Original Article

Distribution of neuronal nitric oxide synthase immunoreactivity in adult male Sprague-Dawley rat brain

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ABSTRACT

Neuronal NOS (nNOS) accounts for most of the NO production in the nervous system that modulates synaptic transmission and neuroplasticity. Although previous studies have selectively described the localisation of nNOS in specific brain regions, a comprehensive distribution profile of nNOS in the brain is lacking. Here we provided a detailed morphological characterization on the rostro-caudal distribution of neurons and fibres exhibiting positive nNOS-immunoreactivity in adult Sprague-Dawley rat brain. Our results demonstrated that neurons and fibres in the brain regions that exhibited high nNOS immunoreactivity include the olfactory-related areas, intermediate endopiriform nucleus, Islands of Calleja, subfornical organ, ventral lateral geniculate nucleus, parafascicular thalamic nucleus, superior colliculus, lateral terminal nucleus, pedunculopontine tegmental nucleus, periaqueductal gray, dorsal raphe nucleus, supragenual nucleus, nucleus of the trapezoid body, and the cerebellum. Moderate nNOS immunoreactivity was detected in the cerebral cortex, caudate putamen, hippocampus, thalamus, hypothalamus, amygdala, and the spinal cord. Finally, low NOS immunoreactivity were found in the corpus callosum, fornix, globus pallidus, anterior commissure, and the dorsal hippocampal commissure. In conclusion, this study provides a comprehensive view of the morphology and localisation of nNOS immunoreactivity in the brain that would contribute to a better understanding of the role played by nNOS in the brain.

Keywords: Neuronal Nitric Oxide Synthase, Immunohistochemistry, Morphology, Neuroanatomy

INTRODUCTION

Nitric oxide (NO) is a free radical signalling molecule that is involved in a broad range of biological functions. These include modulation of immunological responses (Oleszak et al., 1998), synaptic transmission (de Vente et al., 2001), regulation of vascular tone (Greenwood et al., 1997), gene transcription and mRNA translation, as well as post-translational modification of proteins (Brüne et al., 1994). In mammals, NO is synthesized from L-arginine by a family of three nitric oxide synthase (NOS) isoforms, namely the neuronal nitric oxide synthase (nNOS or NOS1), cytokine-inducible nitric oxide synthase (iNOS or NOS2), and the endothelial nitric oxide synthase (eNOS or NOS3) (Moncada et al., 1991; Nathan and Xie, 1994). The nNOS was found to be expressed in specific neurons in the central and peripheral nervous systems (Bredt et al., 1991; Vincent, 2010), while its isoforms were found in non-neuronal cells such as iNOS in macrophages, astrocytes and microglia (Iadecola et al., 1995; Oleszak et al., 1998), as well as eNOS that was mainly detected in the endothelial cells (Marsden et al., 1993). Among the three NOS isoforms, nNOS constitutes the predominant source of NO in neurons as it is involved in various physiological functions (Guix et al., 2005). In earlier studies, the cerebellum has been detected to have the highest NOS activity and nNOS expression, while the brainstem and cerebral cortex exhibit lower levels of nNOS expression (Bredt et al., 1991). Furthermore, the brain nNOS was observed to be present in particulate and soluble forms in animal brain tissues (Hecker et al., 1994). Although localisation of the nNOS-immunoreactivity (nNOS-ir) was predominantly found within the cytoplasm of neurons in rat brain, nuclear localisation of nNOS was observed in some neurons and glial cells without cytoplasmic nNOS staining (Korzhevskii et al., 2008). This article focuses on the localisation of nNOS and its distribution across different regions along the rostro-caudal axis of the rat brain.

Over the past few decades, an increasing number of studies have validated the roles of nNOS in mediating between neuronal signalling and synaptic plasticity. The nNOS signalling was observed to be involved in modulating physiological processes including neurogenesis (Zhu et al., 2006), modulation of nociception, memory formation and learning (Böhme et al., 1993), as well as

regulation of neurotransmitter release via long-term depression and potentiation in the central nervous system (Bon and Garthwaite, 2003). A recent study further revealed that nNOS is also involved as a neurotransmitter modulator in the pathogenesis of obsessive-compulsory disorder (Topaloglu et al., 2016). Numerous neurodegenerative diseases, for example Parkinson's disease, Alzheimer's disease, and multiple sclerosis, have also been shown to associate with excessive and abnormal NO signalling which is triggered by the activation of nNOS (Steinert et al., 2010). Aside from its role in neuronal function, nNOS is also present in the skeletal muscle, cardiac and smooth muscles (Forstermann et al., 1994; Förstermann and Sessa, 2012). NO produced by nNOS is also known to be involved in the regulation of blood pressure (Togashi et al., 1992). Blockade of nNOS activity in the hypothalamus and medulla resulted in systemic hypertension (Toda et al., 2009). Taken together, these further imply that nNOS plays a crucial role in a wide range of physiological functions of both the central and peripheral nervous systems.

The anatomical distribution of nNOS using nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) histochemistry and nNOS immunohistochemistry has been described in various brain regions across species (Bredt et al., 1991; Giraldez-Perez et al., 2008; Menendez et al., 2006; Rodrigo et al., 1994; Vincent, 2010; Vincent and Kimura, 1992). Despite the fact that some data demonstrated the co-localization of NADPH-d with nNOS in certain neurons (Bredt et al., 1991; Dawson et al., 1991), inconsistent findings were found with respect to the histochemical detection of NADPH-d and nNOS-ir in different regions of the rat brain (Sancesario et al., 1996; Spessert and Claassen, 1998; Wang and Morris, 1996) as well as subcellular location of nNOS in the neurons (Rothe et al., 1998). The NADPH-d histochemistry was also reported to detect other NOS isoforms and several types of NADPH-oxidoreductases, suggesting the non-specificity of NADPH-d reaction as a marker for nNOS (Blottner et al., 1995; Rothe et al., 1998). These inconsistent results were probably due to the various methodologies of nNOS-ir density analysis, differences of rodent species, age, gender and weight of the animals used for the investigation, as

well as various staining protocols. Given that nNOS-derived NO is associated with many brain functions (Barkhuizen et al., 2017; Bredt et al., 1991; Guix et al., 2005), localisation of the nNOS-positive neurons and fibres in the brain using antibody specific for rat nNOS would provide fundamental information that would contribute to a better understanding of the role played by nNOS in modulating various physiological functions. Therefore, our current study aims to provide a rigorous analysis and detailed morphological characterization on the rostro-caudal distribution of neurons and fibres expressing positive nNOS-ir in the adult male Sprague-Dawley rat brain.

MATERIAL AND METHODS

Subjects

Two adult male Sprague-Dawley rats (26 weeks-old) were housed in a cage with *ad libitum* access to food and water. These rats were kept under a 12 h dark-light cycle at a controlled temperature (22±1ºC) and humidity (60-70%). The euthanasia procedure was performed after obtaining ethical approval from the *Committee on the Use of Live Animals in Teaching and Research*, The University of Hong Kong.

Histological processing

The animals were anaesthetized with Pentobarbital (75 mg/kg) and perfused transcardially with 4% paraformaldehyde fixative solution. After post-fixation for 48 hrs, the brains were rinsed in PBS, and dehydrated through a series of ethanol and xylene. For paraffin embedding procedures and histochemical staining of NOS, the methodology was performed as previously described with minor modifications (Drobysheva et al., 2008; Shin et al., 2000). In brief, the brains were embedded in paraffin wax at 56-58°C melting point, placed in TissueTek molds and then mounted on TissueTek cassettes. The brain tissues were subsequently sectioned into 10-µm-thickness coronal sections using a microtome, and then deparaffinized by immersing the section slides in xylene and graded ethanol, and finally transferred to deionized water. To block endogenous peroxidase activity, the

section slides were immersed in 0.3% hydrogen peroxide. Prior to staining, the section slides underwent antigen retrieval procedure with 10 mM citrate buffer (pH 6.0) for 10 min at 95-100 °C. Following antigen retrieval, all sections were incubated overnight at room temperature with the sheep polyclonal antibody specific for rat nNOS (1:1000; provided by Dr H.W.M. Steinbusch and Dr. P. Emson, Cambridge University, UK) (Barkhuizen et al., 2017; Herbison et al., 1996), diluted in 0.1% Bovine Serum Albumin (BSA) and Tris Buffered Solution (TBS)-Triton (TBS-T) solution. Negative controls were included by omitting the primary antibody. The tissue sections were then washed 3 times with TBS-T, TBS, and TBS-T solution; followed by 2 hrs incubation with a biotinylated secondary antibody of donkey anti-sheep (diluted 1:100; Mol. Probes, USA) at room temperature. Next, the brain sections were incubated with avidin (diluted 1:400 reagent A in TBS-T) and biotinylated horseradish peroxidase H (diluted 1:400 reagent B in TBS-T; Elite ABC-kit, Vectastain, Burlingame, USA) for 2 hrs

. In between steps, all sections were washed with TBS and TBS-T. To visualise the immune complex of horseradish peroxide (HRP) reaction product, the tissue sections were incubated with 3, 3'-diaminobenzidine tetrahydrochloride (Vector® DAB Substrate Kit; Vector Laboratories, USA). The reaction was stopped after 5 min by washing the sections thoroughly with TBS. All sections were cover-slipped using Permount mounting medium (Thermo Fisher Scientific, Waltham, USA). Brain sections were examined using the Axiophot2 Imaging Microscope (Carl Zeiss Microscopy GmbH, Gottingen, Germany).

To further delineate the brain regions, the coverslips were removed by soaking in xylene.The brain sections were counterstained using the hematoxylin and eosin (H&E) staining to observe the different brain regions. After removing the coverslips, the sections were rinsed with xylene twice for 5 min each, and rehydrated in 100% ethanol twice for 5 min, 95% alcohol and 75% alcohol for 2 min each, washed with distilled water. Then, the sections were stained with Harris hematoxylin solution for 8 min and rinsed in tap water for 5 min. Next, 1% acid alcohol was used to differentiate the tissues for 30 s and the sections were rinsed with tap water for 1 min. In

the bluing step, the sections were soaked in saturated lithium carbonate solution for 30 s, washed with tap water for 5 min and rinsed in 95% ethanol. Counterstaining of the sections was performed with eosin Y ethanol solution for 30 s. The sections were then dehydrated with 95% and 100% ethanol twice for 5 min each, followed by xylene twice for 5 min each. Finally, all sections were cover-slipped using Permount mounting medium (Thermo Fisher Scientific, Waltham, USA). The brain sections were examined again using the Axiophot2 Imaging Microscope (Carl Zeiss Microscopy GmbH, Gottingen, Germany).

Evaluation of nNOS immunoreactive cells in different brain regions

The densities of nNOS-positive neurons and fibres was graded by two independent researchers according to the different levels of their densities: Neuron (not visible – 0; few/low – 1; moderate – 2; and many/high – 3); fibre (not visible – 0; low– 1; scattered/moderate – 2; and dense/high – 3), a method previously used with minor modifications (Rodrigo et al., 1994; Usunoff et al., 2006). The density decribed in this study was based on the degree of distribution of nNOS-ir neurons in the brain region. Photomicrographs of the categorization of nNOS-positive neuronal and fibre densities were presented in Fig. 1C. Low density was described when few nNOS-ir neurons or fibres were observed, while moderate density was described with scattered or clusters of nNOS-ir neurons or fibres present in the brain region. High density was used when clusters of nNOS-positive neurons and dense fibre plexus were observed within the specific region. The nNOS-ir neurons were categorised into small (< 15 µm) and large (15 – 30 µm) size, based on the diameters of the nNOS-ir cell bodies observed in the brain regions. Photomicrographs of small and large nNOS-ir neurons in high magnification were shown in Figure 1D. The specific morphology and cytoarchitecture characteristic of the nNOS-ir neurons and fibres was also described according to their shape (bipolar, multipolar) and processes. Photomicrographs of the nNOS-ir within the areas of interest were taken using an Olympus DP73 digital camera (Olympus, Hamburg, Germany) attached to the bright-field microscope at 4 X, 10 X, and 20 X magnification. The nomenclature on various anatomical regions of interest was adopted according to the rat brain atlas of Paxinos and Watson

(Paxinos and Watson, 2007). Photomicrographs of the nNOS-ir presented in this study were converted into grayscale with the brightness and contrast adjusted with the Adobe Photoshop software (Adobe System, San Jose, USA). The scale bar for the photomicrographs of each complete brain sections in Fig. 1-11 is 500 µm. Note, scale bars in the higher magnification of photomicrographs are 20 µm and 50 µm in Fig. 2, and 50 µm in Fig. 3-8.

RESULTS

The schematic diagram in Fig. 1A shows a sagittal view of the major brain structures in rat brain; while Fig. 1B elucidates the brain in rostro-caudal levels that correspond to the photomicrographs of the respective coronal brain sections shown in Fig. 2-8. Neurons expressing the nNOS-ir showed dark labelling within the cytoplasm and initial segment of the axons/dendrites, while the cell nuclei remained unstained. Neurons and fibres expressing the nNOS staining were present in various regions throughout the rat brain with different density of immunoreactivity. The terms and abbreviations that were used to describe a specific brain region can be found in the list of abbreviations (Table 1).

* Table 1 about here *

Olfactory-related areas

High densities of nNOS-positive neurons and fibres were observed in clusters at the glomerular layer of the olfactory bulb (Fig. 2(A) a & d). Dense fibres were also observed at the granule cell layer of the olfactory bulb (Fig. 2(A) b) and the olfactory tubercule (Fig. 3(A) l). In contrast, the fibre network of nNOS-ir was less substantial in the olfactory nerve (Fig. 2(A) d). Low densities of nNOS-ir neurons and fibres were observed in the granule cell layer of the accessory olfactory bulb (Fig. 2(A) c). Moderate densities of nNOS-positive neurons and dense nNOS-ir fibres were observed at the surrounding area of olfactory ventricle (Fig. 3(A) g). Furthermore, high densities of nNOS-ir neurons and fibres were also observed in layer 2 and 3 of the nucleus of lateral olfactory

tract (Fig. 4(A) l). Majority of these nNOS-ir neurons within the olfactory region exhibited large cell size with bipolar morphology of round and fusiform shape.

Tenia tecta

Low densities of nNOS-positive neurons and fibres were present at the dorsal tenia tecta, dorsal transition zone and navicular nucleus of the basal forebrain (Fig 2(B) g & Fig 3(A) i), ventroposterior part of the anterior olfactory nucleus and ventral tenia tecta (Fig. 2(B) i). The nNOS-ir neurons scattered within the tenia tecta were smaller in size.

* Figure 1 & 2 about here *

Anterior cortical areas

Motor cortex: Moderate densities of nNOS-positive neurons and fibres were found scattered in the primary motor cortex (Fig. 2(B) b) and the secondary motor cortex (Fig. 2(B) a & Fig. 3(A) a). The nNOS-ir neurons were large in size and exhibited either multipolar or bipolar morphology with processes.

Medial prefrontal cortex: Moderate density of nNOS-ir neurons with bipolar and multipolar morphology with dense nNOS-positive fibres were found at the dorsal peduncular cortex (Fig. 3(A) g). Moderate densities of nNOS-positive neurons and fibres were also observed within the prelimbic cortex (Fig. 2(B) c). The nNOS-positive neurons exhibited both multipolar and bipolar morphology with long processes. Similarly moderate densities of nNOS-positive neurons and fibres were observed to be scattered in the infralimbic cortex (Fig. 3(A) d). The nNOS-positive neurons were slightly larger in size and exhibited bipolar morphology. Furthermore, moderate densities of neurons and varicose fibres were also observed in the cingulate cortex (Fig. 3(A) a).

Orbital cortex: Moderate densities of neurons and fibres were observed within the medial orbital cortex (Fig. 2(B) e). The nNOS-positive neurons exhibited both multipolar and bipolar morphology.

Agranular insular cortex: Moderate nNOS-ir neuronal density was observed in the ventral part of the agranular insular cortex which contained nNOS-positive neurons, exhibiting bipolar morphology and dense nNOS-ir fibres (Fig. 3(A) f). Comparatively, few nNOS-positive neurons and fibres were observed in the dorsal part of the agranular insular cortex (Fig. 2(B) d & Fig. 3(A) f).

Somatosensory cortex: Moderate densities of neurons and fibres were observed in the primary somatosensory cortex, jaw region and the primary somatosensory cortex (Fig. 3(A) c & Fig. 4(A) b). These nNOS-ir neurons were large in size and exhibited both multipolar and bipolar morphology. However, low neuronal and fibre densities were observed in the primary somatosensory cortex forelimb region and dysgranular zone (Fig. 3(B) b). Notably, high densities of large nNOS-positive neuron and dense fibre were also observed in the secondary somatosensory cortex (Fig. 4(B) d).

Piriform cortex and endopiriform nucleus: Many nNOS-ir fibres with few neurons were found at layers 1 to 3 of the piriform cortex (Fig. 3(A) k). However, dense nNOS-positive fibres and high density of large nNOS-ir neurons were observed in the dorsal and intermediate endopiriform nucleus (Fig. 2(B) h; Fig. 3(A) h & j; Fig. 4(A) j). Majority of the nNOS-ir cell population observed at the endopiriform nucleus were fusiform or multipolar in shape with long processes, distributed within the dense nNOS-ir plexus. However, moderate density of nNOS-positive fibres with few nNOS-positive neurons were present at the claustrum (Fig. 3(A) e).

Basal ganglia

Nucleus accumbens: Moderate nNOS-positive neuronal and fibre densities were observed within the anterior commissure (Fig. 3(A) h; Fig. 3(B) f & g). In contrast, high densities of nNOS-ir fibres and neurons were observed in surrounding areas of the anterior commissure, including the shell and

core of the nucleus accumbens (Fig. 3(A) h; Fig. 3(B) f & g). The nNOS-ir neurons present in the nucleus accumbens exhibited multipolar and bipolar morphology with long processes and varicose plexus.

Ventral pallidum: Moderate densities of nNOS-positive neurons and fibres were present at the ventral pallidum (Fig. 3(B) k). The cluster of nNOS-ir neurons was observed to be small in size with less dense varicose fibres.

Caudate putamen: A moderate densities of nNOS-positive neurons and fibres were observed in the caudate putamen (Fig. 3(B) h; Fig. 4(A) d). The nNOS-ir neurons exhibited either multipolar or bipolar morphology with long and varicose processes.

Islands of Calleja: High densities of nNOS-positive neurons and fibres were observed in the Islands of Calleja (Fig. 3(B) l).

Globus pallidus: No visible nNOS-ir neurons and low density of nNOS-ir fibres was observed in the globus pallidus (Fig. 4(A) h).

Internal capsule and Entopeduncular nucleus: High density of nNOS-positive neurons and scattered nNOS-ir fibres were observed across the boundary segregating the internal capsule and the entopeduncular nucleus (Fig. 4(B) g).

Septal and diagonal band nuclei

Moderate densities of nNOS-positive neurons and fibres were observed within the lambdoid septal zone (Fig. 3(B) d). Dense nNOS-ir fibres and neurons were observed at the medial septal nucleus, while low densities of nNOS-ir neurons and fibres were observed at the intermediate part of the lateral septal nucleus (Fig. 3(B) e). Clusters of multipolar nNOS-positive neurons with large cell

bodies were also observed at the vertical and horizontal limb of the diagonal band (Fig. 3(B) j). Furthermore, a cluster of high density of small and nNOS-ir neurons with dense plexus was observed in the subfornical organ (Fig. 4(A) e).

* Figure 3 & 4 about here *

Amygdala and bed nucleus of the stria terminalis

Immunoreactivity of nNOS was scattered throughout the amygdaloid complex, with nNOS-ir neurons and a fine network of moderate nNOS-ir fibres observed within this region. Heavily stained nNOS-ir multipolar neurons with large cell bodies were found in the anterior amygdala area (Fig. 4(A) k), amygdalostriatal transition area and dorsolateral part of the lateral amygdaloid nucleus (Fig. 4(B) h) as well as posteroventral part of medial amygdaloid nucleus (Fig. 4(B) k). Low density of nNOS-ir fibre with no nNOS-positive neuron was observed within the central nucleus of amygdala (Fig. 5(A) h). Meanwhile, moderate densities of nNOS-positive neurons and dense fibres were observed in the basolateral amygdaloid nucleus (Fig. 4(B) l & Fig. 5(A) k) and in the ventrolateral part of the lateral amygdaloid nucleus (Fig. 5(A) i). Moderate densities of multipolar neurons and fibres were present within the amygdalopiriform transition area (Fig. 6(B) k), and the posteromedial cortical amygdaloid nucleus (Fig. 5(A) j; Fig. 6(A) l; Fig. 6(B) l). Similarly, low densities of nNOS-positive neuron and fibre were observed within the medial division of posteromedial part of the bed nucleus of the stria terminalis (Fig. 4(A) g).

Thalamus

The nNOS-ir neurons and fibres exhibited weak staining at the anterior part of the paraventricular thalamic nucleus and paratenial thalamic nucleus (Fig. 4(A) f). There were high density of nNOS-positive neurons and moderate density of nNOS-positive fibres within the paraventricular thalamic nucleus (Fig. 4(B) c) as well as clusters of nNOS-positive neurons in the posterior part of the paraventricular thalamic nucleus (Fig. 5(A) e). The rhomboid thalamic nucleus exhibited similar nNOS-ir, with a small number of nNOS-positive neurons (Fig. 4(B) e). Low density of nNOS-

positive fibre plexus was also observed in the reuniens thalamic nucleus, along with moderate density of small size nNOS-ir neurons (Fig. 4(B) f). However, in the medial part of the mediodorsal thalamic nucleus (Fig. 4(B) c) and the submedius thalamic nucleus (Fig. 4(B) e), low density of lightly stained nNOS-positive fibres was observed, but no nNOS-positive neurons. Moderate densities of neurons and fibres were observed in the posterior thalamic nucleus (Fig. 5(B) b; Fig. 6(A) d), subparafascicular thalamic nucleus (Fig. 5(B) f) and posterior intralaminar thalamic nucleus (Fig. 6(A) d). However, high density of neurons and dense nNOS-positive fibres were observed in the parafascicular thalamic nucleus (Fig. 5(B) e).

Hypothalamus

Moderate densities of nNOS-positive neurons and fibres were observed in the anterior part of the paraventricular hypothalamic nucleus and the striohypothalamic nucleus (Fig. 4(A) i), which is similar to those observed in the posterior hypothalamic area and dorsal hypothalamic area (Fig. 4(B) f). Interestingly, nNOS-ir was not observed within the dorsomedial and central part of the ventromedial hypothalamic nucleus, but low densities of nNOS-positive neurons and neuropil were observed at the ventrolateral part of the ventromedial hypothalamic nucleus (Fig. 4(B) j). Low densities of neurons and fibres was also observed in the close proximity of the dorsal hypothalamic area (Fig. 5(A) f). High densities of neurons and fibres were observed at the internal zone of the median eminence (Figure 5(A)). Moderate densities of nNOS-ir neurons and fibres were observed in the penducular part of the lateral hypothalamus (Fig. 5(B) j).

Hippocampal formation

Hippocampus proper: Moderate density of small nNOS-positive neurons and low density of nNOS-positive fibre network were scattered throughout the hippocampus, including CA1 (Fig. 5(A) a & Fig. 6(A) j), CA2 (Fig. 6(A) c) and CA3 (Fig. 6(A) f) of the hippocampus. Most of the nNOS-ir neurons exhibited either the bipolar or multipolar morphology with processes. Furthermore, moderate density of nNOS-positive neurons was observed in the three layers of dentate gyrus in the

granular layer, the polymorph layer and the molecular layer of the dentate gyrus (Fig. 4(B) a; Fig. 5(A) b; Fig. 5(B) a). However, low neuronal density was observed in the lacunosum moleculare layer within the hippocampus proper (Fig. 4(B) a).

Subiculum: Moderate density of small and nNOS-positive neurons was exhibited at the dorsal subiculum, closer to the dorsal hippocampal commissure (Fig. 6(B) a). However, low density of thin fibre plexus was observed at the dorsal hippocampal commissure.

* Figure 5 & 6 about here *

Midbrain / brain stem stuctures

Zona incerta: A dense network of nNOS-positive fibres and moderate density of nNOS neurons were located at the ventral and dorsal part of zona incerta (Fig. 5(A) g). These nNOS-positive neurons were bipolar in shape and the density was relatively higher in the ventral part as compared to the dorsal part.

Ventral lateral geniculate nucleus: High densities of neurons and dense fibres were observed in the ventral lateral geniculate nucleus (Fig. 5(B) g). Most of the nNOS-ir neurons were bipolar and fusiform in shape displaying immunoreactive processes.

Substantia nigra: Low densities of nNOS-positive neurons and fibre plexus were observed in the both the substantia nigra pars compacta and pars reticulata (Fig. 6(A) i & k).

Superior and inferior colliculi: High neuronal and fibre densities were observed in the superficial layer of the superior colliculus (Fig. 6(A) a). However, low density of nNOS-positve neurons were observed at the optic nerve layer of the superior colliculus, while nNOS-ir was not found at the intermediate gray layer of the superior colliculus. High density of nNOS-positive multipolar neurons was observed in the dorsal cortex and external cortex of inferior colliculus. A moderate to

dense network of nNOS-positive varicose fibres was also observed within the dorsal cortex and external cortex of the inferior colliculi (Fig. 7(A) a & c).

Periaqueductal gray: Moderate to high densities of neurons and fibres were observed within the periaqueductal gray area (Fig. 5(B) d; Fig. 7(A) b, d & e). High density of nNOS-positive neurons and dense varicose fibres were observed at the p1 and ventralateral part of periaqueductal gray, close to the dorsal raphe region (Fig. 5(B) d; Fig. 7(A) e). Comparatively, the densities of nNOS-ir neurons and fibres at the dorsal lateral part of the periaqueductal gray appeared slighly higher than those in the dorsal medial and lateral part of the periaqueductal gray (Fig. 7(A) b & d).

Ventral tegmental area: High density of neurons and moderate to dense fibres were observed in the parainterfascicular nucleus and paranigral nucleus of the ventral tegmental area and (Fig. 6(A) k). Similarly, high density of nNOS-positive neurons with moderate fibres were present at the ventral tegmental decussation (Fig. 6(A) h).

Tegmental nuclei of the pons: Interestingly, high density of nNOS-positive neurons with large cell bodies was present in the pedunculopontine tegmental nucleus (Fig. 7(A) g). A dense and puncate nNOS-ir fibre plexus was observed witin the pedunculopontine tegmental nucleus. Similarly, high density of nNOS-positive neurons and thick fibres were observed at the reticulotegmental nucleus of the pons (Fig. 7(A) i).

Raphe nuclei: High density of nNOS-positive neurons and dense varicose fibre plexus were observed in the dorsal raphe region including the lateral, dorsal and ventral part (Fig. 7(A) f). The nNOS-ir neurons within the dorsal raphe region appeared to have large cell bodies with heavily-stained processes. However, high density of small nNOS-positive neurons and dense nNOS-positive punctate fibres were observed at the median raphe nucleus (Fig. 7(A) h).

Superior olive nuclei: Low nNOS-ir was observed within the superior olive (Fig. 7(B) j). Although low density of nNOS-ir fibres was observed, no positive NOS-ir neuron was detected in the lateral superior olive.

Inferior olive nuclei: Moderate neuronal and fibre densities were also observed within subnucleus B and C of the medial nucleus (Fig. 8(A) l). Interestingly, the nNOS-positive fibres and neuropil were mostly found in the subnucleus B and C of the medial nucleus. Besides that, nNOS-positive neurons of moderate density and dense nNOS-ir fibres were found in the dorsal nucleus of inferior olive (Fig. 8(A) k).

Reticular nucleus: The densities of nNOS-positive neurons and fibres were low at the oral part of pontine reticular nucleus (Fig. 7(A) h). High density of nNOS-positive large multipolar neurons, as well as moderate density of nNOS-positive dense fibre network were observed at the intermediate reticular nucleus (Fig. 7(B) i). Similarly, moderate densities of nNOS-positive fibres and neurons were present at the alpha part of the parvocellular reticular nucleus (Fig. 7(B) g). In contrast, the densities of the nNOS-positive neurons and fibres in the dorsal and ventral parts of the medullary reticular nucleus were comparatively lower (Fig. 8(B) e).

Supragenual nucleus: High densities of nNOS-positive neurons and fibres were observed in the supragenual nucleus (Fig. 7 (B) e). The nNOS cell bodies present were small in size with dense fibre plexus.

Nucleus of the trapezoid body: High nNOS-positive neuronal and fibre densities were observed within the nucleus of the trapezoid body (Fig. 7(B) l). The size of the nNOS-positive neurons was large with intense staining.

Lateral terminal nucleus: High densities of nNOS-positive neurons and fibres were observed within the lateral terminal nucleus (Fig. 6(A) e).

Gracile nucleus: The neuronal density of nNOS in the gracile nucleus was comparatively higher compared to that of the median accessory nucleus of the medulla and gracile fasciculus (Fig. 8(B) b). Moderate density of nNOS-positive neurons with few fibres was observed in the gracile nucleus and low densities of nNOS-positive neurons and fibres were observed in the gracile fasciculus. The nNOS-positive neuron and fibre was not observed in the median accessory nucleus of the medulla.

Cuneate nucleus: Low neuronal density of nNOS was also observed in the cuneate nucleus (Fig. 8(B) a) with small number of nNOS-ir fibre plexus present within the region. However, low nNOS-positive neuronal density was observed in the cuneate fasciculus with thin fibre plexus.

Posterior Cortical Areas

Ectorhinal cortex: Moderate densities of nNOS-positive neurons and fibres were observed at the ectorhinal cortex (Fig. 6(B) I; Fig. 7(A) k). Majority of these nNOS-positive neurons were multipolar in shape.

Entorhinal cortex: Moderate densities of nNOS-positive neurons and fibres were observed at the dorsolateral entorhinal cortex (Fig. 6(B) j). Similarly, the caudomedial entorhinal cortex exhibited moderate densities of neurons and fibre plexus (Fig. 7(A) l).

Other areas: Moderate densities of nNOS-positive neurons and fibres were scattered all over other cortical areas in the retrosplenial granular cortex (Fig. 6(B) b), retrosplenial dysgranular cortex (Fig. 8(B) c), primary and secondary visual cortex (Fig. 6(B) d, e & f), primary and secondary auditory cortex (Fig. 8(A) h & i) temporal association cortex (Fig. 6(B) g & h). The nNOS-ir neurons in

these cortical areas exhibited mostly large cell bodies, multipolar morphology with nNOS-positive long processes.

Cerebellum and vestibular complex

High densities of nNOS-ir neurons and fibres were found in the molecular layer and granule cell layer of the cerebellum (Fig. 7(B) a, b & c; Fig. 8(A) a & d). Low neuronal and fibre densities of nNOS-ir were detected in the Purkinje cell layer, paramedian lobule and copula of the pyramid (Fig. 7(B) c; Fig. 8(A) b & c).

Vestibular nucleus: Although there was an absence of nNOS-ir neurons in the vestibular nucleus, low density of nNOS-ir fibre plexus was observed within the medial and superior vestibular nuclei (Fig.7(B) d).

Nuclei in the lower medulla oblongata

Spinal trigeminal nuclei: High density of nNOS-positive neurons and dense fibre plexus were observed within the gelatinous layer of the caudal spinal trigeminal nucleus (Fig. 8(A) g & Fig. 8(B) c). Moderate density of nNOS-positive neurons with few fibres was found in the interpolar and caudal part of the spinal trigeminal nucleus (Fig. 8(A) j; Fig. 8(B) c & f). The nNOS-positive neurons in the caudal part of the spinal trigeminal nucleus were larger in size with dense varicose processes.

Nucleus of the solitary tract: High density of nNOS-positive neurons with varicose processes and fibres was observed in the nucleus of solitary tract and the central cervical nucleus of the spinal cord (Fig. 8(B) d).

* Figure 7 & 8 about here *

An overall summary and photomicrographs of the density of nNOS-ir neurons and fibres distribution across different regions in the rostro-caudal axis of rat brain are presented in Table 2 and Supplementary Fig 1-13.

* Table 2 and Figure 9 about here *

DISCUSSION

In rodents, the distribution of nNOS-expressing neurons has been selectively reported in various regions of the brain using NADPH-d histochemistry, nNOS immunohistochemistry, as well as *in situ* hybridisation of NOS mRNA (Iwase et al., 1998; Liang et al., 2013; Okere and Kaba, 2000; Valtschanoff et al., 1993; Vincent and Kimura, 1992). Although splice variants of nNOS mRNA have been reported (nNOS-α, -β, -γ, -μ, -2), these splice forms result in a single nNOSα protein in the brain (Huber et al., 1998; Ihara et al., 2006). Therefore, the sheep anti-rat nNOS antibody used in our study is directed against the whole recombinant rat nNOS and the specificity of this antibody has been previously characterised for immunohistochemistry (Barkhuizen et al., 2017; Herbison et al., 1996; Wang and Morris, 1996). Although studies have demonstrated the localisation of nNOS-ir neurons and fibres in different brain regions of various rat strains and nNOS immunohistochemistry on free-floating sections (Bredt et al., 1991; Del Moral et al., 2004; Liang et al., 2013; Rodrigo et al., 1994), we examined the rostro-caudal distribution of nNOS-ir neurons and fibres in the adult male Sprague-Dawley rat using paraffin-embedded brain sections in our present study. Therefore, this comprehensive distribution profile of nNOS-ir neurons and fibres in the paraffin-embedded sections would allow a comparative distribution of nNOS-ir neurons and fibres to be made across the brain regions in previous studies (Rodrigo et al., 1994; Vincent and Kimura, 1992) to further identify and understand the role of nNOS in modulating various physiological functions in the brain.

The nNOS-ir was observed across the adult male Sprague Dawley rat brain, with different densities of nNOS-ir neurons and fibre plexus distributed in various nuclei and brain regions. High neuronal densities were observed in the intermediate endopiriform nucleus, lateral terminal nucleus, pedunculopontine tegmental nucleus, ventrolateral periaqueductal gray, and dorsal raphe, as well as nucleus of the trapezoid body. Cluster of nNOS-positive neurons observed in the Islands of Calleja exhibited intense nNOS expression. Moderate neuronal density were observed in many areas, including the striohypothalamic nucleus, dorsolateral part of lateral amygdaloid nucleus, pregeniculate nucleus of the prethalamus, parainterfascicular nucleus of the ventral tegmentum area, inferior colliculus and intermediate reticular nucleus. Furthermore, small clusters of nNOS-ir neurons were detected in the nucleus of the horizontal limb of the diagonal band, nucleus of the vertical limb of the diagonal band, zona incerta, reticulotegmental nucleus of the pons, nucleus of the solitary tract, and cervical nucleus of the spinal cord. The results in the study are in agreement with the distribution of the nNOS-positive neurons and fibre plexus previously described (Blottner et al., 1995; Vincent and Kimura, 1992) as well as in the Wistar rat brain using nNOS immunoreactivity on free-floating sections (Rodrigo et al., 1994).

Olfactory areas

The olfactory bulb is one of the brain regions that demonstrated high immunoreactivity to the nNOS staining, with high densities of nNOS-positive neurons and fibres observed at the glomerular layer and granule cell layer of the olfactory bulb. Low densities of nNOS-positive neurons and fibre plexus were observed in the anterior olfactory nucleus in our study. Clusters of nNOS-ir neurons with dense fibre plexus were similarly observed in the main olfactory bulb with less density in the anterior olfactory nucleus in the Wistar rat (Rodrigo et al., 1994). Low neuronal density was observed within the granule cell layer of the accessory olfactory bulb in our study, which is due to the anterior part of this structure observed with respect to the corresponding coronal rat brain sections (Fig S1). Nevertheless, intense nNOS-ir and NADPH-d histochemsity with dense fibre

plexus has been reported in the accessory olfactory bulb of the rat brain (Rodrigo et al., 1994). In our current study, high densities of neurons and fibres were also observed in the nucleus of the lateral olfactory tract, layer 2 and layer 3. Usunoff et al. reported similar observation using the NADPH-d histochemistry in the Wistar rat (Usunoff et al., 2006). A significant difference of the NADPH-d staining in the three layers of nucleus of the lateral olfactory tract was also observed as the NADPH-d-positive neurons were not detected in the layer 1 (Usunoff et al., 2006). However, low neuronal densitiy was observed in the olfactory nerve layer, similar to that observed in the Wistar rats (Rodrigo et al., 1994). The olfactory bulb consists of cells and neuropil layers that receive, process, and relay olfactory information coming from olfactory receptors in the nasal cavity (Purves et al., 2008). The nNOS-deficient mice demonstrated an impairment in the formation of long-term memory in recognizing olfactory cues, despite showing an intact memory acquitision with short- and intermediate-term memory retention in the social discrimination paradigm (Juch et al., 2009). Given that nNOS is highly expressed in the olfactory bulb (Kosaka and Kosaka, 2007), this implies that nNOS-derived NO is involved in regulating the protein synthesis for the consolidation of olfactory long-term memory within the brain regions (Juch et al., 2009). Furthermore, expression of nNOS mRNA in the accessory olfactory bulb has been found to increase during the formation of olfactory memory recognition in mice (Okere and Kaba, 2000). Therefore, the presence of high neuronal density in the olfactory bulb potentially supports the role of nNOS-derived NO in modulating the formation of memory for olfactory recognition.

High neuronal density was also observed in the Islands of Calleja of olfactory tubercle in the paraffin-embedded sections of the Sprague Dawley rat. This result is in line with most of the previous studies using NADPH-d histochemistry as well as nNOS immunoreactivity in the rat brain (Bredt et al., 1991; Rao and Butterworth, 1996; Rodrigo et al., 1994). Notably, high density of D3 dopamine receptors was also found to be expressed in the Islands of Calleja, which reveals dopaminergic projection from the ventral tegmental area (Bouthenet et al., 1991). Inta *et al.* proposed that alterations of neurogenesis and plasticity in the postnatal stage may lead to

dysfunction of the dopamine and NO systems, thereby contributing to the development of schizophrenia (Inta et al., 2011). More investigations are needed to understand how interaction between nNOS-expressing neurons in the Islands of Calleja and dopamine signalling underpins the pathophysiology of psychiatric disorders.

Cerebral cortex

In our study, moderate to high nNOS positive neuronal density was observed to scatter across the cerebral cortex regions in the Sprague Dawley rat brain with the nNOS-positive neurons exhibited long processes as well as dense varicose fibre plexus. Previous studies have also reported the presence of nNOS-positive neurons of various morphologies with dense varicose and punctate nNOS-positive fibre plexus distributed in the rat cortical areas using both NADPH-d histochemistry and nNOS immunoreactivity (Rodrigo et al., 1994; Vincent and Kimura, 1992). Studies have reported the transient expression of nNOS in the cerebral cortex across the developmental stages of the rodent embryos and neonates, suggesting the role of nNOS in the maturation processes of brain (Ling et al., 2012; Santacana et al., 1998). However, increased nNOS expression was observed in the cerebral cortex of the early postnatal rats exposed to hypoxia during delivery (Fernandez et al., 2003). Interestingly, severe brain damage in human neonates with hypoxic-ischemic encephalopathy showed an increase in the nNOS-positive fibres in the cortex and thalamus, while brain regions ipsilateral to the injury including cerebral cortex, caudate-putamen, and thalamus demonstrated delayed increase in the nNOS-ir (Ishida et al., 2001). Taken together, these studies potentially highlight the role of nNOS in the neuronal maturation during postnatal dveleopment as well as mediating susceptibility to hypoxic-ischemic insult in the neonatal brain and plasticity of the cortical circuitry during recovery (Fernandez et al., 2003; Ishida et al., 2001).

Tenia tecta

In this study, low neuronal density was observed in both the dorsal and ventral tenia tecta with few small nNOS-positive neurons and less dense fibre network. However, the nNOS-ir in the Wistar rats

showed a small number of nNOS-ir neurons in the dorsal tenia tecta while numerous nNOS-ir neurons and fibres were present in the ventral tenia tecta (Rodrigo et al., 1994). Fewer nNOS-positive neurons were also reported in the tenia tecta using the NADPH-d histochemistry (Vincent and Kimura, 1992). This possibly suggests the difference observed in localisation of the nNOS-positive neurons and fibre network in the tenia tecta in comparison with the different rat strains and different NOS antibodies used in previous studies (Rodrigo et al., 1994; Vincent and Kimura, 1992). Although it is reported that the ventral and dorsal tenia tecta receive direct projections from the olfactory bulb (McNamara et al., 2004), their specific functions as well as the role of NO in the this structure remain relatively unknown.

Hippocampus and limbic structures

Furthermore, small to medium-sized nNOS-positive neurons with moderate neuronal density and less intense nNOS-positive fibre network were scattered within the hippocampus in the rat brain. These include the CA1 – CA3 fields as well as the three layers of dentate gyrus. These results are in line with the previous studies on the localisation of nNOS-ir neurons in the rat hippocampal regions (Liang et al., 2013; Rodrigo et al., 1994). NO has been shown to be involved in hippocampus synaptic plasticity (Arancio et al., 1996b; Böhme et al., 1993), as well as in learning and memory (Arancio et al., 1996a; Bernabeu et al., 1995). Furthermore, it has been shown that the increase in nitrite and NOS activity was associated with spatial learning and memory in rats (Bernabeu et al., 1995; Harooni et al., 2009; Zhang et al., 1998). Harooni *et al.* demonstrated that the hippocampal function could be modulated by inhibiting the NOS enzyme, thus, altering memory processes (Harooni et al., 2009). In addition, the induction of long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus along with an increased formation of NO were detected during the learning of foot-shock avoidance task in rats (Bernabeu et al., 1995). These studies therefore have provided evidence for an association between learning process and the activation of nNOS in hippocampus. Interestingly, an increased levels of nNOS and NO activity in the hippocampus have been shown to exhibit both neuroprotective and neurotoxicity effects

respectively along the progression of Alzheimer's disease in the mice model (Chakroborty et al., 2015). This further supports an important role of NO signalling in the modulation of synaptic plasticity of neurons, which is linked to the loss of synaptic function and memory impairment in the pathogenesis of Alzheimer's disease (Asiimwe et al., 2016; Balez and Ooi, 2016).

High densities of neurons and fibres were observed within the septal area as well as the vertical and horizontal limb of the diagonal band in the Sprague Dawley rat. Similarly, Rodrigo et al. described the distribution of nNOS-ir neurons and fibre plexus in the septal area and both the vertical and horizontal limb of the diagonal band of Broca in the Wistar rat (Rodrigo et al., 1994). In the amygdala, moderate to high density of neurons was scattered throughout the amygdaloid nucleus. The presence of nNOS-ir neurons and fibres of different densities have also been reported to scatter within the amygdala region using both NADPH-d histochemisty and nNOS immunohistochemistry in the Wistar rat brain (Rodrigo et al., 1994; Usunoff et al., 2006). A recent finding have shown that NO in the basolateral amygdala induced anxiety and depression in healthy rats, but reduced anxiety and depression in stressed rats, suggesting that NOS might play a modulatory role in basolateral amygdala for the regulation of stress (Nikkar et al., 2019). Futhermore, NOS activity in the central amygdala is recently found to involve in the acquisition and consolidation of conditioned odor aversion (Gonzalez-Sanchez et al., 2019). Interestingly, a study has suggested that the disruption of PSD95/nNOS interation may selectively reduces fear memory in the amygdala, suggesting a potential role of nNOS in the fear-related disorders (Li et al., 2018). In the bed nucleus of the stria terminalis, a small number of nNOS-ir neurons and fibres was observed, which is similar to that shown in Rodigro et al. (Rodrigo et al., 1994).

Hypothalamus

Neurons exhibiting immunoreactivity to nNOS were found in various regions across the hypothalamus in our current study. Clusters of nNOS-positive neurons were observed in the paraventricular nucleus as well as the posterior and dorsal hypothalamic area. Interestingly, the NOS-ir was absent in the dorsomedial and and central part of the ventromedial hypothalamic nucleus in our study. These results are similar to that by Rodrigo et al. (Rodrigo et al., 1994), in

which the dorsalmedial portion of the ventromedial hypothalamic nucleus is consistently unstained in the Wistar rat. However, low neuronal and fibre densities were observed in the ventrolateral part of the ventromedial hypothalamic nucleus in the current study. On the other hand, the absence of nNOS-positive neurons in the suprachiasmatic nucleus in the rat have been previously reported (Rodrigo et al., 1994; Vincent and Kimura, 1992). Although we did not observe any nNOS-ir in the suprachiasmatic nucleus in our current study, nNOS-ir neurons have been detected in the superchiasmatic nucleus in both rats and mice brain using the same nNOS antibody as our study (Aranow et al., 1996). Therefore, the discrepancies observed possibly relates to the different nNOS antibodies used in various studies (Rodrigo et al., 1994) as well as the rodent species used with the same nNOS antibody.

Despite the wide distribution of nNOS-ir neurons in the hypothalamus, subpopulations of nNOS-positive neurons have been linked with the expression of hypothalamic hormones and neuropeptides, such as enkaphalin, substance P, gonadotropin-releasing hormone, corticotropin-releasing hormone, vasopressin, oxytocin, and ß-endorphin, suggesting the role of NO system in modulating various neuroendocrine functions (Herbison et al., 1996; Wolf et al., 1998; Yamada et al., 1996). As a result, alteration in the hypothalamic production of NO has been anticipated to affect the hypothalamo-hypophyseal peptidergic system (Wolf et al., 1998). Besides, the number of nNOS-ir neuron in the paraventricular hypothalamic nucleus was found to be significantly higher in middle and advanced phases of diabetes, implying the possible association between the changes of nNOS activity in paraventricular hypothalamic nucleus and alterations in neuroendocrine and adrenal activity in cases of diabetes (Shen et al., 2003). Therefore, the distribution of nNOS-positive neurons and fibres observed within the hypothalamus suggests the role of nNOS-derived NO in mediating various neuroendocrine functions, such as energy balance and reproduction (Donato et al., 2010; Sica et al., 2009). High neuronal density was also observed in the internal zone of the median eminence. The high nNOS activity in median eminence was reported to involve in

regulating the release of gonadotropin releasing hormone and corticotropin releasing hormone (Kawakami et al., 1998; Knauf et al., 2001; Prevot et al., 1998).

Thalamus

The nNOS-ir was observed in the thalamus and zona incerta, with high neuronal and fibre densities in the posterior part of the paraventricular thalamic nucleus. The presence of nNOS-ir neurons and fibre plexus have also been demonstrated in the thalamus and zona incerta in the Wistar rats (Rodrigo et al., 1994). Furthermore, strong nNOS-ir observed in the parafascicular thalamic nucleus in our study was similar to that observed in the Wistar rats (Rodrigo et al., 1994). Despite the presence of moderate to high density of NOS-positive neurons in the thalamus, majority of the NOS-positive afferents to the thalamus has been reported to originate from the pedunculopontine and laterodorsal tegmental nuclei using retrograde tracing and NOS immunocytochemsitry (Usunoff et al., 1999). This is in line with our study as we observed high densities of nNOS-positive neurons and fibre plexus within the pedunculopontine tegmental nucleus and reticulotegmental nucleus of the pons. Similar observation has also been reported that high densities of neurons and fibres distributed within the pedunculopontine tegmental nucleus of the Wistar rat (Rodrigo *et al.*, 1994). These results therefore support the presence of intense nNOS-ir within the tegmental nuclei of the pons, suggesting that NO may play an important role in mediating the somatosensory transmission (Usunoff et al., 1999).

Nucleus accumbens

In our study, moderate densities of nNOS-positive neurons and fibres were localized in the shell and core of nucleus accumbens, similar to that observed in the Wistar rat (Rodrigo et al., 1994). The presence of nNOS neurons, revealed by NADPH-d histochemistry, has also been shown in subregions of the nucleus accumbens (Hoque and West, 2012). This study further highlighted that the modulation of nNOS activity by the activation of dopamine D1 and D2 receptor in the subregions of nucleus accumbens demonstrates the role of dopamine transmission in the regulation

of nNOS activity (Hoque and West, 2012). A recent study has also shown that an increase in NO through the activation of nNOS-expressing interneurons in the nucleus accumbens is critical for the regulation of cocaine relapse behaviour (Smith et al., 2017). Therefore, more studies are needed to understand the interactive role of nNOS-expressing neurons and dopamine transmission through the nitrergic system in the nucleus accumbens on addiction behaviour.

Substantia nigra

In our present study, low neuronal and fibre densities were observed in the substantia nigra pars compacta and pars reticulata. This is in line with other studies that reported the weak expression of nNOS-containing neurons with a sparse network of nNOS-ir fibres in the substantia nigra as compared to other brain regions (Bredt et al., 1991; Mitkovski et al., 2012; Rodrigo et al., 1994). Albeit the small number of nNOS-ir neurons, nNOS-ir processes were frequently observed in close apposition to tyroxine hydroxylase-positive neurons and processes within the substantia nigra (Matthews et al., 1997). Interestingly, higher nNOS expression was detected in the substantia nigra of the mice model of Parkinson's disease exposed to 1-methyl-4-phenyl-1,2,3,6-tetrehydropyridine treatment (Muramatsu et al., 2002). In 6-hydroxydopamine lesioned rats, inhibition of NOS decreased the toxicity of the dopaminergic neuronal loss and nNOS cell density in subregions of substantia nigra (Gomes et al., 2008). Taken together, these studies suggest that interaction between the dopaminergic system and nitriergic transmission is potentially important for the control of nigrostriatal pathway as well as in the pathology of Parkinson's disease.

Cerebellum

A significant high densities of nNOS-ir neurons and fibres were found within the cerebellum, particularly in the molecular and granule cell layers of the cerebellum. Localisation of the nNOS-ir neurons has also been shown in the molecular and granular cell layers as well as the deep cerebellar nuclei in the Wistar rat using nNOS immunohistochemistry (Rodrigo et al., 1994). However, differences in the nNOS staining pattern in the Purkinje cells and deep cerebellar nuclei has been

previously reported using the NADPH-d histochemistry and nNOS immunohistochemisty (Rodrigo et al., 1994; Vincent and Kimura, 1992). Although previous studies demonstrated no NOS in the Purkinje cell layer and white matter (Rancillac et al., 2006), , we observed low density of fibre plexus in the Purkinje cells and white matter of the cerebellum, suggesting that the nNOS immunohistochemistry is potentially sensitive to the localisation of nNOS-positive fibres within the cerebellum.

As most studies demonstrated that the cerebellum exhibits the highest level of nNOS activity in the brain (Blottner et al., 1995; Rodrigo et al., 1994; Vincent and Kimura, 1992), NO signalling-mediated processes in the cerebellum include synaptic transmission, development and differentiation, learning and memory as well as aging (Abbott and Nahm, 2004; Blanco et al., 2010; Contestabile, 2012; Vincent, 2010). Studies have shown that NO modulating bidirectional synaptic plasticity (LTP or LTD) goes through the postsynaptic Ca^{2+} levels and glutamate-nitric oxide-cGMP pathway in Purkinje cells within the cerebellum, thereby, contributing to cerebellar learning associated with motor control and coordination (Cabrera-Pastor et al., 2016; Contestabile, 2012). On the other hand, human studies have shown that activated nNOS with increased NO transmission in the cerebellum is associated with asphyxia at birth, suggesting that post-asphyxic excitotoxicity is induced by excessive NO release (Gunes et al., 2007; Perlman, 2006). High nNOS activity was also seen throughout the cerebellum in the rat model of perinatal asphyxia, and this study suggested that the inhibition of NO production in specific regions of the preterm brain play a crucial role in development of motor control and coordination. (Barkhuizen et al., 2017).

Brainstem

Moderate to high densities of nNOS-ir neurons and fibres were present within the superior and inferior colliculus. This is in line with the localisation of nNOS-positive neurons in the various laminae of the superior colliculus as well as inferior colliculus in previous studies (Rodrigo et al., 1994; Vincent and Kimura, 1992). In the periaqueductal gray area, high density of nNOS-positive

neurons and dense fibres with were found at the ventrolateral part of the periaqueductal gray area, close to the dorsal raphe region. Similar result was also observed in periaqueductal gray area of the Wistar rat using nNOS immunohistochemistry (Rodrigo et al., 1994).

In our study, high neuronal and fibre densities were observed in the nucleus of traprezoid body, supragenual nucleus and lateral terminal nucleus. High density of nNOS-postive neurons and fibres have also been reported in the nucleus of trapezoid body and supragenual nucleus in the Wistar rat (Rodrigo et al., 1994). Studies have reported that the nNOS-derived NO modulates the K^+ and Ca^{2+} channels in the neurons within the mouse nucleus of trapezoid body (Steinert et al., 2008; Tozer et al., 2012). These potentially suggest an important role of the NO-dependent signalling in mediating the physiological funtions such as neuronal excitability and synaptic plasticity through the modulation of ion channels (Steinert et al., 2008; Tozer et al., 2012). However, the nNOS histochemistry has not been reported previously in the lateral teminal nucleus of the Wistar rat (Rodrigo et al., 1994). Albeit the intense expression of nNOS-positive neurons and fibres in the supragenual nucleus and lateral terminal nucleus, little is still known regarding the role of NO signalling in these brain regions.

High density of nNOS-positive neurons and fibre was observed in the dorsal raphe region in our study. Similarly, abundant nNOS-positive neurons were also found in the dorsal raphe nucleus in other studies (Rodrigo et al., 1994; Vincent and Kimura, 1992). Moderate densities of nNOS-ir neurons and fibres were also found at the median raphe nucleus in our study. High percentage of the nNOS-positive neurons have been shown to colocalise with serotonin immunoreactivity in both dorsal and median raphe nuclei in the adult rat brain (Johnson and Ma, 1993; Wang et al., 1995). In addition, the nNOS-positive axons were in close contact with the cell bodies expressing both nNOS and serotonin immunoreactivity (Wotherspoon et al., 1994). These studies suggest potential interactions between the nitregenic and serotoninergic system in the dorsal raphe region (Blanco et al., 2010). The use of nNOS inhibitors has also been shown to exhibit the antidepressant-like

behaviours in the rat using the forced swim test (Harkin et al., 1999; Sherwin et al., 2017). Therefore, the role of NO signalling pathway is currently implicated in pathophysiology of mood-related disorders, which further suggests the use of nNOS inhibitors as potential therapeutic agents for antidepressant effects (Wegener and Volke, 2010).

Both Vincent and Kimura (1992) and Rodrigo et al. (1994) have performed smilar studies on the mapping NOS in rat brain through different approaches, which include the NADPH-diaphorase histochemical technique and antiserum against cNOS approach, respectively. In our study, we employed a technique similar to Rodrigo et al. (1994) with a different polyclonal antibody targeting the nNOS in the Sprague-Dawley rat brain. Interestingly, the nNOS expression in some of the brain regions reported in our study was inconsistent with that reported in other studies. These regions included accessory olfactory bulb, lateral olfactory tract, tenia tecta and ventromedial hypothalamic nucleus as mentioned previously. The distinct finding of nNOS expression in the tenia tecta and ventromedial hypothalamic nucleus might be potentially due to the use of different antibodies, resulted by different sensitivity or specificity of the antibodies in this study. For example, it has been demonstrated that variations in the fixation protocols of the brain samples affect the sensitivity of the NADPH-d histochemistry that leads to the lack of correlation with NOS immunoreactivity studies in different regions of the brain (Blottner et al., 1995; Rodrigo et al., 1994). The fact that the fixation protocol used in this study is not identical to that used in previous studies might explain the discrepancies in the nNOS distribution observed in this study. Furthermore, variations in the use of rodent strain may contribute to the inconsistency of the finding such as the nNOS expression in the tenia tecta. Additionally, the differential expression of nNOS can be potentially affected by many other factors, such as age and developmental factors. This is supported by a study which suggested that the nNOS expression can be affected by afferent innervation and growth factors at different developmental stages of cerebellar granule neurons (Baader et al., 1997). The translation efficiency of nNOS is also found to be affected by the diversity of nNOS mRNA transcripts (Wang et al., 1999).

CONCLUSION

In summary, this present study documented the distribution of nNOS-ir neurons across various brain regions in the adult male Sprague-Dawley rat using the nNOS immunohistochemistry. High density of nNOS-positive neurons was observed in the olfactory-related areas, intermediate endopiriform nucleus, Islands of Calleja, subfornical organ, ventral lateral geniculate nucleus, parafascicular thalamic nucleus, superior colliculus, lateral terminal nucleus, pedunculopontine tegmental nucleus, periaqueductal gray, dorsal raphe nucleus, supragenual nucleus, nucleus of the trapezoid body, and the cerebellum. Moderate neuronal density was found in the frontal regions, cerebral cortex, caudate putamen, hippocampus, thalamus, hypothalamus, amygdala, and the spinal cord. Finally, mild immunoreactivity of lightly-stained nNOS fibres was also found in the corpus callosum, fornix, globus pallidus, anterior commissure, and the dorsal hippocampal commissure. Overall, the different densities and distinct morphological cytoarchitecture of nNOS localisation play important roles on specific neuronal function in the normal physiological functions of the central nervous system.

Conflict of Interest

The authors declare they have no competing interests.

Acknowledgements

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Authors Contribution

L.W.L. conceptualized and designed the study. P.S.C., C.H.P., W.L.L., & L.W.L. performed histological study and microscopic analysis. P.S.C., C.H.P., W.L.L., & L.W.L. ensured all data were representative of the original raw data. H.W.M.S. & Piers Emson (Cambridge University, U.K.) provided the nNOS antibody. Y.S.C., M.L.F., L.G., & H.W.M.S. contributed to intellectual inputs of the manuscript. L.W.L., P.S.C., & C.H.P., W.L.L. drafted and revised the manuscript text, and all authors reviewed and provided comments on the article.

Ethical Statement

The euthanasia procedure was performed after obtaining ethical approval from the *Committee on the Use of Live Animals in Teaching and Research*, The University of Hong Kong.

LEGENDS

Figure 1: **(A)** Schematic representation of a lateral view of the major neuroanatomical structures in rat brain. **(B)** Representation of the brain in rostro-caudal levels that correspond to the photomicrographs of nNOS-ir localisation within the respective coronal brain section depicted in Figures 2-8. **(C)** Categorization of the nNOS-ir neuronal and fibre densities according to its expression level as follows: Neuron (not visible – 0; few/low – 1; moderate – 2; and many/high – 3); fibre (not visible – 0; low/mild – 1; scattered/moderate – 2; and high/dense – 3). Photomicrographs of Fig. 1C were taken from the brain region as described: Neuron: scale 1 – primary somatosensory cortex; scale 2 - agranular insular cortex; scale 3 – dorsal raphe nucleus; Fibre: scale 1 – anterior part of paraventricular thalamic nucleus; scale 2 – dorsal endopiriform nucleus; scale 3 - subfornical organ. **(D)** Photomicrographs of the nNOS-ir neurons in high magnification, which are categorised into small (< 15 μm) and large (15 – 30 μm) size, measured based on the diameters of nNOS-ir cell bodies observed in the brain regions.

***Figure 2*: (A)** Coronal section of the olfactory-related areas demonstrating the nNOS distribution at the glomerular layer of the olfactory bulb, external plexiform layer of the olfactory bulb and olfactory nerve layer (a, d, & e), as well as granule cell layer of the olfactory bulb (b, e). **(B)** Coronal section of the frontal cortex and olfactory-related areas, illustrating the nNOS distribution at the secondary motor cortex (a), primary motor cortex (b), prelimbic cortex (c), dorsolateral orbital cortex, and frontal cortex area 3 (d), medial orbital cortex (e), dorsal endopiriform nucleus (f), dorsal transition zone, and dorsal tenia tecta (g), intermediate endopiriform nucleus, and layer 3 of the piriform cortex (h), ventroposterior part of the anterior olfactory nucleus, and ventral tenia tecta (i).

***Figure 3:* (A)** Coronal section through the cerebral cortex, demonstrating the nNOS distribution at the secondary motor cortex and area 1 of the cingulate cortex (a), cingulum and forceps minor of the corpus callosum (b), jaw region of the primary somatosensory cortex (c), infralimbic cortex (d), claustrum (e), ventral and dorsal parts of the agranular insular cortex (f), dorsal peduncular cortex, and olfactory ventricle (g), shell and core parts of the nucleus accumbens, anterior part of the anterior commissure, and dorsal endopiriform nucleus (h), dorsal tenia tecta and navicular nucleus of the basal forebrain (i), posterior part of the anterior olfactory nucleus, and intermediate endopiriform nucleus (j), layer 1 to 3 of the piriform cortex (k), olfactory tubercle, and ventral pallidum (l). **(B)** Coronal section through the cerebral cortex showing the nNOS distribution at the area 1 of the cingulate cortex, and secondary motor cortex (a), forelimb region of the primary somatosensory cortex, and dysgranular zone of the primary somatosensory cortex (b), caudate putamen (c & h), lambdoid septal zone (d), medial septal nucleus (e), shell and core parts of the nucleus accumbens (f), core part of the nucleus accumbens (g), dorsal endopiriform nucleus (i), nucleus of the vertical and horizontal limb of the diagonal band (j), ventral pallidum (k), anterior part of the anterior commissure, and Islands of Calleja (l).

Figure 4: **(A)** Coronal section through the cerebral cortex, illustrating the nNOS distribution at the cingulum, and primary motor cortex (a), barrel field of the primary somatosensory cortex (b) dorsal fornix and triangular septal nucleus (c), caudate putamen (d), subfornical organ (e), anterior part of the paraventricular thalamic nucleus, and paratenial thalamic nucleus (f), fornix, and medial division and posteromedial part of the bed nucleus of the stria terminalis (g), globus pallidus (h), anterior parvicellular part of the paraventricular hypothalamic nucleus, and striohypothalamic nucleus (i), dorsal endopiriform nucleus (j), medial part of the interstitial nucleus of the posterior limb of the anterior commissure, anterior amygdaloid area, and intercalated nuclei of the amygdala (k), and nucleus of the lateral olfactory tract (l). **(B)** Coronal section through the forebrain, demonstrating the nNOS distribution at the radiatum layer of the hippocampus, lacunosum moleculare layer of the hippocampus, molecular, granular and polymorph layers of the dentate gyrus (a), CA2 field of the hippocampus (b), dorsal 3rdventricle, paraventricular thalamic nucleus, and medial part of the mediodorsal thalamic nucleus (c), caudate putamen, external capsule, and secondary somatosensory cortex (d), central medial thalamic nucleus, rhomboid thalamic nucleus, and submedius thalamic nucleus (e), reuniens thalamic nucleus, dorsal part of the posterior hypothalamic area, and dorsal hypothalamic area (f), internal capsule (g), amygdalostriatal transition area, and dorsolateral part of the lateral amygdaloid nucleus (h), dorsal endopiriform nucleus (i), dorsomedial, central, and ventolateral parts of the ventromedial hypothalamic nucleus (j), posteroventral part of the medial amygdaloid nucleus, and posterior part of the basomedial amygdaloid nucleus (k), and ventral part of the basolateral amygdaloid nucleus, and ventral endopiriform nucleus (l).

Figure 5: **(A)** Coronal section through the forebrain, showing the nNOS distribution at the CA1 field of the hippocampus (a), molecular, granular and polymorph layers of the dentate gyrus (b), CA2 field of the hippocampus (c), barrel field of the primary somatosensory cortex, and secondary somatosensory cortex (d), posterior part of the paraventricular thalamic nucleus (e), dorsal hypothalamic area, and A11 dopamine cells (f), dorsal and ventral parts of the zona incerta (g),

central nucleus of amygdala (h), ventrolateral part of the lateral amygdaloid nucleus, dorsal endopiriform nucleus, and posterior part of the basolateral amygdaloid nucleus (i), posteroventral part of the medial amygdaloid nucleus (j), posterior and ventral parts of the basolateral amygdaloid nucleus (k), ventromedial hypothalamic nucleus (l). **(B)** Coronal section through the forebrain, illustrating the nNOS distribution at the polymorph layer, inner and outer blades of the dentate gyrus (a), mediocaudal part of the lateral posterior thalamic nucleus (b), posterior commissure, subcommissural organ, and precommissural nucleus (c), P1 periaqueductal gray (d), P1 periaqueductal gray, and parafascicular thalamic nucleus (e), parvicellular part of the subparafascicular thalamic nucleus, and medial lemniscus (f), ventral geniculate nucleus (g), ectorhinal cortex (h), fasciculus retroflexus, and rostral interstitial nucleus (i), peduncular part of lateral hypothalamus, and cerebral peduncle (j), posterior part of the basolateral amygdaloid nucleus (k), fornix, medial and lateral parts of the medial mammillary nucleus (l).

Figure 6: **(A)** Coronal section through the midbrain, showing the nNOS distribution at the superficial gray layer, optic nerve layer and intermediate gray layer of the superior colliculus (a), commissure of the superior colliculus and dorsomedial periaqueductal gray (b), p1 reticular formation (c), triangular part of the posterior thalamic nucleus group and the posterior intralaminar thalamic nucleus (d), peripeduncular nucleus (e), field CA3 of the hippocampus (f), ectorhinal cortex and perirhinal cortex (g), ventral tegmental decussation (h), compact part and reticular part of substantia nigra (i) field CA1 of the hippocampus (j), lateral subnucleus of interpeduncular nucleus, mammillary peduncle, reticular part of substantia nigra, paranigral nucleus and parainterfasicular nucleus of the ventral tegmental area (k), posteromedial part of amydalohippocampal area and posteromedial cortical amygdaloid nucleus (l). **(B)** Coronal section through the midbrain, showing the nNOS distribution at the dorsal hippocampal commissure and dorsal subiculum (a), b region of retrosplenial granular cortex (b), retrosplenial dysgranular cortex (c), mediolateral area of secondary visual cortex (d), primary visual cortex (e), lateral area od secondary visual cortex (f), primary auditory cortex (g), ventral area of secondary auditory cortex (h), temporal association cortex and

ectorhinal cortex (i) dorsolateral entorhinal cortex (j), amygdalopiriform transition area (k) and posteromedial cortical amygdaloid nucleus.

Figure 7: **(A)** Coronal section through the midbrain, showing the nNOS distribution at the dorsal cortex of the inferior colliculus (a), dorsolateral and dorsomedial periaqueductal gray (b), external cortex of the inferior colliculus (c), lateral periaqueductal gray (d), ventrolateral periaqueductal gray, lateral part of the dorsal raphe nucleus and posterodorsal raphe nucleus (e), medial longitudinal fasiculus, dorsal and ventral part of dorsal raphe nucleus (f), pedunculopontine tegmental nucleus (g), median raphe nucleus, paramedian raphe nucleus and oral part of pontine reticular nucleus (h), reticulotegmental nucleus of the pons (i), lateral area of secondary visual cortex and forceps major of the corpus callosum (j), ectorhinal cortex and perirhinal cortex (k), and caudomedial entorhinal cortex (l). **(B)** Coronal section through the cerebellum and pons, showing the nNOS distribution at the 4^{th} and 5^{th} cerebellar lobule (a), simple lobule B (b), 1^{st} cerebellar lobule (c), parvicellular part of medial vestibular nucleus, superior vestibular nucleus and superior cerebellar peduncle (d), alpha part of central gray, supragenual nucleus and facial nerve (e), spinal trigeminal tract, vestibular root of the vestibulocochlear nerve and the anterior part of ventral cochlear nucleus (f), facial nerve and the alpha part of parvocellular reticular nucleus (g), trapezoid body and the cochlear root of the vestibulocochlear nerve (h), intermediate reticular nucleus (i), lateral superior olive (j), pyramidal tract, raphe pallidus nucleus and medial lemnicus (k), and the nucleus of the trapezoid body (l).

Figure 8: **(A)** Coronal section through the cerebellum and medulla, showing the nNOS distribution at the 8^{th} cerebellar lobule (a), paramedian lobule (b), copula of the pyramid (c), 10^{th} cerebellar lobule (d), area postrema, subbrachial nucleus and commissural part of the nucleus of the solitary tract (e), gracile nucleus (f), paratrigeminal nucleus and gelatinous layer of the caudal spinal trigeminal nucleus (g), central canal, vagus nerve and the root of hypoglossal nucleus (h), medial longitudinal fasciculus and raphe obscurus nucleus (i), rubrospinal tract, interpolar part of the spinal

trigeminal nucleus and gelatinous layer of the caudal spinal trigeminal nucleus (j), dorsal nucleus of inferior olive (k), medial lemniscus, pyramidal tract and subnucleus B and C of medial nucleus of inferior olive (l). **(B)** Coronal section through the spinal cord, showing the distribution of nNOS at the cuneate fasciculus, cuneate nucleus and interstital nucleus of the medulla 4th and 5th cerebellar lobule (a), gracile nucleus, gracile fasciculus and median accessory nucleus of the medulla (b), spinal trigeminal tract, caudal part of spinal trigeminal nucleus and gelatinous layer of the caudal spinal trigeminal nucleus (c & g), medial and commissural part of the nucleus of the solitary tract, central canal and central cervical nucleus of the spinal cord (d), pyramidal decussation, dorsal and ventral part of medullary reticular nucleus (e), dorsal part of medullary reticular nucleus and caudal part of spinal trigeminal nucleus (f), pyramidal decussation (h) and ventral part of medullary reticular nucleus (i).

Figure 9: Photomicrograph of the NOS-ir **(A)** olfactory bulb and **(B-D)** cerebral cortex counterstained with H&E (left), and the delineation of specific regions within the olfactory bulb and the cerebral cortex (right).

Figure 10: Photomicrograph of the NOS-ir **(A)** cerebral cortex and **(B-D)** forebrain counterstained with H&E (left), and the delineation of specific regions within the cerebral cortex (right).

Figure 11: Photomicrograph of the NOS-ir **(A-B)** midbrain, **(C)** cerebellum and pons, **(D)** cerebellum and medulla and **(E)** spinal cord counterstained with H&E (left), and the delineation of specific regions within the cerebral cortex (right).

Table 1: A table of abbreviations for nomenclature of various brain regions used in this study. The list of abbreviation is virtually a verbatim copy from the Rat Brain Atlas Edition 6 of Paxinos and Watson.

Table 2: Summary of the densities of nNOS-ir neuron and fibre distribution in selected brain regions of interest. The density was measured according to a scale of nNOS-ir expression as the following: (a) *Neuron* (not visible – 0; few/low – 1; moderate – 2; and many/high – 3); and (b) *fibre* (not visible – 0; low – 1; scattered/moderate – 2; and dense/high – 3).

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Table 1: List of Abbreviations

10Cb	10th cerebellar lobule
10N	vagus nerve
12N	hypoglossal nucleus
12n	root of hypoglossal nucleus
1Cb	1st cerebellar lobule (lingula)
2Cb	2nd cerebellar
3Cb	3rd cerebellar
4Cb	4th cerebellar lobule
5Cb	5th cerebellar lobule
6Cb	6th cerebellar
7Cb	7th cerebellar
7n	facial nerve
8Cb	8th cerebellar lobule
8cn	cochlear root of the vestibulocochlear nerve
8vn	vestibular root of the vestibulocochlear nerve
9Cb	9th cerebellar
a	artery
A11	A11 dopamine cells
AA	anterior amygdaloid area
aca	anterior commissure, anterior part
AcbC	accumbens nucleus, core
AcbSh	accumbens nucleus, shell
acp	anterior commissure, posterior part
AHiPL	amygdalohippocampal area, posterolateral part
AHiPM	amygdalohippocampal area, posteromedial part
AID	agranular insular cortex, dorsal part
AIP	agranular insular cortex, posterior part
AIV	agranular insular cortex, ventral part
AOL	anterior olfactory nucleus, lateral part
AOP	anterior olfactory nucleus, posterior part
AOVP	anterior olfactory nucleus, ventroposterior part
AP	area postrema
APir	amygdalopiriform transition area
APT	anterior pretectal nucleus
Aq	aqueduct
arcH	arcuate hypothalamic nucleus
ASt	amygdalostriatal transition area
Au1	primary auditory cortex
AuV	secondary auditory cortex, ventral area
BL	basolateral amygdaloid nucleus
BLA	basolateral amygdaloid nucleus, anterior part
BLP	basolateral amygdaloid nucleus, posterior part
BLV	basolateral amygdaloid nucleus, ventral part
BM	basomedial amygdaloid nucleus
BMA	basomedial amygdaloid nucleus, anterior part
BMP	basomedial amygdaloid nucleus, posterior part
CA1	field CA1 of the hippocampus
CA2	field CA2 of the hippocampus
CA3	field CA3 of the hippocampus
CB	cell bridges of the ventral striatum
cbw	cerebellar white matter
CC	central canal
cc	corpus callosum
CeCv	central cervical nucleus of the spinal cord
CEN	central nucleus of amygdala
CEnt	caudomedial entorhinal cortex
cg	cingulum
Cg1	cingulate cortex, area 1
Cg2	cingulate cortex, area 2
CGA	central gray, alpha part
cic	commissure of the interior colliculus
CIC	central nucleus of the inferior colliculus
Cl	claustrum
CM	central medial thalamic nucleus
Com	commissural nucleus of the inferior colliculus
Cop	copula of the pyramid
CPu	caudate putamen (striatum)
cp	cerebral peduncle
Crus 1	crus 1 of the ansiform lobule
Crus 2	crus 2 of the ansiform lobule
csc	commissure of the superior colliculus
cu	cuneate fasciculus
Cu	cuneate nucleus
CuR	cuneate nucleus, rotundus part
D3V	dorsal 3rd ventricle
DA	dorsal hypothalamic area
DCIC	dorsal cortex of the inferior colliculus
dcw	deep cerebral white matter
DEn	dorsal endopiriform nucleus
df	dorsal fornix
DI	dysgranular insular cortex
dhc	dorsal hippocampal commissure
DLEnt	dorsolateral entorhinal cortex
DLO	dorsolateral orbital cortex
dlPAG	dorsolateral periaqueductal gray
DMC	dorsomedial hypothalamic nucleus, compact part
DMD	dorsomedial hypothalamic nucleus, dorsal part
DMV	dorsomedial hypothalamic nucleus, compact part
dmPAG	dorsomedial periaqueductal gray
DP	dorsal peduncular cortex
DpG	deep gray layer of the superior colliculus
DpWh	deep white layer of the superior colliculus
DRD	dorsal raphe nucleus, dorsal part
DRL	dorsal raphe nucleus, lateral part
DRV	dorsal raphe nucleus ventral part
DS	dorsal subiculum
DTr	dorsal transition zone
DTT	dorsal tenia tecta
E	ependyma and subependymal layer
E/OV	olfactory ventricle (olfactory part of lateral ventricle)
EAC	sublenticular extended amygdala, central part
ECIC	external cortex of the inferior colliculus
Ect	ectorhinal cortex
EP	entopeduncular nucleus
EPl	External plexiform layer of the olfactory bulb
f	fornix
F	nucleus of the fields of Forel
fmi	forceps minor of the corpus callosum
fmj	forceps major of the corpus callosum
fr	fasciculus retroflexus
Fr3	frontal cortex, area 3
Ge5	gelatinous layer of the caudal spinal trigeminal nucleus
GI	granular insular cortex
Gl	glomerular layer of the olfactory bulb
GP	globus pallidus
gr	gracile fasciculus
Gr	gracile nucleus
GrA	granule cell layer of the accessory olfactory bulb
GrDG	granular layer of the dentate gyrus
GrO	granule cell layer of the olfactory bulb
HDB	nucleus of the horizontal limb of the diagonal band
IB	interstitial nucleus of the medulla
IBl	inner blade of the dentate gyrus
ic	internal capsule
ICj	island of Calleja
IEn	intermediate endopiriform nucleus
IF	interfascicular nucleus
IL	infralimbic cortex
InC	interstitial nucleus of Cajal
InG	intermediate gray layer of the superior colliculus
InWh	intermediate white layer of the superior colliculus
IOA	inferior olive, subnucleus A of medial nucleus
IOB	inferior olive, subnucleus B of medial nucleus
IOC	inferior olive, subnucleus C of medial nucleus
IOD	inferior olive, dorsal nucleus
IP	interpeduncular nucleus
IPACM	interstitial nucleus of the posterior limb of the anterior commissure, medial part
IPL	interpeduncular nucleus, lateral subnucleus
IRt	intermediate reticular nucleus
isRt	isthmic reticular formation
LaDL	lateral amygdaloid nucleus, dorsolateral part
LaVL	lateral amygdaloid nucleus, ventrolateral part
LaVM	lateral amygdaloid nucleus, ventromedial part
Ld	lambdoid septal zone
LD	laterodorsal thalamic nucleus
lf	lateral funiculus
lfp	longitudinal fasciculus of nucleus
LH	lateral hypothalamus
LMoL	lacunosum moleculare layer of the hippocampus
lo	lateral olfactory tract
LO	lateral orbital cortex
LOT2	nucleus of the lateral olfactory tract, layer 2
LOT3	nucleus of the lateral olfactory tract, layer 3
lPAG	lateral periaqueductal gray
LPMC	lateral posterior thalamic nucleus, mediocaudal part
LPT	lateral posterior thalamic nucleus
LRt	lateral reticular nucleus
LSD	lateral septal nucleus, dorsal part
LSI	lateral septal nucleus, intermediate part
LSO	lateral superior olive
LT	lateral terminal nucleus (pretectum)
Lth	lithoid nucleus
LV	lateral ventrical
M1	primary motor cortex
M2	secondary motor cortex

MCPC	magnocellular nucleus of the posterior commissure
MDC	mediodorsal thalamic nucleus, central part
MdD	medullary reticular nucleus, dorsal part
MDM	mediodorsal thalamic nucleus, medial part
MdV	medullary reticular nucleus, ventral part
Me	medial amygdaloid nucleus
MEnt	medial entorhinal cortex
MePD	medial amygdaloid nucleus, posterodorsal part
MePV	medial amygdaloid nucleus, posteroventral part
MGD	medial geniculate nucleus, dorsal part
MGV	medial geniculate nucleus, ventral part
MH	medial habenular
Mi	mitral cell layer of olfactory bulb
ml	medial lemniscus
ML	medial mammillary nucleus, lateral part
mlf	medial longitudinal fasciculus
MM	medial mammillary nucleus, medial part
MnA	median accessory nucleus of the medulla
MnR	median raphe nucleus
MO	medial orbital cortex
MoDG	molecular layer of the dentate gyrus
mp	mammillary peduncle
MPOM	medial preoptic nucleus, medial part
MS	medial septal nucleus
mt	mammillothalamic tract
MTu	medial tuberal nucleus
MVeMC	medial vestibular nucleus, magnocellular part
MVePC	medial vestibular nucleus, parvicellular part
Nv	navicular nucleus of the basal forebrain
OBl	outer blade of the dentate gyrus
och	optic chiasm
ON	olfactory nerve layer
Op	optic nerve layer of the superior colliculus
opt	optic tract
Or	oriens layer of the hippocampus
P1PAG	p1 periaqueductal gray
p1Rt	p1 reticular formation
PA	preoptic area
Pa5	paratrigeminal nucleus
PaAP	paraventricular hypothalamic nucleus, anterior parvicellular part
PaS	parasubiculum
PBP	parabrachial pigmented nucleus of the VTA
pc	posterior commissure
PCRtA	parvocellular reticular nucleus, alpha part
PDR	posterodorsal raphe nucleus
PF	parafascicular thalamic nucleus
PH	posterior hypothalamic nucleus
PHA	posterior hypothalamic area
PHD	posterior hypothalamic area, dorsal part
Pi	pineal gland
PIF	parainterfascicular nucleus of the ventral tegmental area
PIL	posterior intralaminar thalamic nucleus
Pir1	piriform cortex, layer 1
Pir2	piriform cortex, layer 2
Pir3	piriform cortex, layer 3
Pk	purkinje cell layer of the cerebellum
PL	paralemniscal nucleus
PLd	paralambdoid septal nucleus
PLH	peduncular part of lateral hypothalamus
PM	paramedian lobule
PMCo	posteromedial cortical amygdaloid nucleus
PMD	premammilary nucleus, dorsal part
PMnR	paramedian raphe nucleus
PN	paranigral nucleus of the ventral tegmental area
PnC	pontine reticular nucleus, caudal part
PnO	pontine reticular nucleus, oral part
Po	posterior thalamic nuclear group
PoDG	polymorph layer of the dentate gyrus
Post	postsubiculum
PoT	posterior thalamic nucleus group, triangular part
PP	peripeduncular nucleus
Pr5	principal sensory trigeminal nucleus
PrC	precommissural nucleus
PrG	pregeniculate nucleus of the prethalamus
PRh	perirhinal cortex
PrL	prelimbic cortex
PT	paratenial thalamic nucleus
PTg	pedunculopontine tegmental nucleus
PtPD	parietal cortex, posterior area, dorsal part
PtPR	parieral cortex, posterior area, rostral part
PV	paraventricular thalamic nucleus
PVA	paraventricular thalamic nucleus, anterior part
PVP	paraventricular thalamic nucleus, posterior part
Py	pyramidal cell layer of the hippocampus
py	pyramidal tract
pyx	pyramidal decussation
Rad	radiatum layer of the hippocampus
Re	reuniens thalamic nucleus
rf	rhinal fissure
Rh	rhomboid thalamic nucleus
RI	rostral interstitial nucleus
RIP	rape interpositus nucleus
RLi	rostral linear nucleus of the raphe
ROb	raphe obscurus nucleus
RPa	raphe pallidus nucleus
rs	rubrospinal tract
RSD	retrosplenial dysgranular cortex
RSGa	retrosplenial granular cortex, a region
RSGb	retrosplenial granular cortex, b region
RSGc	retrosplenial granular cortex, c region
Rt	reticular thalamic nucleus
RtTg	reticulotegmental nucleus of the pons
S1BF	primary somatosensory cortex, barrel field
S1DZ	primary somatosensory cortex, dysgranular zone
S1FL	primary somatosensory cortex, forelimb region
S1HL	primary somatosensory cortex, hindlimb region
S1J	primary somatosensory cortex, jaw region
S1Tr	primary somatosensory cortex, trunk region
S1ULp	primary somatosensory cortex, upper lip region
S2	secondary somatosensory cortex
SCh	suprachiasmatic nucleus
SCO	subcommissural organ
scp	superior cerebellar peduncle (brachium conjunctivum)
SF	septofimbrial nucleus
SFO	subfornical organ
SGe	supragenual nucleus
SimA	simple lobule A
SimB	simple lobule B
sm	stria medullaris of the thalamus
SNC	substantia nigra, compact part
SNR	substantia nigra, reticular part
SolC	nucleus of the solitary tract, commissural part
SolM	nucleus of the solitary tract, medial part
Sp5	spinal trigeminal tract
Sp5C	spinal trigeminal nucleus, caudal part
Sp5I	spinal trigeminal nucleus, interpolar part
SPFPC	subparafascicular thalamic nucleus, parvicellular part
SPH	subparafascicular thalamic nucleus
st	stria terminalis
StHy	striohypothalamic nucleus
STMPM	bed nucleus of the stria terminalis, medial division, posteromedial part
Sub	submedius thalamic nucleus
SubP	subbrachial nucleus
SuG	superficial gray layer of the superior colliculus
SuVe	superior vestibular nucleus
TeA	temporal association cortex
TS	triangular septal nucleus
Tu	olfactory tubercle
Tz	nucleus of the trapezoid body
tz	trapezoid body
V1	primary visual cortex
V1B	primary visual cortex, binocular area
V2L	secondary visual cortex, lateral area
V2ML	secondary visual cortex, mediolateral area
V2MM	secondary visual cortex, mediomedial area
VA	ventral anterior thalamic nucleus
VCA	ventral cochlear nucleus, anterior part
VDB	nucleus of the vertical limb of the diagonal band
VEn	ventral endopiriform nucleus
VG	ventral geniculate nucleus
vhc	ventral hippocampal commissure
VIEnt	ventrak intermediate entorhinal cortex
VL	ventrolateral thalamic nucleus
VLPAG	ventrolateral periaqueductal gray
VM	ventromedial thalamic nucleus
VMH	ventromedial hypothalamic nucleus
VMHC	ventromedial hypothalamic nucleus, central part
VMHDM	ventromedial hypothalamic nucleus, dorsomedial part
VMHVL	ventromedial hypothalamic nucleus, ventrolateral part
VO	ventral orbital cortex
VP	ventral pallidum
VPL	ventral posterolateral thalamic nucleus
VPM	ventral posteromedial thalamic nucleus
vtgx	ventral tegmental decussation
VTT	ventral tenia tecta
ZI	zona incerta
ZID	zona incerta, dorsal part
ZIV	zona incerta, ventral part

Table 2

Brain Regions	Density	
	Neuron	Fibre
Olfactory-related Areas		
Olfactory bulb		
- Glomerular Layer	3	3
- Granule Cell Layer	2	3
Olfactory Tubercule	2	3
Olfactory Nerve	0	1
Olfactory Ventricle	2	3
Nucleus of Lateral Olfactory Tract	3	3
Anterior Olfactory Nucleus	1	1
Accessory Olfactory Bulb	1	1
Tenia Tecta		
- Dorsal	1	1
- Ventral	1	1
Dorsal Transition Zone	1	1
Navicular Nucleus	1	1
Anterior Cortical Areas		
Motor Cortex	2	2
Medial Prefrontal Cortex		
- Prelimbic Cortex	2	2
- Infralimbic Cortex	2	2
- Dorsal Peduncular Cortex	2	3
Cingulate cortex	2	2
Orbital Cortex	2	2
Agranular Insular Cortex		
- Dorsal	1	1
- Ventral	2	3
Somatosensory Cortex		
- Primary Region	2	2
- Jaw Region	2	2
- Forelimb Region	1	1
- Dysgranular Zone	1	1
- Secondary Region	3	3
Piriform Cortex	1	3
Endopiriform Nucleus		
-Dorsal	3	2
-Intermediate	3	3
Claustrum	1	2
Basal Ganglia		
Nucleus Accumbens (Shell)	3	3
Nucleus Accumbens (Core)	3	3
- Anterior Commissure (within the Nucleus Accumbens)	1	1
Ventral Pallidum	2	2
Caudate Putamen	2	2
Islands of Calleja	3	3
Globus Pallidus	0	1
Internal Capsule	3	2

Brain Regions	Density	
	Neuron	Fibre
Medial Septum		
Medial Septal Nucleus	3	3
Lateral Septal Nucleus	1	1
Lambdoid Septal Zone	2	2
Paralamboid Septal Nucleus	1	1
Diagonal Band Nuclei		
Vertical Limb	2	2
Horizontal Limb	2	2
Subfornical Organ	3	3
Amygdala		
Anterior Amygdala Area	3	2
Central Nucleus of Amygdala	0	1
Amygdalostriatal Transition Area	3	2
Lateral Amygdaloid Nucleus		
- Dorsolateral	3	2
- Ventrolateral	2	2
Medial Amygdaloid Nucleus	3	3
Basolateral Amygdaloid Nucleus	2	2
Amygdalopiriform Transition Area	2	2
Posteromedial Cortical Amygdaloid Nucleus	1	2
Bed Nucleus of the Stria Terminalis	2	1
Thalamus		
Paraventricular Thalamic Nucleus		
- Anterior	1	1
- Posterior	3	2
Paratenial Thalamic Nucleus	1	1
Rhomboid Thalamic Nucleus	2	1
Reuniens Thalamic Nucleus	2	1
Mediodorsal Thalamic Nucleus	0	1
Submedius Thalamic Nucleus	0	1
Posterior Thalamic Nucleus	2	2
Parafascicular Thalamic Nucleus	3	3
Subparafascicular Thalamic Nucleus	2	2
Posterior Intralaminar Thalamic Nucleus	2	2
Ventral Anterior Thalamic Nucleus	0	0
Ventrolateral Thalamic Nucleus	0	0
Hypothalamus		
Paraventricular Hypothalamic Nucleus	2	2
Striohypothalamic Nucleus	2	2
Hypothalamic Area		
- Posterior	2	2
- Dorsal	2	2
Ventromedial Hypothalamic Nucleus		
- Dorsomedial	0	0
- Central	0	0
- Ventrolateral-	1	1
Lateral Hypothalamus	2	2
Medial Tuberal Nucleus	2	2

Brain Regions	Density	
	Neuron	Fibre
Hippocampus		
Hippocampus Proper		
- CA1 Subfield	2	2
- CA2 Subfield	2	2
- CA3 Subfield	2	2
- Dentate Gyrus	2	1
Subiculum	2	1
Midbrain / Brain Stem		
Substantia Nigra		
- Pars Reticulata	1	1
- Pars Compacta	1	1
Superior Colliculus		
- Superficial Layer	3	3
- Optic Nerve Layer	1	2
- Intermediate Gray Layer	0	0
Inferior Colliculus		
- Dorsal Cortex	3	3
- External Cortex	3	3
Periaqueductal Gray		
- P1	3	3
- Ventrolateral Part	3	3
- Dorsal Lateral Part	2	2
- Dorsal Medial Part	1	1
- Lateral Part	2	2
Ventral Tegmental Area		
- Parainterfascicular Nucleus	3	3
- Paranigral Nuclues	3	2
- Ventral Tegmental Decussation	3	2
Tegmental Nuclei of the Pons		
- Pedunculopontine Tegmental Nucleus	3	3
- Reticulotegmental Nucleus of the Pons	3	3
Raphe Nuclei		
- Dorsal Raphe	3	3
- Median Raphe	3	3
Superior Olive		
- Lateral Superior Olive	0	1
Inferior Olive		
- Dorsal Nucleus	2	3
- Subnucleus B of Medial Nucleus	2	2
- Subnucleus C of Medial Nucleus	2	2
Intermediate Reticular Nucleus	3	2
Ventral Lateral Geniculate Nucleus	3	3
Zona Incerta	2	3
Lateral Terminal Nucleus	3	3

Brain Regions	Density	
	Neuron	Fibre
Supragenual Nucleus	3	3
Nucleus of the Trapezoid Body	3	3
Gracile Nucleus	2	1
Gracile Fasciculus	1	1
Median Accessory Nucleus	0	0
Cuneate Nucleus	1	1
Posterior Cortical Areas		
Ectorhinal Cortex	2	2
Entorhinal Cortex	2	2
Retrosplenial Granular Cortex	2	2
Retrosplenial Dysgranular Cortex	2	2
Primary Visual Cortex	2	2
Secondary Visual Cortex	2	2
Primary Auditory Cortex	2	2
Secondary Auditory Cortex	2	2
Temporal Association Cortex	2	2
Cerebellum		
Molecular Layer	3	3
Granule Layer	3	3
Purkinje Cell Layer	1	1
Vestibular Nucleus		
- Medial Vestibular Nucleus	0	1
- Superior Vestibular Nucleus	0	1
Nuclei in the Spinal Cord		
Spinal Trigeminal Nuclei		
- Gelatinous Layer	3	3
- Interpolar part	2	1
- Caudal part	2	1
Nucleus of the Solitary Tract	3	2
Central Cervical Nucleus of Spinal Cord	3	2

Categorization of neuronal and fibre densities according to the nNOS-ir expression levels as the following:

(a) ***Neuron*** (not visible – 0; few/low– 1; moderate – 2; many/high – 3);

(b) ***Fibre*** (not visible – 0; low– 1; scattered/moderate – 2; dense/high – 3).

Distribution of neuronal nitric oxide synthase immunoreactivity in adult male Sprague-Dawley rat brain

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Author Statement

L.W.L. conceptualized and designed the study. P.S.C., C.H.P., W.L.L., & L.W.L. performed histological study and microscopic analysis. P.S.C., C.H.P., W.L.L., & L.W.L. ensured all data were representative of the original raw data. H.W.M.S. & Piers Emson (Cambridge University, U.K.) provided the nNOS antibody. Y.S.C., M.L.F., L.G., & H.W.M.S. contributed to intellectual inputs of the manuscript. L.W.L., P.S.C., & C.H.P., W.L.L. drafted and revised the manuscript text, and all authors reviewed and provided comments on the article.