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Sub-synaptic localization of Ca_v3.1 T-Type calcium channels in the thalamus of normal and parkinsonian monkeys

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Abstract

T-type calcium channels (Ca_v3) are key mediators of thalamic bursting activity, but also regulate single cells excitability, dendritic integration, synaptic strength and transmitter release. These functions are strongly influenced by the subcellular and subsynaptic localization of Ca_v3 channels along the somatodendritic domain of thalamic cells. In Parkinson's disease, T-type calcium channels dysfunction in the basal ganglia-receiving thalamic nuclei likely contributes to pathological thalamic bursting activity. In this study, we analyzed the cellular, subcellular, and subsynaptic localization of the Ca_v3.1 channel in the ventral anterior (VA) and centromedian/parafascicular (CM/Pf) thalamic nuclei, the main thalamic targets of basal ganglia output, in normal and parkinsonian monkeys. All thalamic nuclei displayed strong Ca_v3.1 neuropil immunoreactivity, although the intensity of immunolabeling in CM/Pf was significantly lower than in VA. Ultrastructurally, 70–80% of the Ca_v3.1-immunoreactive structures were dendritic shafts. Using immunogold labeling, Ca_v3.1 was commonly found perisynaptic to asymmetric and symmetric axo-dendritic synapses, suggesting a role of Ca_v3.1 in regulating excitatory and inhibitory neurotransmission. Significant labeling was also found at non-synaptic sites along the plasma membrane of thalamic neurons. There was no difference in the overall pattern and intensity of immunostaining between normal and parkinsonian monkeys, suggesting that the increased rebound bursting in the parkinsonian state is not driven by changes in Ca_v3.1 expression. Thus, T-type calcium channels are located to subserve neuronal bursting, but also regulate glutamatergic and non-glutamatergic transmission along the whole somatodendritic domain of basal ganglia-receiving neurons of the primate thalamus.

Keywords

centromedian; parafascicular; immunogold; primate; Parkinson's disease; MPTP

Introduction

In the thalamus, low-threshold calcium spikes (LTS) and burst firing are mediated by low voltage activated (LVA) T-type calcium channels (Steriade and Llinás, 1988; Huguenard, 1996) which play key roles in sleep, arousal, and sensory gating (McCormick and Bal, 1997). Changes in T-type calcium channel function have been implicated in the pathophysiology of various neurological disorders including Parkinson's disease (PD), absence epilepsy, neuropathic pain, and various neuropsychiatric disorders (Buzsaki et al., 1990; Jeanmonod et al., 2001; Llinás et al., 2001; Nelson et al., 2006; Belardetti and Zamponi, 2012; Cain and Snutch, 2013; Bladen et al., 2015). In addition to their long-expected role in the regulation of thalamic burst firing, several new functions have recently been assigned to these channels, such as the regulation of single cells excitability, dendritic integration, transmitter release and synaptic strengthening (Lambert et al., 2014).

The onset and progression of PD motor symptoms have been closely linked to the loss of striatal dopamine and the resulting aberrant activity within basal ganglia-thalamocortical circuits (Albin et al., 1989; DeLong, 1990; Bergman et al., 1990), including increased thalamic bursting (Zirh et al. 1998; Magnin et al., 2000; Pessiglione et al., 2005; Devergnas et al., 2015). A characteristic type of bursts in the mammalian thalamus is termed "rebound bursts" (Llinas and Jahnsen, 1982), which are characterized by low threshold calcium spike bursts (LTS) that result from hyperpolarization-induced de-inactivation of T-type calcium channels (Perez-Reyes, 2003). Alterations in burst kinetics of T-type calcium channels in the motor thalamus induce changes in rebound LTS bursting that may underlie motor dysfunction in PD (Pare et al., 1990; Yang et al., 2014). Increased rebound bursting has, indeed, been documented in the ventral motor thalamus of parkinsonian monkeys (Zirh et al. 1998; Magnin et al., 2000; Pessiglione et al., 2005; Devergnas et al., 2015).

Based on differences in their pore-forming $\alpha 1$ -subunit, T-type calcium channels are classified as $\text{Ca}_v3.1$, $\text{Ca}_v3.2$, and $\text{Ca}_v3.3$ (Perez-Reyes, 2003, 2006; Perez-Reyes and Lory, 2006). Although these subtypes of T-type calcium channels have similar functional characteristics (Perez-Reyes 2003; Catterall et al. 2005), they differ in their distribution across brain regions and in their activation or inactivation kinetics. In rodents, $\text{Ca}_v3.1$ is the most strongly expressed T-type calcium channel subtype in thalamocortical projection neurons, while $\text{Ca}_v3.3$ is abundantly expressed in reticular thalamic neurons (Talley et al. 1999). Although the cellular expression of these channels has been studied throughout the brain, very little is known about their spatial localization at the single cell level, and about potential changes in the localization of the channels in the parkinsonian state. Thus, using high resolution electron microscopy procedures, we studied in detail the subcellular and subsynaptic localization of the $\text{Ca}_v3.1$ channel in the basal ganglia-receiving thalamic nuclei, the parvocellular and magnocellular ventral anterior nucleus (VApC/VAMc) and centromedian/parafascicular (CM/Pf) nuclei, in normal and parkinsonian monkeys.

Material and Methods

Animals

Six female adult (4–8 years old) Rhesus monkeys (*Macaca mulatta*, 5–10 kg) from the Yerkes primate center breeding colony were used in this study. Three of these animals were used as control and three were rendered parkinsonian by weekly injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). All animals were pair-housed, and had *ad libitum* access to food and water. All experiments were performed in accordance with the United States Public Health Service Policy on the humane care and use of laboratory animals, including the provisions of the “Guide for the Care and Use of Laboratory Animals” (Garber et al., 2011). All studies were approved by the Biosafety and Animal Care and Use Committee of Emory University.

MPTP treatment

Animals were treated with MPTP (0.2–0.6mg/kg i.m.; Sigma, St. Louis, MO; cumulative doses: 2.8 – 8.8 mg/kg) once per week, until they reached a state of stable moderate parkinsonism. The degree and stability of the MPTP-induced motor disability was assessed, as described in many previous studies from our laboratory (Galvan et al., 2011; 2014; Masilamoni et al., 2011; Bogenpohl et al., 2013; Villalba et al., 2014; Devergnas et al., 2014; Mathai et al., 2015). Briefly, animals were evaluated weekly in an observation cage that was equipped with infrared beams, allowing us to measure their body movements as infrared beam break events. A parkinsonism rating scale was also used to quantify impairments in ten aspects of motor function (bradykinesia, freezing, extremity posture, trunk posture, action tremor, the frequency of arm and leg movements, finger dexterity, home cage activity, and balance), each scored on a 0 to 3 scale (maximal score 30). For the three MPTP-treated monkeys used in this study, the final parkinsonism rating score ranged between 13 to 20, corresponding to moderately severe parkinsonism. The severity of the parkinsonian motor signs had to be stable for a period of at least 6 weeks after the last MPTP injection before the decision was made to sacrifice the animal.

Perfusion of Animals and sectioning of tissue

At the end of the experiment, the animals were sacrificed with an overdose of sodium pentobarbital (100 mg/kg, i.v.) and transcardially perfused with cold oxygenated Ringer's solution, followed by a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in a phosphate buffer (PB) solution. After perfusion, the brains were removed from the skull, cut coronally into 10 mm thick blocks, and post-fixed overnight in 4% paraformaldehyde. The blocks were then cut into 60µm-thick coronal sections using a vibrating microtome and stored at –20 °C in an anti-freeze solution, containing 30% ethylene glycol and 30% glycerol in PB, until the time of the immunohistochemistry studies.

Immunohistochemistry

Antibodies used—For the localization of Ca_v3.1, we used a highly specific monoclonal antibody (NINDS/NIMH NeuroMab, Davis, CA). According to the supplier, this antibody reacts with the >250kDa molecular weight protein associated with Ca_v3.1, does not cross-

react with other subtypes of Ca_v3 channels, and does not result in any significant immunostaining or Western blot band labeling in tissue from Ca_v3.1 knockout mice (NeuroMab; Accession # Q9WUT2). The quality and specificity of this antibody for its use in the present study was demonstrated via Western blot analysis performed on fresh monkey brain tissue (Devergnas et al., 2016). Overall, the pattern of immunostaining throughout the monkey brain with this antibody was consistent with findings from previous anatomical and electrophysiological studies suggesting the functional expression or lack of T-type calcium channels (Cavelier and Bossu, 2003; McKay et al., 2006; Hildebrand et al., 2009; Isope et al., 2012).

Light microscopic pre-embedding immunoperoxidase procedures—Sections of thalamic tissue containing the VA and CM/Pf thalamic nuclei were chosen based on maps from the rhesus monkey stereotaxic brain atlas (Paxinos et al., 1999). Sections containing the basal ganglia-recipient regions of VA corresponded approximately to sections at the interaural 11.55 plane, while CM/Pf sections were at the interaural plane 7.95 in the stereotaxic atlas. Sections of tissue to be processed were removed from the anti-freeze solution and then placed in phosphate-buffered saline (PBS, 0.01M, pH 7.4). They were immersed in sodium borohydride (1% in PBS) for 20 minutes and incubated for 1 hour in PBS containing 1% normal horse serum (NHS), 1% bovine serum albumin (BSA), and 0.3% Triton X-100, followed by incubation in the primary antibody (anti-Ca_v3.1-NeuroMab; Accession # Q9WUT2) solution containing 1% NHS, 1% BSA, and 0.3% Triton X-100 in PBS for 24 hours at 4°C. Sections were then rinsed three times in PBS and subsequently incubated in the secondary antibody solution containing 1% NHS, 1% BSA, 0.3% Triton X-100, and biotinylated horse anti-mouse IgGs (Vector Laboratories, Burlingame, CA; used at 1:200 dilution) for 90 minutes at room temperature. After three rinses in PBS, the sections were incubated for 90 minutes in avidin-biotin peroxidase complex (ABC) solution (Vectastain standard ABC kit, Vector Laboratories; used at 1:100 dilution) including 1% BSA. To reveal the antigenic sites, the sections were first rinsed with PBS and Tris buffer (50 mM; pH 7.6), and then incubated in a solution containing 0.025% 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Sigma), 10 mM imidazole, and 0.005% hydrogen peroxide in Tris buffer for 10 min. The sections were subsequently washed several times in PBS, mounted on gelatin-coated glass slides, dehydrated, and coverslipped with Cytoseal XYL™ (Richard-Allan Scientific).

The slides were scanned at 20X using a ScanScope CS scanning light microscope system (Aperio Technologies, Vista, CA). Digital representations of the slides were saved and analyzed using the ImageScope software (Aperio). To help with the delineation of the nuclear borders between the basal ganglia- and cerebellar-receiving territories of the ventral motor nuclei, some adjacent sections were immunostained for the vesicular glutamate transporter 2 (vGluT2) (Kuramoto et al., 2011), a reliable marker of cerebellar glutamatergic terminals, using highly specific antibodies (Mab Technologies, Atlanta, GA, USA) and immunohistochemical procedures described in detail in our previous studies (Villalba et al., 2006; Raju et al., 2008). Additional adjacent sections from representative control and MPTP-treated monkeys were immunostained for tyrosine hydroxylase (TH) using specific monoclonal antibodies (Catalog nos. MAB 318; Millipore, Billerica, MA) and

immunohistochemical procedures detailed in our previous studies (Mazloom and Smith, 2006; Masilamoni et al., 2011; Mathai et al., 2015) to demonstrate the substantial reduction in dopamine conferred by MPTP treatment of the monkeys used in this study.

Electron microscopic pre-embedding immunoperoxidase protocol—Sections were immersed in sodium borohydride (1% in PBS) for 20 minutes, rinsed in PBS, and placed in a cryoprotectant solution (0.05M phosphate buffer [PB], pH 7.4, 25% sucrose and 10% glycerol) for 20 minutes prior to being frozen at -80°C for 20 minutes and thawed to permeabilize cell membranes. Then, sections were put through a graded series of cryoprotectant solution (100%, 70%, 50%, and 30% in PBS), and finally washed in PBS. The subsequent tissue processing was identical to that used for light microscopy, up to the point of the use of DAB, with two important differences: Triton X-100 was omitted from all solutions, and sections were incubated in the primary antibody solution for 48 hours at 4°C . After DAB exposure, the tissue was rinsed in PB (0.1 M, pH 7.4) and treated with 1% osmium tetroxide for 20 minutes. The sections were then dehydrated through an increasing gradient of ethanol (50%, 70%, 90% and 100%) with the initial incubation done in 70% solution containing 1% uranyl acetate for 35 minutes to increase contrast under the EM. The sections were then placed in propylene oxide and subsequently embedded in epoxy resin (Durcupan, ACM; Fluka, Buchs, Switzerland) for 24 hours. Then, the sections were mounted on microscope slides, dabbed with epoxy resin, coverslipped with mineral oil-coated coverslips, and put in the oven at 60°C for 48 hours to cure the resin. After polymerization, the coverslips were taken off, and small blocks of tissue from the basal ganglia-receiving area of the VA and CM/Pf nuclei were cut from the slides and glued onto resin blocks with cyanoacrylate glue. The blocks were cut into 60nm sections using a diamond knife ultramicrotome (Ultracut T2; Leica, Nussloch, Germany) and collected on single-slot Pioloform-coated copper grids. The sections were then stained with lead citrate for 5 minutes, rinsed in distilled water, and viewed under a transmission EM (JEM-1011; JEOL USA Inc., Peabody, MA).

Electron microscopic pre-embedding immunogold protocol—The tissue was prepared as described above, except that sections were pre-incubated in PBS containing 5% milk, rinsed in Tris Buffer Saline-Gelatin (TBS-Gelatin). On the next day, sections were first incubated for 90 minutes with secondary goat anti-mouse Fab' fragments conjugated to 1.4-nm gold particles (1:100; Nanoprobes, Yaphank, NY) and 1% dry milk in TBS-gelatin. Sections underwent incubation for approximately 10 minutes in the dark with aHQ Silver Kit (Nanoprobes) to increase gold particle sizes to 30–50-nm through silver intensification, as described in our previous studies (Kuwajima et al., 2007; Mitrano et al., 2010; Gonzales et al., 2013).

Densitometric Analysis of Light Microscopy Material—Using Imagescope image viewing software (Aperio) and the NIH Image J software (Schneider et al., 2012), we quantified the intensity of Cav3.1 immunostaining from digital 0.4x magnification images of immunostained tissue slides containing the motor thalamus in a manner similar to that reported in our previous studies (Galvan et al., 2011). The images were converted into 16-bit grayscale format and inverted. The intensity of Cav3.1 immunoreactivity was determined by

measuring the optical density of Ca_v3.1 immunoperoxidase labeling in the basal ganglia-receiving territory of the VA and CM/Pf complex of control and MPTP-treated monkeys. Adjacent vGluT2-immunostained sections were used to help separate the cerebellar- from the basal ganglia-receiving regions of the ventral motor nuclei (Kuramoto et al. 2011; Devergnas et al., 2016). In each selected nuclear region of the three control and three MPTP-treated monkeys, measurements of optical density were taken from three immediately adjacent 2.08 mm² areas of Ca_v3.1-immunostained tissue and averaged, yielding three values for each nucleus per treatment condition. The average optical density measurements in VA, CM and Pf for a single animal were calculated by taking the mean of these measurements from the three sampled areas/nucleus. Additional optical density measurements were taken in the internal capsule immediately adjacent to thalamic borders in each section in the same manner and averaged to reflect background labeling. The average values for background labeling were subtracted from measurements made in each thalamic nucleus. Student's t test was used to compare the background-corrected values between control and MPTP-treated monkeys, and between VA, CM and Pf.

Analysis of Electron Microscopy Material—At the electron microscopic level, immunoperoxidase labeling could be identified as a dark, amorphous deposit within neuronal elements, while immunogold labeling appeared as small dark, round particles within neuronal elements. To assess the localization of Ca_v3.1 labeling in normal and parkinsonian animals, 50 digital micrographs of randomly encountered Ca_v3.1-labeled neuronal elements were captured in each animal at 40,000X magnification and saved with a CCD camera (Orius 78; Gatan, Inc., Pleasanton, CA) that was controlled by DigitalMicrograph software (Gatan Microscopy Suite), yielding 2214 μm² of tissue analyzed per nucleus per animal.

Immunoperoxidase Material—Elements labeled with the peroxidase deposit were classified, based on ultrastructural features (Peters et al., 1991), and the relative distribution of Ca_v3.1 immunoreactivity among neuronal elements was compared between normal and MPTP-treated animals. Labeled dendrites were categorized into small (< 0.5μm), medium (0.5μm–1μm), or large (> 1μm) profiles, based on their cross-sectional diameter. The same immunoperoxidase-stained sections were used to assess the relative prevalence of Ca_v3.1-labeled dendrites over the total population of dendritic profiles in the VA, CM, and Pf. Values obtained for all measures were compared between control and MPTP-treated animals using Student's or Welch's t-test.

Immunogold Material—The immunogold-stained sections were used to elucidate the specific localization of Ca_v3.1 labeling in relation to synaptic and non-synaptic sites along the plasma membrane of VA and CM/Pf neurons. Asymmetric (putative excitatory) and symmetric (putative inhibitory) synapses were differentiated based on the presence or absence of thick post-synaptic densities, respectively (Gray, 1959; Uchizono, 1965; Guillery, 2000). The gold particles that were directly on or within 20 nm of the plasma membrane were identified as “plasma membrane-associated”, and categorized as either perisynaptic or extrasynaptic, based on their localization relative to postsynaptic specializations. In this manuscript, the term “perisynaptic” refers to gold particles found within 20 nm of the edges

of postsynaptic specializations. All other plasma membrane-bound gold particles were categorized as “extrasynaptic”. The 20-nm cut-off point was chosen based on the assertion that the distance between the immunoreactive substrate and gold particle, bridged by the primary and secondary antibodies, can be approximate to 20 nm (Blackstad et al., 1990). Statistical analyses were performed using the same procedures described for the immunoperoxidase data.

Results

Light microscopic (LM) immunohistochemical staining for Ca_v3.1 in the monkey thalamus

At the LM level, a high degree of Ca_v3.1 immunoreactivity was found throughout the neuropil of the monkey thalamus, except in the reticular nucleus, which was completely devoid of labeling (Fig. 1). Although the VA, CM and Pf nuclei displayed Ca_v3.1 immunoreactivity, the intensity of immunostaining was significantly lower in the CM and Pf than in the VA of normal and MPTP-treated monkeys ($p < 0.001$ control; $p < 0.05$ post-MPTP; t-test) (Fig. 1E). Overall, there was no significant difference in the intensity of immunostaining between the normal and parkinsonian animals for each thalamic nucleus. CM and Pf displayed comparable level of immunoreactivity in either state ($p = 0.39$ control; $p = 0.40$ post-MPTP; t-test) (Fig. 1E). At high magnification, immunoreactivity was apparent throughout the neuropil, with clear localization in perikarya and dendritic processes (Fig. 1A',C',C'')

EM immunoperoxidase localization of Ca_v3.1 in the VA, CM and Pf

At the EM level, ~80–90% of Ca_v3.1 channel-immunoreactive elements comprise postsynaptic dendrites of various sizes in all three thalamic nuclei examined in both, the normal and parkinsonian states. Immunoreactivity was rarely encountered in unmyelinated axons (~3–9%), glial processes, or axon terminals (<4%) (Fig. 2). MPTP treatment did not significantly alter the relative prevalence of Ca_v3.1-immunoreactive elements in the VA, CM, and Pf (Fig. 2C,F,I). The only statistically significant difference was a reduction in the proportion of labeled glial processes (from 3.4% to 1.92%) in the CM of MPTP-treated animals compared with controls ($p = 0.014$; t-test) (Fig. 2 H). The proportion of large, medium and small immunoreactive dendrites in VA, CM and Pf was also not changed in MPTP-treated animals (Fig. 3D). None of the labeled dendritic profiles had the ultrastructural features of putative GABAergic interneurons (ie vesicle-filled and pre-synaptic to axo-dendritic synapses; Ralston 1971; Hamos et al., 1985; Ohara et al., 1989; Jones, 2007). Although the peroxidase deposit in most labeled elements was diffusely distributed without any clear interactions with specific subcellular organelles (Fig. 2A,B,D,E,G,H), there were some dendrites in which aggregates of labeling were confined to the post-synaptic densities of putative asymmetric synapses (Fig. 5A).

EM immunogold localization of Ca_v3.1 in the VA and CM/Pf

Because of the diffuse and amorphous nature of the peroxidase deposit, the immunoperoxidase method is not a reliable approach to assess the subcellular and subsynaptic localization of Ca_v3.1. We used the pre-embedding immunogold method to further address these issues in normal and parkinsonian monkeys. The general pattern of

immunoreactivity amongst neuronal elements was similar to that found in the immunoperoxidase-stained sections of VA and CM/Pf, ie gold labeling was localized predominantly in dendrites of various sizes, with additional sparse labeling in axons, terminals and glia. In immunoreactive dendrites, 75–87% of the Ca_v3.1 immunogold labeling was found in direct association with plasma membranes (Fig. 4 A–E). This pattern was the same in all nuclei examined, and was not significantly different in MPTP-treated animals (Fig. 4E).

Of the plasma membrane-bound gold particles in dendrites of VA, CM and Pf neurons, 12–17% were located within 20nm of the edge (ie perisynaptic) of symmetric and asymmetric synapses (Fig. 5 A–D), while the remainder was expressed extrasynaptically (Fig 6 A,B). Perisynaptic labeling was found in dendrites of all sizes (Fig. 5B–D). When normalized to the portion of membrane occupied by the individual subsynaptic domains (perisynaptic vs extrasynaptic) in each of the nuclei, the relative density of perisynaptic Ca_v3.1 immunogold labeling was significantly higher than would be expected from a random distribution across all three nuclei (Fig. 6 C).

Discussion

The results of this study demonstrate that the Ca_v3.1 subtype of T-type calcium channel immunoreactivity is strongly expressed in neurons of the VA, CM, and Pf thalamic nuclei, albeit to a higher level in VA than in CM/Pf, in primates. At the subcellular and subsynaptic level, Ca_v3.1 channels are expressed along the whole somatodendritic domain of thalamic neurons, being preferentially aggregated extrasynaptically or perisynaptically to putative glutamatergic and non-glutamatergic synapses (Fig. 7). Overall, the pattern of cellular and subcellular localization of the channel was not affected in MPTP-treated parkinsonian animals. Together, these findings indicate that Ca_v3.1 channels are located to subserve widespread influences over burst firing activity, but also modulation of specific glutamatergic and non-glutamatergic synapses, in basal ganglia-receiving nuclei of the primate thalamus in normal and parkinsonian state.

Uniform dendritic expression of Ca_v3.1 immunoreactivity in the monkey thalamus

The strong level of Ca_v3.1 immunoreactivity throughout the whole monkey thalamus is consistent with results of previous *in situ* hybridization and light microscopic immunohistochemical studies in rodents and cats (Talley et al., 1999; McKay et al., 2006; Kovacs et al., 2010; Parajuli et al., 2010; Liu et al., 2011). Similarly, the predominant dendritic localization of the Ca_v3.1 channels found in this study is consistent with previous data from the cat reticular nucleus (Kovacs et al., 2010) or different brain regions including the hippocampus, cerebellum (Christie et al., 1995; Kavalali et al., 1997; Gauck et al., 2001) and subthalamic nucleus (Song et al., 2000). However, their relative distribution along the somatodendritic axis of thalamic neurons has not yet been fully elucidated and remains a matter of debate. Although previous electrophysiological, pharmacological and neuronal modeling studies have suggested different patterns of T-type channels expression in various populations of thalamic neurons (Munsch et al., 1997; Zhou et al, 1997; Destexhe et al., 1998; Williams and Stuart, 2000; Rhodes and Llinas, 2005), these findings must be

interpreted with caution because of the lack of selective T-type calcium channel blockers, the limited ability to detect calcium signaling in small-sized distal dendrites and the equivocal criteria used to define T-type currents. Our ultrastructural findings indicate that T-type calcium channels are expressed along both the proximal and distal parts of the dendritic tree of thalamic cells in VA, CM and Pf neurons in normal and parkinsonian monkeys. A similarly uniform dendritic expression of $\text{Ca}_v3.1$ immunoreactivity has also been reported in thalamocortical neurons of the lateral geniculate nucleus in mice (Parajuli et al., 2010). However, another study that used immunofluorescence as labeling method suggested that Cav3.1 immunoreactivity is confined to the cell bodies and proximal dendrites of thalamocortical neurons in the rat lateroposterior and ventral posterolateral nuclei (McKay et al., 2006). The sensitivity of different Cav3.1 antibodies combined with species and nuclear specificity in the distribution of channels along the dendritic tree of thalamic cells may explain these differences. The recent development of highly specific T-type calcium channel blockers (Shipe et al., 2008; Yang et al., 2008; Dreyfus et al., 2010; Ardashov et al., 2011; Xiang et al., 2011) will help elucidate the specific roles of T-type channels on the different parts of the dendritic tree of these thalamic neurons. In that regard, electrophysiological studies have demonstrated that an important role of spatially and temporally regulated T-type calcium channel-mediated dendritic $\text{Ca}(2+)$ signaling properties throughout the whole dendritic tree of rat thalamocortical neurons is to mediate regenerative propagation of low threshold spikes in a behavioral state-dependent manner (Errington et al., 2010). It has also been shown that T-type calcium channel-mediated dendritic properties are highly conserved between population of thalamocortical and reticular low-threshold spiking neurons, and that these properties underlie a whole-cell somato-dendritic spike generation mechanism that makes low threshold spikes a unique global electrical and biochemical signal in neurons (Connelly et al., 2015).

Differential expression level of $\text{Ca}_v3.1$ immunoreactivity in CM/Pf and VA

The intensity of labeling was significantly lower in CM/Pf than in VA in both normal and parkinsonian monkeys. This difference in staining intensity may be due to striking morphological differences between projection neurons in the CM/Pf and other thalamic nuclei (Lacey et al., 2007; Jones 2007). In contrast to most thalamic neurons, including those in VA, which display a profuse “bushy” dendritic arbor, CM/Pf neurons harbor a much less arborized “reticular-like” dendritic tree (Lacey et al., 2007; Jones, 2007). In rats, the total dendritic length of single “bushy” neurons of the centrolateral nucleus can be as much as two times larger than neurons of the Pf (Lacey et al., 2007). The differential level of T-type channel expression between the Pf and other thalamic nuclei may have functional consequences. It is, indeed, noteworthy that rodent Pf neurons rarely discharge low-threshold calcium spikes during cortical slow-wave activity *in vivo*, compared with neurons in the centrolateral nucleus (Lacey et al., 2007). These observations suggest that the lower expression of T-type calcium channels may confer CM/Pf neurons specific physiological properties that allow them to respond differently to different states of vigilance or sleep compared with other thalamic nuclei. Other factors, such as the level of membrane depolarization of CM/Pf neurons compared with VA may also account for their differential propensity in developing LTS. If such is the case, these observations add further evidence to the concept put forward in our previous studies that CM/Pf neurons are a unique

subpopulation of thalamic neurons endowed with anatomical and functional properties distinct from most other thalamic neurons (Galvan et al., 2011; Smith et al., 2004, 2014).

Ca_v3.1 at glutamatergic and non-glutamatergic synapses

Our immunogold data revealed two major structural features related to the subsynaptic localization of Ca_v3.1 in the monkey thalamus. They showed that most gold particles were bound to the plasma membrane of small-, medium- and large-sized dendrites in VA and CM/Pf. This predominant membrane localization of Ca_v3.1 can be interpreted as channel insertion in the plasma membrane, while the low level of intracellular immunoreactivity likely represents newly synthesized or internalized proteins being trafficked to intracellular organelles or different parts of the dendritic tree. Thus, these observations provide further support that “functional” membrane-bound T-type calcium channels are expressed along the entire somatodendritic domain of thalamic neurons in VA and CM/Pf. These observations are consistent with those reported in the rodent lateral geniculate nucleus (Parajuli et al., 2010). However, because these immunohistochemical data are not reliable indicators of the relative amount and physiological responsiveness of the channels at different dendritic sites, future in vitro slice studies are needed to further characterize the properties of T-type channels along the somatodendritic domain of these neurons.

Our immunogold data also showed that a subset of Ca_v3.1 channels selectively aggregates perisynaptically at putative glutamatergic and non-glutamatergic synapses in the VA, CM and Pf of rhesus monkeys. To our knowledge, this is the first direct evidence for perisynaptic expression of T-type calcium channels in the mammalian thalamus. Although the source(s) of terminals associated with these channels was not determined, their ultrastructural features suggest that those forming asymmetric synapses likely originate from the cerebral cortex, while those forming symmetric synapses may come from GABAergic reticular neurons or interneurons (Liu et al., 1995; Ilinsky et al., 1999; Jones, 2007). However, we cannot rule out that some of these inputs may also arise from monoaminergic or cholinergic ascending brainstem afferents. Because pre-embedding immunostaining may lead to false negative results due to limited access of antibodies to their antigenic sites, our data do not allow to quantify the exact percentage of total symmetric and asymmetric synapses that express perisynaptic Cav3.1 labeling. However, based on the high prevalence of labeled synapses encountered during our electron microscopy analyses, we suggest that T-type calcium channels are frequently associated with synaptic junctions in the basal ganglia-receiving nuclei of the monkey thalamus.

While the physiological role of T-type calcium channels at these synapses was not studied, it is worth discussing hypotheses regarding the function of these channels, based on rodent data from other brain regions. One possibility is that they allow calcium influx to boost depolarization induced by specific cortico-thalamic glutamatergic synapses, a mechanism for signal amplification that has been documented for other calcium channels at axo-spinous glutamatergic synapses on cortical and hippocampal pyramidal neurons (Markram and Sakmann, 1994; Magee et al., 1995; Isope et al., 2012; Lambert et al., 2013). Another possibility is that the Ca_v3.1 channels functionally interact and couple with group I metabotropic glutamate receptors to regulate fast calcium signaling pathway at specific

cortico-thalamic synapses. A similar phenomenon has recently been described for parallel fiber synapses on Purkinje cells in the rodent cerebellar cortex (Isope et al., 2012, Hildebrand et al., 2009). This hypothesis is further supported by the fact that group I mGluRs display a perisynaptic pattern of localization similar to that described here for $\text{Ca}_v3.1$ channels in many thalamic nuclei and other brain regions (Baude et al., 1993; Liu et al., 1998; Hanson and Smith, 1999; Paquet and Smith, 2003). Because of its perisynaptic localization (Villalba et al., 2006), GABAB receptors may be another target of relevance to consider as possible functional partners with T-type channels at glutamatergic and non-glutamatergic synapses in the monkey thalamus, although functional evidence for such interaction in the thalamus and other regions remain weak and poorly understood (Guyon and Leresche, 1995; Huang et al., 2015).

Thalamic expression of $\text{Ca}_v3.1$ immunoreactivity in normal and parkinsonian states

In line with our recent findings (Devergnas et al., 2015), results of the present study did not reveal any major changes in the overall pattern of $\text{Ca}_v3.1$ expression in the VA and CM/Pf of MPTP-treated parkinsonian monkeys. Thus, despite clear evidence for increased thalamic rebound bursting in the parkinsonian state (Zirh et al., 1998; Magnin et al., 2000; Pessiglione et al., 2005; Devergnas et al., 2016), these functional changes are unlikely to result from abnormal cellular, subcellular and subsynaptic expression of $\text{Ca}_v3.1$ channels. However, we cannot rule out that functional properties of T-type channels might be affected in parkinsonism. On the other hand, the fact that intracerebral injections of a specific T-type calcium channel blocker in the basal ganglia-receiving region of the thalamus did not significantly affect the rate and pattern of neuronal activity of BGMT neurons in our previous study (Devergnas et al., 2016) suggests that altered T-type channel activity may not be the sole source of alterations in thalamic firing in the parkinsonian state. A thorough assessment of the functional properties of T-type calcium channels in thalamic slices from normal and dopamine-depleted animals is warranted to further address this issue.

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Figure 1.

(A–D) Cav3.1-immunostained coronal brain sections at different rostrocaudal levels of the thalamus in control (A,C) and MPTP-treated (B,D) monkeys. (A',C',C'') show high-power micrographs of Ca_v3.1 immunoreactivity in the VA, CM, and Pf of a control animal. The delineation of thalamic nuclear boundaries was based on *The Rhesus Monkey Brain in Stereotactic Coordinates* (Paxinos et al., 2000) and a coronal atlas of the macaque brain (Lanciego and Vazquez, 2012). (E) Measurements of optical intensity taken in the VA, CM and Pf (values are mean ± SEM). Significance was assessed with the Mann-Whitney Rank Sum Test and MPTP-treatment related changes were found to be insignificant at the $\alpha = 0.05$ significance level. Both the CM and Pf expressed a significantly lower optical density than VA in normal and MPTP-treated monkeys (asterisks). Scale bars: A=2mm (valid for B, C, D). A'=50 μ m (valid for C', C''). Abbreviations: CM – centromedian thalamic nucleus, CN – caudate nucleus, GPe – external segment of the globus pallidus, GPi – internal segment of the globus pallidus, IC – internal capsule, Pf – parafascicular thalamic nucleus, PUT – putamen, Rt – reticular nucleus, VAmc- ventroanterior nucleus, magnocellular division; VApC- ventroanterior nucleus, parvocellular division; VL- ventrolateral nucleus; VLo – ventrolateral thalamic nucleus, pars oralis; VPL- ventroposterior nucleus, pars lateralis.

Figure 2. Immunoperoxidase localization of $Ca_v3.1$ in the thalamus of control and MPTP-treated monkeys. (VA-A,B; CM-D,E; Pf-G,H) Representative electron micrographs of $Ca_v3.1$ immunoreactivity in thalamic nuclei of control (A, D, G) and MPTP-treated (B, E, H) monkeys. Immunoreactive dendritic and unmyelinated axonal processes are labeled. (C, F, I) Distribution of $Ca_v3.1$ -immunoreactive elements in the VA, CM and Pf of control and MPTP-treated animals. Values are mean $\pm$ SEM and comparison between control and MPTP-treated monkeys tested with either student's t-test or Welch's t-test, depending on the variance of the groups as determined by the F-test. In E, note a statistically significant

difference (*) between treatment groups in the density of immunoreactive glia in the CM ($p = 0.014$; t-test). Values are mean $\pm$ SEM. Abbreviations: U.Ax – unmyelinated axon, Den – dendrite. Scale bar in A= $0.5\mu\text{m}$ (valid for all micrographs).

Figure 3.

EM immunoperoxidase localization of Ca_v3.1 labeling in dendrites of thalamic neurons in control and MPTP-treated monkeys. (A–C) Representative examples of Ca_v3.1 immunoreactivity in small (≤0.5μm), medium (0.5μm–1μm), and large (≥1μm) dendrites of thalamic nuclei of control and MPTP-treated monkeys. (D) Percent of small, medium, and large dendrites out of all Ca_v3.1-immunoreactive dendrites. The MPTP treatment did not alter the general distribution of Ca_v3.1 immunoreactivity among dendritic profiles of different sizes in the VA, CM, and Pf ($p > 0.05$; t -test). Values are mean $\pm$ SEM. Abbreviations: Den – dendrite. Scale bar in A = 0.5μm (valid for B,C).

Figure 4.

Pre-embedding immunogold labeling for Cav3.1 in dendrites of VA, CM, and Pf nuclei in control and MPTP-treated monkeys. (A–D) Immunogold labeling along the plasma membrane of dendrites of various sizes in all thalamic nuclei. (E) Relative percentage of plasma membrane-bound gold labeling in dendrites of VA, CM and Pf neurons in control and MPTP-treated monkeys. In all nuclei, MPTP treatment did not significantly affect the plasma membrane localization of Cav3.1 immunoreactivity. Values are mean $\pm$ SEM.

Abbreviations: Den – dendrite, Te – terminal. Scale bars: A= 0.5 μ m (valid for B); C= 0.1 μ m (valid for D).

Figure 5.

EM immunoperoxidase and immunogold localization of Cav3.1 at asymmetric and symmetric synapses in dendrites of thalamic neurons in control and MPTP-treated monkeys. (A-D) High powered immunoperoxidase (A) and immunogold (B-D) Cav3.1 dendritic labeling. Note in (A) the dense immunoperoxidase deposit at two post-synaptic densities of putative asymmetric axo-dendritic glutamatergic synapses (*) compared with the lack of labeling at another asymmetric synapse on the same dendrite (X). (B-D) show high powered

micrographs of perisynaptic Cav3.1 immunogold labeling at asymmetric (arrows) or symmetric (+) axo-dendritic synapses. Scale bar: A=0.5 μ m (valid for B-D).

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Figure 6.

Quantification of $\text{Ca}_v3.1$ channel immunogold labeling associated with different sectors of the plasma membrane of thalamic neurons in VA, CM and Pf. (A-B) Comparison between the percentages of $\text{Ca}_v3.1$ gold particle labeling at extrasynaptic and perisynaptic sites (black bars) and the proportion of total plasma membrane occupied by these two subsynaptic domains based on random observations (white bars). Data are expressed as means $\pm$ SE of measurements in 6 animals. Asterisks in B indicate that the percent plasma membrane-bound gold particles perisynaptic labeling is significantly higher than that expected from a random distribution of labeling in this domain along the plasma membrane (Mann-Whitney U test; $p < 0.05$). (C) Relative density of $\text{Ca}_v3.1$ immunogold labeling on different portions

of the plasma membrane. Normalized values were calculated as the percentage of labeling for $\text{Ca}_v3.1$ in a specific subsynaptic domain divided by the percentage of dendritic membrane that contributes to that domain along the plasma membrane. Note that the relative density of labeling for $\text{Ca}_v3.1$ is higher at perisynaptic sites than on the extrasynaptic plasma membrane.

Figure 7. Summary diagram of the subsynaptic localization of Cav3.1 labeling in thalamic neurons of VA, CM and Pf. Perisynaptic labeling at asymmetric (putatively glutamatergic, green terminals with thick post synaptic density) and symmetric (unknown neurotransmitter, blue terminal with thin post synaptic density) synapses, as well as extrasynaptic and intracellular labeling are depicted (black circle). Modified from: Ladyof-tats (2007), Diagram of neurons (nerve cells), Wikimedia Commons.