalamic projection of layer 6 cells can innervate both types of thalamic relay neurons, i.e. the first-order (core, parvalbumin-positive) and the higher-order (matrix, calbindin-positive) cells. Both types of relay neurons not only lie in *first-order relay nuclei* but also *higher-order or associative nuclei*—the relative number of first-order relay cells being predominant in first-order relay nuclei and vice-versa. Regardless their thalamic address, the first-order relay cells are driven by ascending axons to the thalamus and successively innervate the spiny stellate cells and small pyramidal cells of layers 4 and 3b of precise sites of corresponding cortical areas. The higher-order relay cells widely innervate principal cells of layers 1, 2 and 3a of several cortical areas [48,51]. The first-order relay neurons are driver cells to their innervated cortical cells whereas higher-order relay neurons would be modulator cells to their innervated cortical cells. In turn, cells of layer 6a of cortico-thalamic projection are modulatory cells to the core cells of *first-* and *higher order nuclei* of the thalamus whilst leaving collaterals to the thalamic reticular nucleus and cortical layer 4 cells. (It should be remembered here that the type II cells of layer 5 are driving cells to the core cells of the *higher-order nuclei* of the thalamus and do not leave collaterals to the thalamic reticular nucleus.) Layer 6b cells of cortico-thalamic projection innervate the core and matrix cells of *first-* and *higher order nuclei* of the thalamus and leave collaterals to cortical layer 5 and 6 layer. By this way, cortical output will influence the synchronous ascending first-order and high order thalamo-cortical pathways. In Ray W. Guillery's words, this is "*how anatomical pathways link perception and action*" [59]. At least in the visual primary area, the cortico-thalamic cells of layer 6a dominate numerically over cortico-thalamic cells of layer 6b. Cortico-thalamic cells of layer 6 have been estimated to be between 30%-50% of all cells of layer 6 neurons (see [60] for review).

### 3. Cortical intrinsic circuits

*Principal cells.* A solitary action potential in a single principal cell of layer 2/3 can trigger polysynaptic chains of activity, detected as excitatory postsynaptic potentials and inhibitory postsynaptic potentials in recorded neurons [61]. This reveals an extremely efficacious means of activity propagation in the cortical network. In human brain slices, a relatively high proportion of basket (20%) and chandelier (33%) neurons could be driven to threshold by a single principal cell spike, in stark contrast to an estimated 1% likelihood of finding polysynaptic events in rats [61,62]. The fact is that the intralaminar circuit of layer 2/3 is highly recurrent and dominates its own cells. Principal cells of layer 2/3 extend collaterals for several millimetres to form patchy connections in the layer in cats and monkeys [63,64]. On the contrary, the intralaminar circuit of layer 5 depends little of the input from its own layer [58]. Intralaminar circuits exist basically on all species studied. However, the weight of the layer 2/3 circuit could be lower in rodents and rabbits because they do not have a patchy pattern connection in layer 2/3 but in layer 5b-6a [27,65].

Only the type I of principal cells occurs in layer 2/3. These cells extend their axon to the cortex, striatum, claustrum and other telencephalic centres. The probability of reciprocal connections

between projection subtypes of all of these intratelencephalic cells is similar [57]. In layer 5 there are several types of projection cells. Synapses occur reciprocally between cells of similar type, e.g., intratelencephalic with intratelencephalic cells, type II cells with type II cells, etc. There are connections between type I and type II cells of layer 5 too, but they are hierarchical, that is, projections from type I cells to type II cells are more probable than projections in opposite way [44,54-56]. Principal cells of layer 6 of intratelencephalic projection have a higher probability of extending reciprocal connections between one another than with other type of projection cells. Next, these cortico-cortical cells have a higher probability of extending collaterals to cortico-thalamic cells than vice-versa [60]. Cells of layer 6 receive collaterals from cells of any other layer except layers 2 and 4 and extend collaterals to cells of layers 5-6. The exception being the layer 6a cell of cortico-thalamic projection to the core cells of thalamic nuclei that also extend collaterals to the thalamic reticular nucleus. This cell originates cortico-cortical collaterals only to cortical layer 4 cells [60].

Considering all cortical layers, the weight of the local intrinsic circuit in cat and monkey neocortex is estimated to be 80%-85% of excitatory inputs on principal neurons [58,66] whereas the weight of short- and long-range cortico-cortical circuits are 15% and 3%; on the same cells, the weight of the cortico-thalamic and intriguing cortico-claustral circuits is 1.3% and 3% respectively [66]. Finally, although cells of layer 2/3 probably connect more with cells of the same layer, some cells of layer 2/3 send collaterals to both, type I and type II cells of layers 5 and 6.

Principal cells are approximately 80% of the total of cortical neurons; interneurons make up the remaining  $\approx 20\%$ . Over 71% of the synapses in the cortex are derived from principal cells. However, this number does not truly reflect the relative balance between excitation and inhibition in cortex. When the larger efficacy of inhibitory synapses is taken into account, the dominance of the principal cells is reduced to 24%. Thus, the spiny cells provide the basic framework of long-distance excitation in both the vertical and lateral dimensions of the cortex, which is moulded by local inhibitory neurons [67].

*Interneurons.* Cortical interneurons innervate mainly principal cells, but they also innervate interneurons [16]. Interneurons form distinct intralaminar and interlaminar networks [16,53]. The probability of reciprocal synaptic connections between principal cells and interneurons varies with the type of interneuron. The intralaminar reciprocal connections between fast-spiking interneurons (chandelier cells and basket parvalbumin-positive cells) and principal cells are significantly higher than the probability of reciprocal connections between non fast-spiking interneurons and principal cells [53]. On the contrary, the interlaminar reciprocal connection between principal cells and interneurons is more frequent with non fast-spiking interneurons [53].

Another distinctive feature of the network of interneurons is its coupling through gap junctions. Electrical coupling of neocortical interneurons is firmly established by anatomical studies and electrophysiological experiments since [68]. The coupling is generally between interneurons of the same type [69]. Chandelier cells are electrically coupled too, as disclosed recently [62]. However, neurogliaform cells are electrically coupled to other neurogliaform

cells but also to basket cells, regular-spiking interneurons and chandelier cells, among others interneurons. Thus, the neurogliaform cell links networks of particular classes of interneurons, each network being in turn electrotonically coupled itself [70].

Fast-spiking interneurons target the somatic and perisomatic domains of the principal cell; thus, these interneurons strongly regulate the output of the principal cell [4,18]. Reciprocal connections between pyramidal cells and fast-spiking interneurons act as a recurrent feedback inhibition that can regulate the timings of pyramidal cell firing [5]. In turn, spike timings of fast-spiking interneurons are correlated with the local field potential in the network of principal neurons during gamma oscillations that depend on the electrical and chemical coupling between fast-spiking, parvalbumin-positive interneurons [17,71]. Parvalbumin-positive basket cells fire counter-phase with principal cells and in the same phase but slightly delayed to chandelier cells [17,72].

#### **4. The axon initial segment**

Electrically, the AIS bridges dendritic and axonal compartments, converting graded dendritic inputs into all-or-none action potentials. Molecularly, the AIS maintain neuronal polarity preserving the molecular distribution between the axonal and somatodendritic domains [73]. Recent studies have revealed an ever-expanding complexity in the molecular components and in the types and distribution of ion channels embedded in the AIS. This complexity underlies what is now being recognized as a highly dynamic structure [73]. AIS structure and composition vary considerably across, and even within, neuronal classes [74,75], seemingly tuned to the computational demands of the cell. A recent activity of the cell can affect AIS ion channel kinetics and availability, thus altering action potential waveform, timing, and probability. Over long timescales, even the location and size of the AIS can change to compensate for alterations in neuronal activity.

##### **4.1. Ion channels, anchoring proteins and cytoskeletal components of the axon initial segment**

*Ion channels.* Although multiple neuronal sites can support action potential generation, the high density of Na<sup>+</sup> channels inherent to the AIS makes it the lowest threshold site for it. Immunostaining, imaging of spike-dependent Na<sup>+</sup> flux and electrophysiological studies suggest a similar density of Na<sup>+</sup> channels throughout the AIS [73,76]. However, a recent study revealed that Na<sup>+</sup> channels at the distal AIS and the adjacent axon have a much lower half-activation voltage (up to 14 mV) than those at the proximal AIS and the soma [77]. Accordingly, the use of newly developed voltage imaging techniques combined with careful analysis of the site of initiation and propagation of the action potentials, show that they preferentially initiate at the distal end of the AIS [77-79]. Consistent with these electrophysiological data, immunostaining results revealed a segregation of two Na<sup>+</sup> channel subtypes at the AIS: high-threshold Nav1.2 channels and low-threshold Nav1.6 channels, targeted preferentially to the proximal and the distal AIS, respectively [77]. Immunostaining intensity of Nav1.6 peaked at the distal end of the AIS, corresponding well

to the action potential initiation zone (see [73] for review). Voltage-threshold for action potential initiation seems to be largely determined by the density of Nav1.6, whereas Nav1.2 channels in the proximal AIS support action potential back-propagation into the somato-dendritic compartment. Indeed, axonal action propagation fails to back propagate when the somatic membrane potential is hyperpolarized [77]. Spike-evoked  $\text{Ca}^{2+}$  influx in the AIS of different neurons in the SNC has been revealed by fluorescence imaging techniques [73]. These cells fire spike bursts typified by the recruitment of sub threshold  $\text{Ca}^{2+}$  influx through low voltage-activated  $\text{Ca}^{2+}$  channels, which include T- and R-type isoforms [80]. Traditionally, burst evoked  $\text{Ca}^{2+}$  influx was thought to be restricted to the dendrites. Indeed, these same low voltage-activated channels mediate  $\text{Ca}^{2+}$  influx in the AIS. Since low voltage-activated channels activate at hyperpolarized potentials, they contribute to sub threshold depolarization of the AIS. Thus, their activity can determine when and if a given stimulus evokes an action potential.  $\text{Ca}^{2+}$  influx through cartwheel cell T-type channels of the AIS, is down regulated by a type 3 dopamine receptor (D3R)-dependent pathway [81]. This neuromodulatory pathway is remarkably specific for just AIS T-type channels; neither dendritic T-type channels nor AIS  $\text{Na}^+$  or  $\text{K}^+$  channels were affected by D3R signalling [73]. Serotonin<sub>1A</sub> receptor mediates neuronal hyperpolarization by activating potassium channels in the AIS. In human and monkey neocortex the serotonin<sub>1A</sub> receptor have been reported to be highly concentrated in the membrane of the AIS of principal neurons [82,83].

*Associated anchoring proteins and cytoskeletal components.* The structure of the AIS of multipolar neurons possesses a dense layer of finely granular material undercoating the plasma membrane, scattered clusters of ribosomes and fascicles of microtubules. The fascicles of microtubules occur only in the axon hillock and initial segment. An undercoating of the plasma membrane of the axon occurs in the node of Ranvier [84-86]. Interestingly, the plasma membrane of AIS and nodes is surrounded by a large extracellular space containing dense material; this similarity between nodes and AIS is coincident with the presence of voltage gated channels and specialization of the cytoskeleton present therein [73,76]. In addition, the dense material undercoating the plasma membrane of the AIS is separated 15-25 microns from the internal surface of the AIS membrane [84]. The neuronal cytoskeleton, consisting of interacting spectrins and actins, forms the neuronal structural scaffold and is a spatial delimiter for neuronal membrane proteins; the membrane undercoating is a specialized cytoskeletal element, found only in the AIS. The  $\beta$ IV isoform of spectrin (an actin-binding protein) and ankyrin G (a spectrin-binding protein) mutually confine each other to the AIS [14,76]. Ankyrin G provides a specific anchor for many AIS-specific proteins, including the  $\text{Na}^+$  and  $\text{K}^+$  channel subunits KCNQ2 and 3. PSD-93, other scaffold protein, binds to the  $\text{K}_v1$  channels found at the AIS. In addition  $\text{K}_v1$  channels are associated to the adhesion molecule Casppr2 in the layer 2/3 of the human cerebral cortex [76]. Silencing of PSD-93 expression in cultured hippocampal neurons blocks the recruitment of  $\text{K}_v1$  channels to the AIS but not  $\text{Na}^+$  channels [87]. The AIS is also enriched in the cell adhesion molecules Nr-CAM and Neurofascin-186, and the cytoskeletal linker  $\beta$ IV spectrin [76]. Recruitment of these proteins to the AIS also depends on ankyrin G [73,88]. Together, these results point to ankyrin G as the master regulator of AIS assembly. Silencing of AIS proteins in mature neurons in culture reveals that as for development, ankyrin G is required

to maintain ion channels at the AIS [89]. Intriguingly, these and other experiments also showed that ankyrin G functions not only to cluster and maintain ion channels, but also to maintain neuronal polarity [89].

## 4.2. Length of the axon initial segment

AIS-length of cortical principal cells ranges between 17-40 microns as counted from the axon hillock [76,84,86]. This AIS length approximately coincides with the electrophysiological zone of initiation of the action potential, estimated to be  $\approx 35$ -50 microns from the soma [78,79,90,91]. Interneurons have a shorter AIS than principal cells [15]. Granule cells of the fascia dentata give rise to the smallest unmyelinated fibres of the CNS. In these cells the action potentials are initiated at the distal axon but at  $\approx 5$ -15 microns from soma with Na<sup>+</sup> channel density specialized for robust action potential initiation and propagation with minimal current flow [91,92]. The length of the AIS of granule cells of the hippocampus resembled that of the large basket cells of the neocortex, which on average are 6 microns length although the axons of large basket cells are myelinated [15]. Hippocampal granule cells may be an exception because they are not connected between themselves as it occurs in all cortical neurons [5]. It would be of interest to explore if differences in the length of AIS are functionally relevant. In the AIS of hippocampal granule cells the Na<sub>v</sub>1.6 channel is the predominant  $\alpha$ -subunit whilst the Na<sub>v</sub>1.2 cannot be detected [92]; this suggests that Na<sub>v</sub>1.6 channels are enough to generate the axon potential, and therefore that the mechanisms governing this generation are different in the thin non-myelinated axon of the granule cell than in other myelinated or unmyelinated neurons having also a short AIS length.

## 5. Study with electron microscopy of the axon initial segment of the inverted variety of cerebral cortical principal cell (of intratelencephalic projections)

Our studies following the use of retrograde tracers in cerebral cortex of rats, rabbits and cats showed that spiny inverted neurons of layers 5 and 6 originate intra-telencephalic projections [23,27,28,33]. Spiny inverted neurons are odd because of having not only circumscribed projections and an inverse somatodendritic orientation but also for the sites from which their axon arises. As seen with Golgi-method impregnation, retrograde tracers and intracellular filling (for a review, see [28]), these cell sites may be (1) the basal surface of the soma, or even the basal dendrite portion next to the soma, (2) the (lateral) surface of the soma and (3) the apical dendrite, sometimes from a sector more remote from the soma than the emergence site of the first dendrite branching [23]. Of the 127 Golgi-impregnated spiny inverted neurons in the occipital and temporal cortices of rabbits which we examined, 29% of the axons arises from (1), 9.5% from (2) and 61.5% from (3) [23]. This distribution is similar to that in rat visual and sensory-motor cortices [28]: of 28 Golgi-impregnated spiny inverted neurons, 32% of the axons emerges from (1), 21.5% from (2) and 46.5% from (3). Hence, the probability of axon emergence from the apical dendrite is higher both in rabbits and rats. There, inputs on the apical dendrite and its branches can be more operational for the generation of cell outputs, as they do not pass



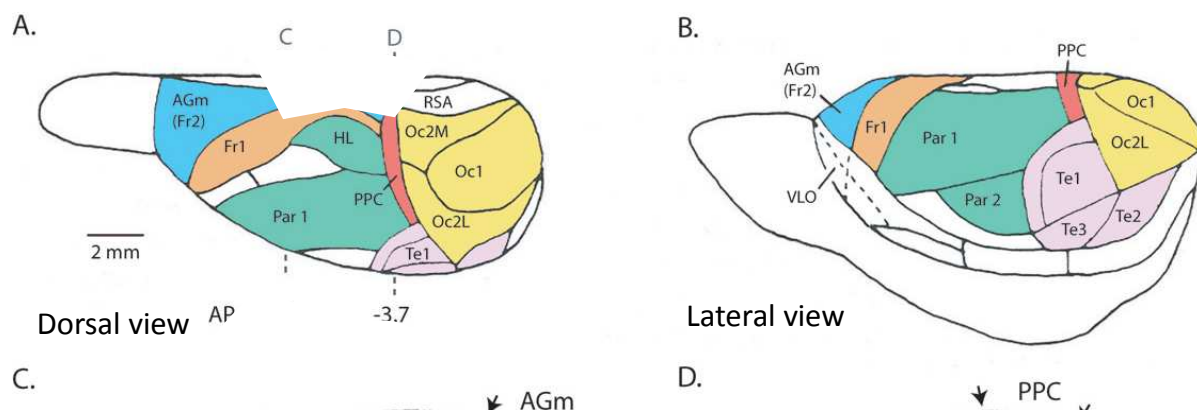
the somatic robust inhibitory barrier; in turn, the back-propagation of a generated axon potential can more effectively result in changes of the membrane potential of the apical dendrite and its branches. Thus, two physically segregated input integrations may simultaneously operate in the cells in which the axon arises from the apical dendrite: one at the immediate apical-tree membrane domain, the other at the somatic and remaining dendritic membrane domain. In the cat visual cortex, the orientation of apical dendrites of a spiny inverted neuron depends on the position of the parent cell within the gyri [28]. Accordingly, the incidence of truly reversed spiny inverted neurons decreased from the top of the gyri to the bottom of the sulci, while that of almost horizontally orientated pyramidal neurons increased. Most axons were found to arise from the apical dendrite or from one of its first branches in cat spiny inverted neurons too.

We reconstructed by means of electron-microscopy serial photography the AIS of eleven spiny inverted neurons of unknown projection (though they probably were cortico-cortical, cortico-claustral, or cortico-striatal, see Table 1). The length of the AIS of these neurons ranged between 32.0 and 101.2 microns, and its thickness varied between 0.58 and 1.04 microns. The range of the AIS length described by others shows some variability, indicating that cells of different subtypes of projection neurons, or cells within the subtypes themselves, may have different AIS length; e.g. the commissural and cortico-thalamic cells show more variability in the length of the AIS than the cortico-cortical associative ones [86]. Importantly, the number of synaptic boutons received by the AIS of spiny inverted neurons was found to be 24.4 on the average, but the range was also wide (11-37 boutons). Analysis of the spiny inverted neurons as classified in terms of the emergence site of the axon revealed that AIS which proceed from the apical dendrite were the shortest, thinnest and less innervated, whereas AIS arising from the somatic flank were the longest, widest and most innervated. Thus, AIS originated on apical dendrites averaged a length of 38.8 microns (range 37.1–45.4 microns), a diameter of 6.0 microns (range 0.58–0.62 microns) and a number of 15 apposed boutons (range 11–18 boutons). AIS of lateral somatic emergence averaged a length of 95.5 microns (range 93.2–97.8), a diameter of 1.04 microns (range 1.04–1.04) and 34 boutons (range 31–37). In turn, six AISs of somatic basal emergence averaged 60.9 microns in length, 0.83 microns in diameter and 26 boutons (ranges being 32.0–101.2 microns, 0.71–0.96 microns and 21–29 boutons, respectively) [28]. These features of the AIS may support an output generation that is peculiar to subclasses of spiny inverted neurons. Work is currently in progress in our laboratory to refine to which extent differences in the number of synapses received by the AIS are related to the type of axon projection of spiny inverted cells.

## **6. Study with electron microscopy of the axon initial segment of the upright variety of cerebral cortical principal cell with experimentally identified backward ipsilateral and commissural projections in visual cortex**

We present in the following a preliminary report on AIS of twelve pyramidal neurons of cortico-cortical projection to primary visual cortex; these cells, amid other projection cells,

were recently studied in our laboratory [93]. All these twelve cells had a typical upright somatodendritic orientation; they sited in the lateral partition of secondary visual cortex of rats (Figure 1). The axons of six of these neurons projected to the ipsilateral primary visual cortex that bears the cortical representation of the vertical central meridian of binocular visual field. The axons of the remaining six neurons projected to the same region of primary visual cortex but contralaterally through the callosum. In each of these two groups, three neurons lay in layer 3 and the remaining three in layer 5. Thus, the neurons reported here were distributed in four subgroups in agreement with their layer address and cortico-cortical projection.

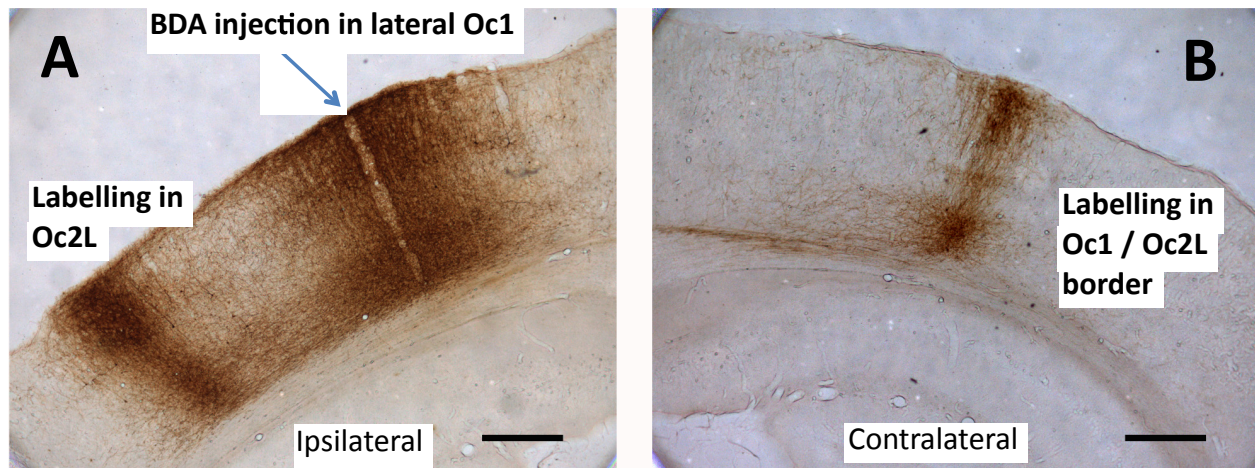


**Figure 1.** Schematic drawings of the dorsal view (A) and lateral view (B) of the left hemisphere of rat cerebral cortex. Oc1 corresponds with the primary visual area; Oc2M and Oc2L do so with the medial and lateral parts of the secondary visual area, respectively. The border between Oc1 and Oc2L is the cortical representation of the vertical central meridian of binocular visual field. Biotinylated dextran-amide (BDA) microinjections were placed in the Oc1 side of this border. Cells labelled with BDA by retrograde axon transport were taken from the ipsilateral Oc2L and homotopic contralateral border between Oc1 and Oc2L.

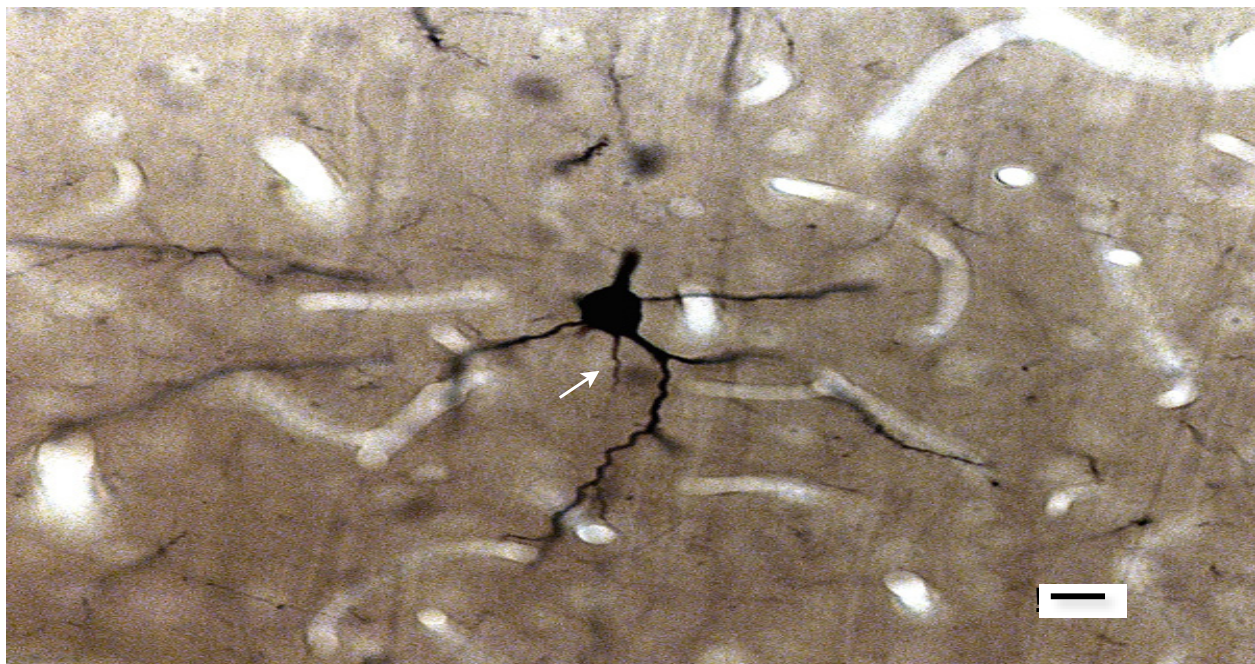
### 6.1. Material and methods

To study all these twelve neurons we combined axon track-tracing methods and serial electron microscopy. We injected biotinylated dextran-amide (BDA) in primary visual cortex, in order to identify projection pyramidal neurons under light microscopy (Figures 1, 2); then, we performed serial ultrathin-cutting and -photography to study anatomical parameters of labelled AISs under electron microscopy (Figures 3-5). Studied AIS parameters were length, thickness, and number of apposed synaptic boutons and distribution of these boutons along the AIS membrane. Student's *t* tests ( $p\text{-value} \leq 0.05$ ) and linear regressions statistics were used to compare measured AIS parameters (simple linear regression and non-parametric multiple tests;  $p\text{-value} \leq 0.05$ ).

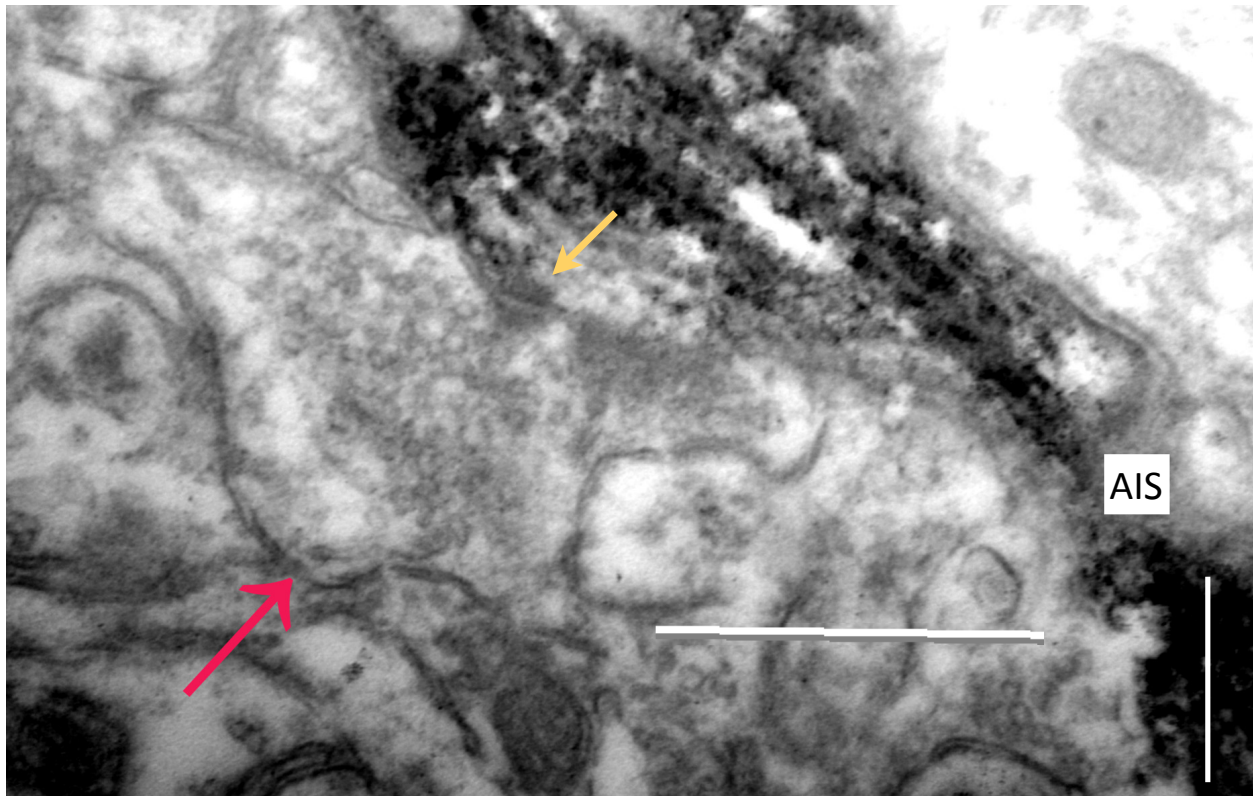




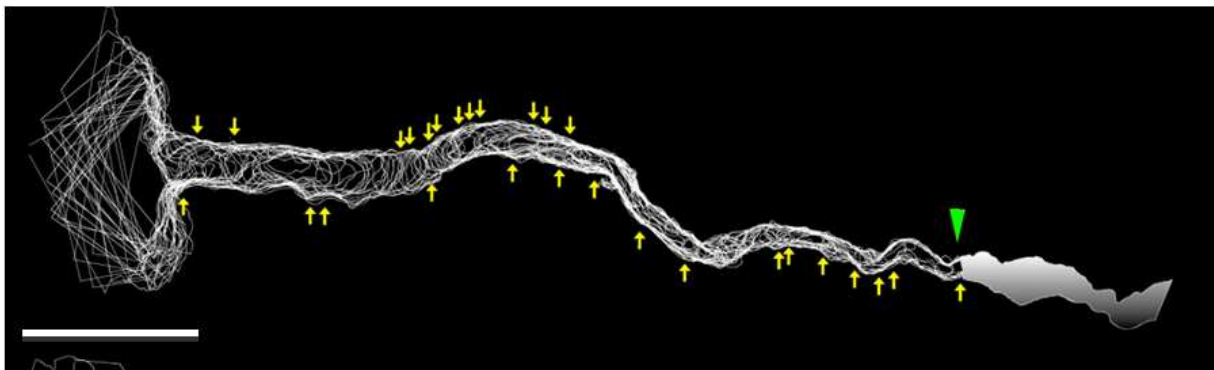
**Figure 2.** Microphotographs of paired coronal sections of left (A) and right (B) hemispheres of rat visual cerebral cortex. See columns of labelling in ipsilateral Oc2L (lateral partition of secondary visual area) and contralateral border between Oc2L and Oc1 after a biotinylated dextran-amide (BDA) microinjection (blue arrow) in Oc1 (primary visual area). Projection cells were selected for the present study among BDA-labelled cells of these or similar columns following comparable injections of BDA in other rats. Scale bars, 500 microns.



**Figure 3.** Microphotograph of a cell sited in layer 3 of Oc2L (lateral partition of secondary visual area); the cell was labelled after a biotinylated dextran-amide injection to ipsilateral Oc1 (primary visual area) in the cerebral cortex of a rat. The cell was flat embedded and the resultant slice was glued on top of a resin capsule. The axon initial segment (arrow) underwent serial ultrathin cutting and then it was examined under electronic microscopy. Scale bar, 15 microns.



**Figure 4.** Electron-microscopy photograph of an axonal bouton (between red and yellow arrows) synapsing on the axon initial segment (AIS) of the cell shown in Figure 3. Most other boutons synapsing on this and other AIS contained flat vesicles. AIS was occupied by biotinylated dextran-amide, which gave it its black, grainy aspect. Notwithstanding this filling, see the pre- and postsynaptic densities, which are pointed at by the yellow arrowhead. Scale bar, 0.5 micron.



**Figure 5.** Electron-microscopy serial reconstruction of the axon initial segment (AIS) of the cell shown in Figures 3-4. The cell sited in layer 3 and furnished the associative backward projection to most-lateral (next to the border with lateral secondary cortex) primary visual cortex. The AIS was 22.27-micron-long, averaged a diameter of 0.93 microns and received 28 synaptic boutons (yellow arrows). Note the uneven distribution of these boutons along AIS: boutons were more abundant in the central tier and then in the distal one. Not all studied neurons in [93] and reported here had this bouton distribution along their AIS. The green arrowhead points to the site at which the myelin sheet begun. Scale bar, 5 microns.



## 6.2. Results

As a group, upright principal cells furnishing the associative backward projection from ipsilateral lateral secondary visual area to most-lateral primary visual area in the rat cerebral cortex had, on the average, shorter and thicker an AIS than similar cells of projection to the same area but through the callosum; these differences were however non-significant (Box 1). Importantly, AIS of cells of the associative backward projection averaged more synaptic boutons than AIS of cells of projection through the callosum; thus, the synaptic-bouton density per AIS length-unit was higher for AIS of cells of the associative backward projection; these differences were significant (Box 1).

Results also showed that the neurons sited in layer 3 had, on the average, shorter and thinner an AIS than the neurons sited in layer 5, these differences being non-significant except for the AIS-length comparison. Importantly, the AIS of layer 3 cells averaged more synaptic inputs than that of layer 5 cells; thus, the bouton density per AIS length-unit was higher for layer 3 cells. These differences were significant (Box 1).

### AVERAGED VALUES OF PYRAMIDAL-NEURON AIS AND THEIR COMPARISON AS GROUPED BY AXON PROJECTION

Projection	Neurons	AIS Length ( $\mu\text{m}$ ) $\pm$ Standard Deviation	AIS $\varnothing$ ( $\mu\text{m}$ ) $\pm$ Standard Deviation	Number of Boutons $\pm$ Standard Deviation	Syn. Bouton Density per AIS Length ( $\mu\text{m}$ )
Ipsilateral	6	26.73 $\pm$ 5.08	0.98 $\pm$ 0.10	26 $\pm$ 5.25	1.02 $\pm$ 0.33
Contralateral	6	28.38 $\pm$ 4.41	0.96 $\pm$ 0.15	18 $\pm$ 3.10	0.66 $\pm$ 0.20

### AS GROUPED BY SOMA LOCATION

Location	Neurons	AIS Length ( $\mu\text{m}$ ) $\pm$ Standard Deviation	AIS $\varnothing$ ( $\mu\text{m}$ ) $\pm$ Standard Deviation	Number of Boutons $\pm$ Standard Deviation	Syn. Bouton Density per AIS Length ( $\mu\text{m}$ )
Layer 3	6	24.50 $\pm$ 4.01	0.92 $\pm$ 0.13	25.50 $\pm$ 5.75	1.06 $\pm$ 0.28
Layer 5	6	30.61 $\pm$ 2.91	1.02 $\pm$ 0.12	18.50 $\pm$ 3.62	0.62 $\pm$ 0.17

Differences between values in gray boxes were statistically significant (Student's t-test,  $p < 0.05$ )

**Box 1.** Averaged values of AIS parameters and their comparison as grouped by projection and layer address.

In human cortex, the length of AIS of layer 3 neurons ranges between 11-47 microns, as calculated by immunocytochemistry to  $\beta$ IV spectrin, GAT-1 and  $\text{Na}^+$  channels [76]. However the length of the AIS is not correlated with the size of the perikarion [84,86, but see 94]. By means of regression tests we compared (a) the lengths versus the diameters, and (b) the lengths versus the numbers of received synaptic boutons for the twelve separate AIS presented here. The first comparison revealed a linear and significant relation ( $p$ -value = 0.009), by which the AIS diameter variation explained the 52% the AIS length variation. The second comparison, i.e. the lengths versus the numbers of received synaptic boutons, revealed an inverse, non-significant relation ( $p$ -value = 0.136). With a non-parametric multiple regression test we compared for the twelve AIS, as taken separately, the numbers of received synaptic boutons versus the layer addresses of the parent cell somata and the projection of the on-going axons. Direct and highly significant correlations emerged from these comparisons ( $p$ -values being  $9.3 \times 10^{-5}$  and 0.00025, respectively). Thus, AIS with a high

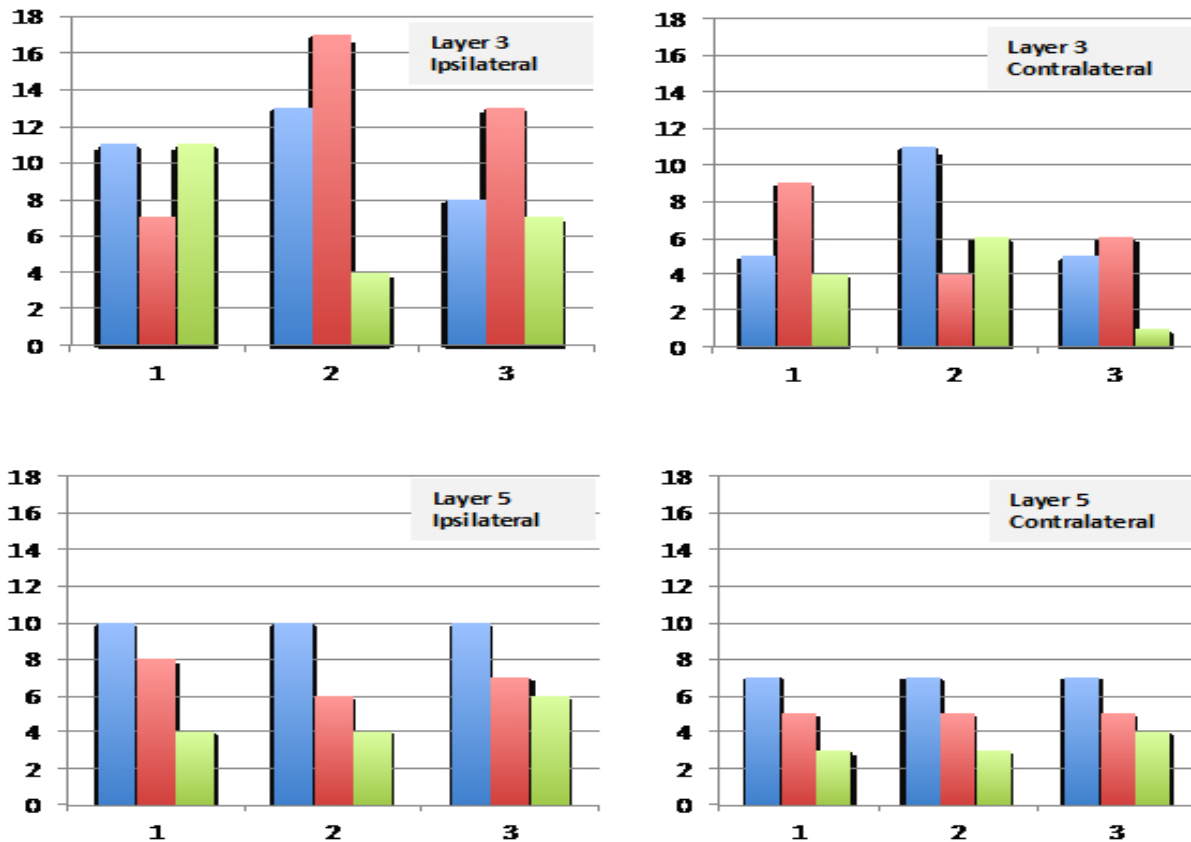


number of apposed synaptic boutons has up to 90% of probabilities of being part of a cell sited in layer 3, on the one hand, or projecting backwards to the primary visual cortex, on the other hand.

Though the number of measured cells in the present study is admittedly low, we believe it is satisfactory for an electron microscopy study. The numbers of synaptic boutons on AIS of upright principal cells of cortico-cortical projection reported here are in general agreement with others of different cortical areas and mammals, if differences between animals, area addresses and axon projections have to be taken into account. Major discrepancies are with cat visual cortex [85] and monkey sensory-motor cortex [95] (Table 2). It has to be said however that the number of boutons revealed for the cat visual cortex in [85] exceeds by a large amount the number of boutons found for the cat visual cortex in [96] and in [86]—though in [86] the layer address of the cells must be considered too. More quantitative studies on AIS innervation of subtypes of principal cells of identified axon projection in different animals and cerebral cortical areas are clearly needed.

Reference	Animal	Type of cortex	Layer – projection	Cells	Boutons
[96]	Cat	Visual	2/3 – unknown	18	24 (average)
[85]	Cat	Visual	2/3 – unknown	3	42-44
[86]	Cat	Area 17	2/3 – * <sup>a</sup>	18	16-28* <sup>a</sup>
[95]	Monkey	Sensory-motor	2/3 – callosal (2 cells) and unknown	8	2-52
[93]	Rat	Visual	2/3 – * <sup>b</sup>	6	19-34* <sup>b</sup>
[95]	Monkey	Sensory-motor	5 – unknown	8	2-26
[28]	Rat	Visual	5/6 – +	11	11-37 <sup>+</sup>
[93]	Rat	Visual, secondary & primary	5/6 – * <sup>c</sup>	6	15-23* <sup>c</sup>
[97]	Rabbit	Visual	5 – **	1	10**
[86]	Cat	Visual	5 – ***	10	1-5***

**Table 2.** Comparison between numbers of synaptic boutons apposed to the AIS membrane as found in [93] and other studies. Note that the boutons seen on AIS of axons projecting to the thalamus and the colliculi are far fewer than those seen on AIS of axons projecting to intratelencephalic projections. Differences concerning types of projection: (\*) cortico-cortical. <sup>(a)</sup> Contralateral projection from primary visual area to secondary visual area, 16-23 boutons; ipsilateral projection from primary visual area to secondary visual area, 22-28 boutons. <sup>(b)</sup> Contralateral projection from primary visual area to the border between primary visual area and lateral secondary visual area, 19-22 boutons; ipsilateral projection from lateral secondary visual area to the border between primary visual area and lateral secondary visual area, 28-34 boutons. <sup>(c)</sup> Contralateral projection from primary visual area to the border between primary visual area and lateral secondary visual area, 15-16 boutons; ipsilateral projection from lateral secondary visual area to the border between primary visual area and lateral secondary visual area, 20-23 boutons. \*\* Cortico-collicular projection. \*\*\* Cortico-thalamic projection. Difference concerning anatomical subtypes of principal cells: + Spiny inverted neuron AIS.



**Figure 6.** Distribution of synaptic boutons along each of twelve AIS of the present study [93]. Cells sited either in layer 3 or layer 5 and furnished either the associative backward (ipsilateral) projection or the callosal (contralateral) projection, both to primary visual cortex (3 neurons per layer and projection). Each AIS was divided into three segments of equal length (blue bars, closest tiers to the cell somata; green bars, closest tiers to the axon). X-axes show the bouton distribution for each AIS. Y-axes show the number of boutons counted per tier per AIS. Notice that, whilst all six AIS of layer 5 cells had a comparable decreasing pattern, AIS of layer 3 cells had uneven distributions even among cells of the same axon projection.

In order to know on the distribution of synaptic boutons along AIS, we divided each of the twelve AIS reported here in three equal longitudinal tiers and then we assigned boutons to tiers. As averaged for layer addresses and axon projection cell-subgroups, the distribution was decreasing towards the axon but for the subgroup of layer 3 cells of associative backward (ipsilateral) projection; there, the intermediate tier had more boutons (not shown). Most importantly, we compared one another the AIS of the twelve neurons (Figure 6). Then different patterns emerged not only for cells sited in layer 3 of ipsilateral projection but also for cells sited in layer 3 of commissural projection. Still, each cell sited in layer 5 had the decreasing pattern, notwithstanding if the cell was of ipsilateral or contralateral projection. Cells of layer 3 had uneven distributions, which can be tentatively grouped in two presumptive patterns: a 'reel' one, with more boutons apposed to the proximal and distal tiers of the AIS, and a 'barrel' type, with less boutons on the intermediate tier (Figure 6).

The variety in the distribution observed for the principal cells of layer 3 suggests either the presence in this cell group of examples of minor cell subpopulations occurring within those

studied in [93], or activity-dependent AIS plasticity, or both. Ion channels composition at the AIS can vary considerably across different neuronal types [74] and diversity in the AIS length and position in the axon may also underlie interneuron variation in firing properties. For example, classes of retinal ganglion cells with different visual properties have initial segments that differ in length, and in their position within the axon [98]. In the avian magnocellularis and laminaris nuclei, which are respectively the second- and third-order nuclei in the auditory pathway [99], the length and the location of the AIS vary with the tuning frequency of neurons [100]. Activity-dependent AIS plasticity has been observed in cultured hippocampal neurons. Increasing chronically neuronal activity over 48 hours resulted in a significant shift in AIS position with the entire structure (ankyrin G,  $\beta$ IV spectrin, and  $\text{Na}^+$  channels) moving distally away from the soma [75]. Importantly, hippocampal AIS relocation is a bidirectional phenomenon because the AIS can shift proximally after neurons are returned to baseline activity conditions. In this way, on-going neuronal activity can fine-tune AIS position. Moreover, a lack of auditory input causes a change in AIS length of neurons of chick auditory nucleus magnocellularis [101]. All these studies showed that the AIS plasticity is coupled with changes in neuronal excitability. The distinct length of the AIS of hippocampal pyramidal neurons ( $\approx 30$  microns) and the neurons of the nucleus magnocellularis ( $\approx 10$  microns) has been suggested to be the cause of these different plastic changes in the AIS. Indeed, cell type-based variability in AIS plasticity is seen within hippocampal cultures, where GAD 65-expressing interneurons display little or no shift in AIS location upon high potassium stimulation [75]. Dopaminergic neurons in dissociated cultures of rat olfactory bulb show inverse AIS plasticity: their initial segments move proximally after 48 hours depolarization.

Although these plastic changes have not been shown for neocortical neurons to the best of our knowledge, nor is clear to which extent are associated with shifts on the distribution of presynaptic boutons apposed to the membrane of the AIS, it will be fascinating in the future to see how different types of neuron of diverse brain regions use different forms of AIS plasticity in response to perturbations in their electrical activity. Nonetheless, it follows from our study that at least for associative and commissural projections to primary visual cortex in the rat, the innervation of AIS of principal cells of typical somatodendritic orientation is steadier in layer 5 than in layer 3.

## 7. Conclusion

By presenting data on parameters such as the length and diameter of the AIS and, of no lesser importance, the number and distribution of presynaptic boutons apposed to the AIS membrane, this study advances the knowledge on the control of membrane potential and initiation of axon potential of visual cortex neurons, and particularly of the principal cells with cortico-cortical axon projection from layers 3 and 5 to primary visual area. It unveils that the bouton density on the AIS of principal cells would correlate significantly with the layer address and axon projection of the parent neuron rather than with the length and thickness of AIS proper. The present study additionally shows that there was a repeated decrease in the number of synaptic boutons apposed along the AIS of neurons of layer 5 but

not of layer 3. It might be that neurons of layer 3 can be subdivided in subgroups within the groups of cortico-cortical ipsilateral or contralateral projection we have considered in the study. These subgroups might possess undisclosed structural and functional cell features associated to permanent or transitory synaptic-bouton distributions along the AIS.

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