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Baseline striatal and nigral interneuronal protein levels in two distinct mice strains differ in accordance with their MPTP susceptibility

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ABSTRACT

Epidemiological studies reveal an ethnicity-based bias in prevalence of Parkinson's disease (PD), deriving from the differences that exist between Caucasians and African or Asian populations. Experimental mice models provide a scope to analyse the cellular mechanisms of differential susceptibility to PD. C57BL/6J mice, for instance, are more susceptible to MPTP-induced Parkinsonism whereas CD-1 mice are resistant. In PD-pathogenesis, interneuronal contribution is also likely, although they comprise only 5–10% of the striatal cells. The interneurons harbour calcium binding proteins, like calretinin (Cal-R) and parvalbumin (PV), which are crucial in Ca^{2+} homeostasis for preventing calcium-induced excitotoxicity. GAD-67-immunoreactive interneurons are the other prominent set of GABAergic interneurons. In PD, dopamine loss up-regulates GAD-67 expression in striatal projection neurons and other basal ganglia circuit. We studied the possible contribution of interneurons in determining variable susceptibility by assessing the expression of calretinin, PV and GAD-67 in both striatum and substantia nigra pars compacta (SNpc) in two distinct mice strains, i.e. C57BL/6J and CD-1 under normal conditions, using unbiased stereology for quantification of immunoreactive cells and immunoblotting. The vulnerable C57BL/6J had lesser basal parvalbumin expression in both nigra and striatum whereas the calretinin levels were low only in the striatum. GAD-67 expression showed no perceptible differences in the striatum or SNpc of either of the strains. Differential expression of calcium buffering/binding proteins under normal physiological condition proffers a role for interneurons in the differential susceptibility to PD. Thus, even the baseline susceptibility indices i.e. without using the neurotoxin; can provide vital mechanistic insights into PD pathogenesis.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, which manifests following a loss of 50–60% of dopaminergic neurons in substantia nigra pars compacta (SNpc). The loss results in the cardinal motor symptoms of bradykinesia, resting tremor, rigidity and postural instability (Jankovic, 1987; Fahn, 2000). In addition to the genetic predisposition, environment and dietary patterns as well as aging are prominent risk factors. Interestingly, the prevalence of PD varies among different ethnicities worldwide, the basis for which are the door-to-door studies conducted in different countries. For example, the prevalence of PD is higher in the Caucasians than in non-whites (Tanner and Goldman, 1994; Schoenberg et al., 1988; Das et al., 2010; Razdan et al., 1994; Bharucha et al., 1988; Muthane et al., 1998). It is proposed that understanding the underlying molecular mechanisms, may explain the etiopathogenesis of PD.

The neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), induces Parkinsonian symptoms in mice, non-human primates as well as humans (Davis et al., 1979; Langston et al., 1983). In rhesus monkeys, administration of NMPTP (*N*-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine) destroyed the dopaminergic neurons of pars compacta resulting in Parkinsonian symptoms (Burns et al., 1983; Chiueh et al., 1983, 1985a). In a striking contrast, in rats NMPTP caused acute retropulsion, immobilization, piloerection, clonic movements of the forepaws (Chiueh et al., 1984). Therefore MPTP is a more favoured neurotoxin to mimic PD-like symptoms. Interestingly, very similar to the humans, the C57BL/6J mice strain that has lesser nigral neurons, shows higher susceptibility to the MPTP while CD-1 that bears more

Abbreviations: BSA, bovine serum albumin; Cal-R, calretinin; Cal-B, calbindin; DAB, 3′-3′-diaminobenzidine; GABA, γ-amino butyric acid; GAD, glutamate acid decarboxylase; H_2O_2, hydrogen peroxide; IHC, immunohistochemistry; MPTP, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; MSN, medium spiny projection neurons; NMPTP, N- methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; Nurr1, nuclear receptor related protein 1; PBS, phosphate buffered saline; PBS-TX, phosphate buffered saline- TritonX; PD, Parkinson's disease; PFA, paraformaldehyde; PV, parvalbumin; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulate; TBST, Tris buffered saline- tween 20; TH, tyrosine hydroxylase

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neurons is resistant. Thus, the human phenomenon of differential vulnerability and the role of neurons can be reminisced using these mice strains (Vidyadhara et al., 2016a,b). However, there is insufficient information on the involvement of interneurons; the specialized cells releasing the neurotransmitters like γ-amino-butyric acid (GABA) and glycine. They are vital during the formation of the circuitry in the developing nervous system of vertebrates and invertebrates.

The striatum is a major integrative component of the basal ganglia, in control of motor activities and reward behaviours (Graybiel, 1998; Parent and Hazrati, 1995; Schultz, 2002). Among the striatal cell types 90–95% are GABAergic medium spiny neurons (MSNs) and remaining 5–10% comprise of interneurons (Gerfen and Bolam, 2010; Rymar et al., 2004; Petryszyn et al., 2017). Despite being a small population, striatal interneurons exert a powerful pre- and post-synaptic striatal modulation (Goldberg et al., 2012) implicating them in many movement and psychiatric disorders (Ding et al., 2011; Kataoka et al., 2010; Pisani et al., 2007).

Although majority of the striatal cells use GABA as a neurotransmitter, they can be distinguished from one another by their morphological features and due to the expression of different sets of neuropeptides or proteins (Cicchetti et al., 2000; Kawaguchi et al., 1995). In-situ hybridization studies in rats show abundant GAD-67 expression in dorsal and ventral striatum, but only few cells stain heavily suggesting the prominence of non-cytoplasmic localisation (Chesselet et al., 1987; Lindefors et al., 1989). One of the hallmarks in PD is that dopamine loss up-regulates GAD-67 expression in striatal projection neurons and other basal ganglia circuit. Studies evaluating alterations in GAD-67 expression have mostly been performed in other nuclei of basal ganglia, like- globus pallidus, subthalamic nuclei where GAD-67 mRNA expression was up-regulated following toxin administration (Billings and Marshall, 2004; During et al., 2001). Manganese-exposure induced chronic or partially acute neurotoxicity involves the activation of SN GABAergic neurons (Yang et al., 2011). An in-situ hybridization study in transgenic mouse model (R6/2) of the Huntington's disease showed normal expression of GAD-67 in striatum, cerebellum and septum contrary to the decreased expression in the frontal cortex, parietal cortex and pars reticulata of SN (Gourfinkel-An et al., 2003). CB1 i.e. cannabinoid receptor mutant mice displayed higher levels of substance P, enkephalin, dynorphin and GAD-67 mRNAs in the output pathways that project from striatum to substantia nigra and globus pallidus (Steiner et al., 1999).

Other interneuronal proteins such as calretinin (Cal-R), calbindin-D28K (Cal-B), parvalbumin (PV) assist calcium homeostasis. As for the small Cal-R cells, a decreasing gradient is seen along the anteroposterior and dorsoventral axes in mice (Revishchin et al., 2010; Ming and Song, 2011). In monkeys as well as humans, most of the striatal interneurons express Cal-R (Cicchetti et al., 2000; Parent et al., 1995; Wu and Parent, 2000) whereas both neurons and interneurons express Cal-B in different regions of brain (Liu and Graybiel, 1992). Immunohistochemistry and double-labelled fluorescence microscopy studies report that the Cal-R and TH- co-immunoreactive neurons survive better than only TH alone-immunopositive cells and are less vulnerable to 6-OHDA in the SNpc of the Parkinsonian rats (Kim et al., 2000). Selective preservation of Cal-R positive cells in SNpc renders protection to a particular population of dopaminergic cells (Mouatt-Prigent et al., 1994). In human midbrain tissues, majority of the nigral cells did not express Cal-R and Cal-B. Few Cal-R positive neurons were present in the dorsal aspect while Cal-B immunoreactive neurons were present in the ventral part of SN (Korzhevskii et al., 2017). In another study, Lee and Tepper (2007) reported that distinct electrophysiological or morphological properties to differentiate PV and Cal-R neurons in SN are lacking. Changes in the inhibitory activity of PV expressing neurons in the cortex, increases the animal's susceptibility to epileptic seizures (Defelipe et al., 1999; Schwaller et al., 2004). In the striatum, the PV immunoreactive neurons primarily downregulate the activity of medium spiny projection neurons via monosynaptic inhibition in a feed forward fashion (Burguière et al., 2013; Mallet et al., 2005; Qi et al., 2016). Postnatal alterations in neurons, interneurons and glia were studied in the mouse substantia nigra, which provide valuable information about the developmental aspect of different cells in SN and reported that neurons expressing neuronal nitric oxide synthase develop prior to PV neurons (Abe et al., 2010). Studies on autopsied brain showed more PV expressing neurons in SNpr (Hardman et al., 1996). In SN, excitoxicity and oxidative stress lead to raised intracellular calcium levels. In this situation, increase in PV expression was protective (Soós et al., 2004).

Thus, though some studies report about these calcium-binding proteins in basal ganglia, there is insufficient literature about their contribution to susceptibility or resilience against MPTP or PD. The present study investigates the baseline expression pattern of calcium binding proteins i.e., Cal-R and PV as well as GAD-67 to determine the possible contribution of interneurons in imparting variable susceptibility to MPTP under normal non-toxic conditions. We compared the differences in two mice strains, i.e. C57BL/6J and CD-1 mice under normal conditions, using immunohistochemistry and Western blotting.

2. Materials and methods

2.1. Mice strains

Adult male mice of 15–17 weeks age, of two genetically distinct strains i.e. C57BL/6J and CD-1 mice were studied under normal conditions (n = 6 in each group). We housed the mice in polypropylene cages and maintained under standard laboratory conditions, with ad-libitum access to food and water. All the experiments were carried out during the light period (08:00–18:00h) in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, which adheres to the guidelines of National Institute of Health, USA; Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. We made all efforts to minimize the number of animals used and their suffering.

2.2. Tissue processing and immunolabeling

The adult mice were anaesthetized using halothane and perfused intracardially with 0.9% saline followed by 4% buffered paraformaldehyde (0.1 M phosphate buffer; pH 7.4) for 30 min. We dissected the brains and post-fixed them in the paraformaldehyde solution for two days. Following cryopreservation in sucrose gradients (15% and 30%), we collected 40 µm thick cryosections of the striatum and the nigral region on gelatin-subbed slides.

The immunoperoxidase labeling protocol was identical to that reported earlier (Alladi et al., 2010a). In brief, we subjected the sections to antigen unmasking using sodium citrate buffer (pH-6, 10 mM) followed by quenching using 0.1% H_2O_2 in 70% methanol for 30 min. Thereafter we exposed the sections to 3% buffered bovine serum albumin for 4 h at room temperature to reduce non-specific staining and thereafter labeled with one of the primary antibodies in 0.1 M PBS-TX (Goat anti-Calretinin, Mouse anti-Parvalbumin or Mouse anti-GAD-67 Table 1) for 72 h at 4 °C. We sourced the primary antibodies from Merck as per recent literature (Ding et al., 2014; McKenna et al., 2013; Aryal et al., 2011). The initial 4 h of incubation was at room temperature to facilitate binding. Following buffer washes, we incubated the sections with biotinylated secondary antibody (1:200; Vector Laboratories, USA) for 8 h at 4 °C overnight. Thereafter, we applied avidin- biotin complex for 4 h at room temperature. Between the changes of antibodies, we washed the sections four times with 0.1 M PBS (pH 7.4). We visualized the staining using 0.05% DAB (3′-3′-diaminobenzidine) as a chromogen solubilized in imidazole buffer (pH 7.4) alongwith 0.1% H_2O_2. We mounted the sections with DPX, after dehydration in an alcohol series.

For immunofluorescence based labeling, the sections were