



## Research Paper

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# Fluoxetine caused cerebellum dysfunction via reducing the Purkinje cells — A proposed mechanism *via* 'hepatic encephalopathy'

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### ABSTRACT

The use of antidepressant treatment during pregnancy is increasing, and selective serotonin reuptake inhibitors (SSRIs) are the most widely prescribed antidepressants in pregnant women. Much of literature has indicated the beneficial effects of Fluoxetine (Prozac), including myelination, a process that shapes brain connectivity contributing to the remediation of symptoms of psychiatric disorders, such as anxiety. On the contrary, many side effects of Fluoxetine are emerging, which include serotonin syndrome and cardiovascular etc. Purkinje neuronal cells are found within the cerebellar cortex that participates in the processes of motor control and learning. Seldom has been cited for the damaging effect of Fluoxetine on the Purkinje cells. Fluoxetine reduced locomotor activities with poorer motor performance. When the cerebellum is damaged, motor movement is impaired. In addition, Fluoxetine induces liver damage and mediates free radical reactions. To understand whether the early exposure of fetus to Fluoxetine could damage the cerebellar Purkinje cells and how is the associated mechanism, we performed this study. Results indicated that i.p. Fluoxetine (10  $\mu$ M) induced severe ataxia with poorer motor performance in day-1 chicks. H&E stain revealed severe swollen and scattered damaged Purkinje cells in cerebellum. The relevant action mechanism is proposed to be associated with 'the Hepatic Encephalopathy', the damaging of synaptic receptor mGlu1, and the disturbance of melatonin in secretion. Conclusively, Fluoxetine induced ataxia of the newborns via reducing the Purkinje cells. The action mechanism is proposed to be mainly associated with the Hepatic Encephalopathy. The early Fluoxetine exposure and its long term effects should be concerned.

**Key words:** Fluoxetine (Prozac), cerebellar Purkinje cells, locomotor activities, selective serotonin reuptake inhibitors (SSRIs), hepatic encephalopathy, melatonin.

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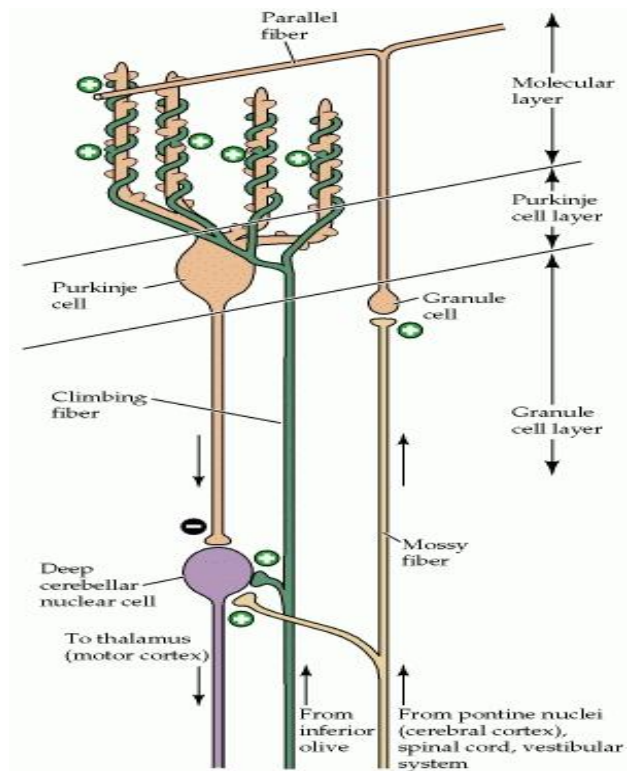
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### INTRODUCTION

Purkinje cells (Purkinje neurons) (PC) are neurons found within the Purkinje layer in the cerebellar cortex of the brain that participate in the processes of motor control and learning (Minai, 2014). Purkinje cells extend many threadlike extensions (dendrites) to receive impulses from other neurons called granule cells. Each PC also has a single projection called an axon, which transmits impulses to the part of the brain that controls movement, the cerebellum (Minai, 2014) (Figure 1). Two kinds of neuronal fibers carry

input to the Purkinje cells: mossy fibers and climbing fibers. Mossy fibers, originated in the spinal cord and brain stem, influence Purkinje cells by way of granule cells (Minai, 2014) (Figure 1).

The use of antidepressant treatment during pregnancy is increasing, and selective serotonin reuptake inhibitors (SSRIs) are the most widely prescribed antidepressants in pregnant women (Kaiholo et al., 2016). Much of literature has indicated the beneficial effects of Fluoxetine (Prozac)



**Figure 1:** Excitatory and inhibitory connections in the cerebella cortex and the deep cerebellar nuclei (Depicted from Purves D. "Neuron Science" 2<sup>nd</sup> edn. 2001. Purkinje Cells).

(Di Rosso, 2018; Kroeze et al., 2015), such as chronic Fluoxetine exposure caused long-term changes in hippocampal expression of ciliary neurotrophic factor and other genes linked to myelination, a process that shapes brain connectivity and could contribute to the remediation of symptoms of psychiatric disorders, such as anxiety (Kroeze et al., 2015). On the contrary, Fluoxetine has shown to exhibit many side effects (Messiha, 1993; Ferguson, 2001), which include the "serotonin syndrome", cardiovascular complications, extrapyramidal side effects such as akathisia, dyskinesias, and parkinsonian-like syndromes and an apparently increased risk of suicidality, mania and hypomania, seizures, sexual disorders, hematological changes, and inappropriate secretion of the antidiuretic hormone (Messiha, 1993). More recently, Fluoxetine was found to reduce locomotor activities and poorer motor performance of rats (Lee and Lee, 2012). When the cerebellum is damaged, motor movement is impaired, often in the form of ataxia and uncoordinated movement (Scott et al., 2018). Examples of these cerebella-based motor deficits can be found in well-known diseases such as Parkinson's (Wu and Hallett, 2013) and fragile X syndrome (Ellegood et al., 2010; Roy et al., 2011).

More attractively, the reduced number of PCs can affect the locomotor activities with poorer motor performance. Literature has indicated that the cerebellum is

disproportionately small in the Ts65Dn mouse and individuals with Down Syndrome and has a reduced number of granule neurons and Purkinje cells (Roper et al., 2006). Antidepressant pharmacotherapy is to date the most often used treatment for depression, but the exact mechanism of action underlying its therapeutic effect is still unclear. Many theories have been put forward to account for depression, as well as antidepressant activity, but none of them is exhaustive (Antonioli et al., 2012).

Circadian rhythm disturbances can occur as part of the clinical symptoms of major depressive disorder and have been found to resolve with antidepressant therapy (Reiersen et al., 2009). The pineal gland is relevant to circadian rhythms as it secretes the hormone melatonin following activation of the cyclic adenosine monophosphate (cAMP) signaling cascade and of arylalkylamine N-acetyltransferase (AA-NAT), the rate-limiting enzyme for its synthesis (Reiersen et al., 2009). Chronic fluoxetine treatment increases daytime plasma melatonin and pineal AA-NAT gene expression (Reiersen et al., 2009).

During and following pregnancy, women are at high risk of experiencing depression (Kiryanova et al., 2013). Growing evidence from human and animal studies has shown adverse consequences of maternal usage of antidepressants in their newborn babies (Lee and Lee, 2012). To understand whether or not the early exposure to Fluoxetine during the fetus could reduce the cerebellar PCs, and more importantly, the associated mechanism of how Fluoxetine induces the reduction of PC population in cerebellum, the present study was conducted.

The excitatory input from the Mossy fibers and climbing fibers to Purkinje cells (PCs) and deep nuclear cells is basically the same. Additional convergent input onto the PC from local circuit neurons (basket and stellate cells) and other PCs establishes a basis for the comparison of ongoing movement and sensory feedback from it. The PC output to the deep cerebellar nuclear cell thus generates an error correction signal that can modify movements already begun. The climbing fibers modify the efficacy of the parallel fiber-PC connection, producing long-term changes in cerebellar output (after Stein, 1986).

## MATERIALS AND METHODS

### *Ex vivo* model of chicken embryos

This animal experiment project has been approved by the University Ethic Committee in accordance with the Declaration of Helsinki with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. In summary, day zero fertilized eggs purchased from the local farm were divided into the control and the experimental groups, each 12 eggs. These selected fertilized eggs were incubated at 37°C and RH 72–82% until HH stage-22 (day 3.5) was reached. Different doses of Fluoxetine at 0.0 (control) and 10.0 µM

(experimental) were aseptically applied. The incubation was continued and observed at HH stage 46 (day-1) chicks (Hamburger and Hamilton, 1951). The morphology and activity of day-1 chicks were recorded, and then CO<sub>2</sub>-anesthetized, brain was dissected, rinsed twice with 4°C PBS buffer, and immediately frozen for Hematoxylin-Eosin staining.

## RESULTS

### The behavior and activity of hatched day-1 chicks

The Fluoxetine treated day-1 chicks showed outer legs with weak muscles, severe ataxia, difficult to stand up, loss of appetite, and listless, intention tremors, lack of menace reflex, high-stepping gait.

### Hematoxylin-Eosin staining of the brain tissues

Fluoxetine reduced the PCs in cerebellum, as compared with the normal chicks (Figure 2a), the cerebellum of affected chicks exhibited much less Purkinje cells, and the PCs were seen randomly scattered and severely swollen (Figure 2b).

### Proposed mechanism of action associated with the Fluoxetine-induced reduction of Purkinje cells

It was found that, as compared with the control (Figure 2a), the population of PCs in the day-1 chick was severely reduced after Fluoxetine treatment (Figure 2b). As well documented, Fluoxetine has been accumulatively cited for its neuroprotective effects (Jin et al., 2009 and Vizi et al., 2013), why could it have killed the cerebellar PCs in the fetus during embryogenesis? The proposed mechanism of action is presented in Figure 3.

Such a proposed mechanism is partly based on the so-called “Hepatic Encephalopathy” (García-Lezana et al., 2017), which will be discussed in the following section.

## DISCUSSION

The neuroprotective effects of Fluoxetine hydrochloride have been reported by numerous studies (Jin et al., 2009; Vizi et al., 2013). Purkinje cell (PC) dysfunction or degeneration is the most frequent finding in animal models with ataxic symptoms. However, its structural, histological, and physiological adverse effects are seldom cited, and in some cases PC dysfunction precedes the onset of ataxic signs (Mitoma et al., 2016; Jaber, 2017; Hoxha et al., 2018).

mGlu receptors are widely distributed in the CNS, where they are localized at synaptic and extra synaptic levels in

neurons and glia. Group I mGlu receptors are generally localized postsynaptically, surrounding ionotropic receptors, and they modulate depolarization and synaptic excitability (Spampinato et al., 2018). A correct function of metabotropic glutamate receptor 1 (mGlu1) receptor and healthy climbing fiber maturation are necessary to avoid ataxia (Hoxha et al., 2018).

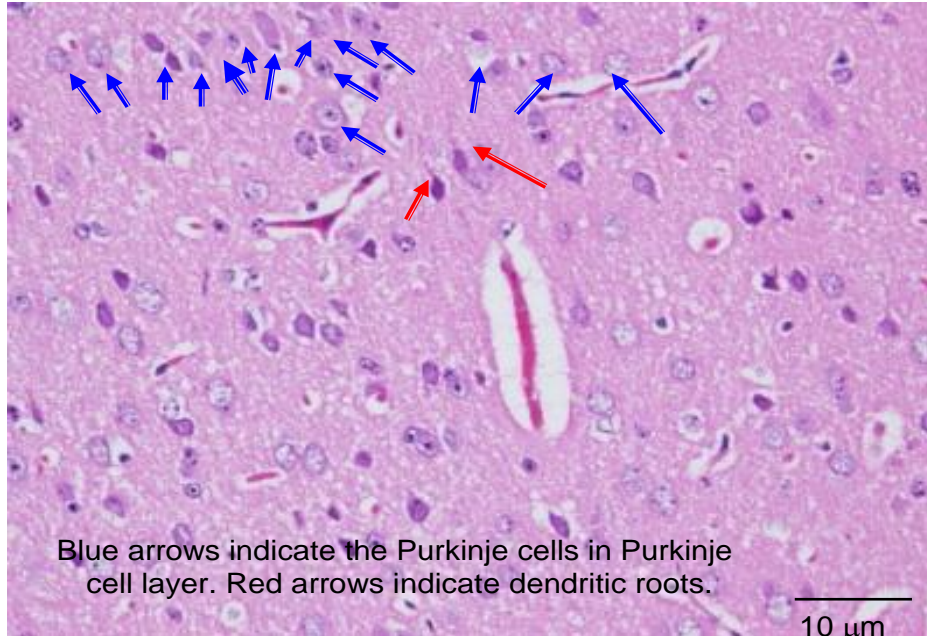
In humans, PCs can be harmed by a variety causes: toxic exposure, e.g. to alcohol or lithium; autoimmune diseases; genetic mutations causing spinocerebellar ataxias, Unverricht-Lundborg disease, or autism; and neurodegenerative diseases that are not known to have a genetic basis, such as the cerebellar type of multiple system atrophy or sporadic ataxias (Mitoma et al., 2016; Jaber, 2017).

Some domestic animals can develop a condition where the PCs begin to atrophy shortly after birth, called cerebellar abiotrophy (Axelrad et al., 2008; Lee and Lee, 2012; Scott et al., 2018), which can lead to symptoms such as ataxia, intention tremors, hyperreactivity, lack of menace reflex, stiff or high-stepping gait, apparent lack of awareness of foot position (sometimes standing or walking with a foot knuckled over), and a general inability to determine space and distance (Lee and Lee, 2012). A similar condition known as cerebellar hypoplasia occurs when PCs fail to develop *in utero* or die off before birth (Axelrad et al., 2008; Lee and Lee, 2012).

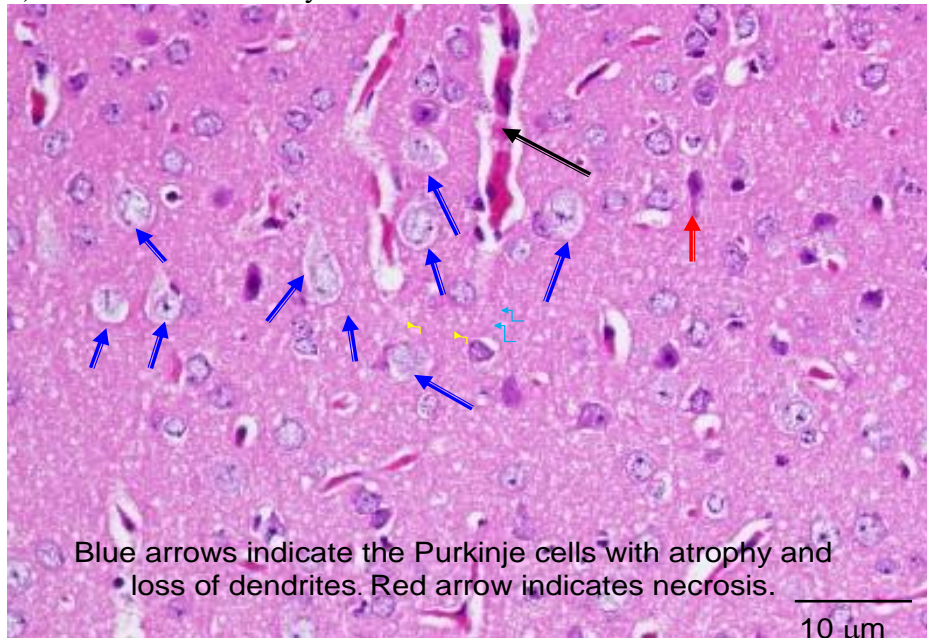
Fluoxetine hydrochloride induced various deleterious changes in the histological structure of the cerebellar cortex of albino rat offspring of treated mothers (Abo-Ouf, 2018), and altered cortical expression of multiple heat shock protein 60 forms along with neurofilaments and related proteins that are critical determinants of synaptic structure and function (Guest et al., 2004). Fluoxetine also induced degeneration and necrosis of the cerebellar cells and nerve fibers, in a dose and duration-dependent manner (Abo-Ouf, 2018), in the form of cytoplasmic vacuoles, dilated rough endoplasmic reticulum, swollen mitochondria with destructed cristae, degenerated mitochondria and nuclear changes in the form of karyolysis, pyknosis and karyorrhexis (Abo-Ouf, 2018). Also, there was decrease in the number of PCs (Abo-Ouf, 2018). The density of dendritic spine in medial spiny neurons of striatum and Layer 5 pyramidal neurons in the primary motor cortex (M1), as well the dendritic complexity, were reduced in Fluoxetine-rats (Lee and Lee, 2012). The structure and function of the motor system are affected by early Fluoxetine exposure, which might account for the poorer motor performances (Lee and Lee, 2012).

During and following pregnancy, women are at high risk of experiencing depression (Kiryanova et al., 2013). It is worth noting that the structure and function of the motor system are affected by early Fluoxetine exposure, as a consequence thereof, the long-term effects of early exposure to SSRI-type antidepressants should be concerned.

a) Normal rat cerebellum



b) Fluoxetine treated day-1 chick cerebellum

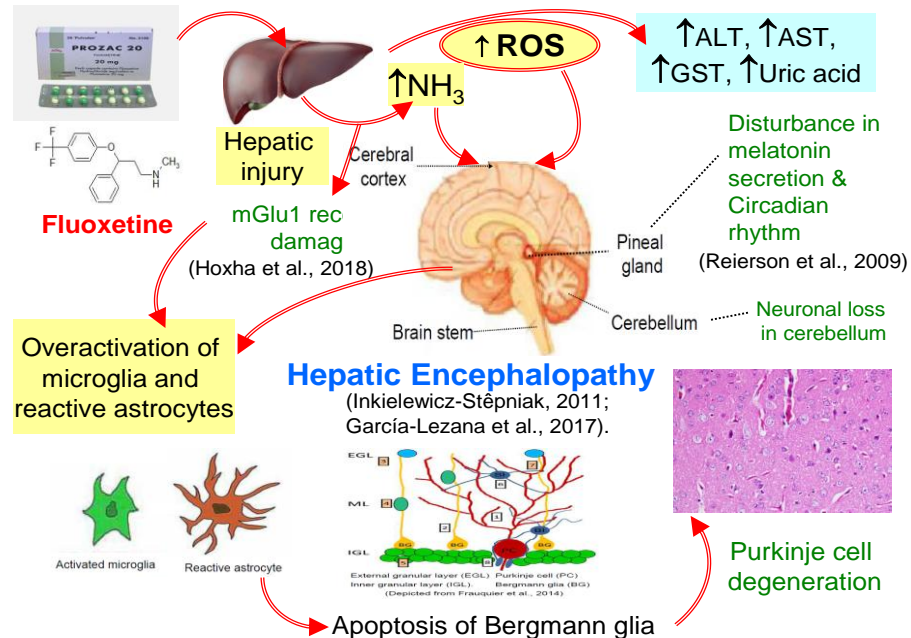


**Figure 2: The cerebella of normal and Fluoxetine-treated day-1 chicks.** (HE stain,  $\times 400$ ). a) Normal group: PCs are well located in Purkinje layer, normal sulcus with normal pia mater distribution and dense population of granule cells. b) Fluoxetine-treated group: PCs are damaged with atrophy and loss of dendrites, narrowed and elongated sulcus with reduced pia matter (black arrow), less dense of the internal granular cell population, vacuole formation in granule cells (H&E  $\times 40$ ).

### Why the population of Purkinje cells was reduced by Fluoxetine therapy?

Fluoxetine induces liver damage and mediates free radical reactions (Inkielewicz-Stêpniak, 2011). Fluoxetine treatment for 24 h increased the levels of carbonyl groups,

thiobarbituric acid reactive species (TBARS) and uric acid in the liver (Inkielewicz-Stêpniak, 2011), and simultaneously, the activities of serum alanine transaminase (ALT), aspartate transaminase (AST) and glutathione-S transferase (GST) all increased (Inkielewicz-Stêpniak, 2011).



**Figure 3: ‘Hepatic Encephalopathy’, ‘Damage of mGlu1’, and Disturbance of melatonin secretion’ are the mechanisms associated with reducing cerebellar Purkinje cells by Fluoxetine.** Fluoxetine induced hepatic injury, producing a tremendous production of reactive oxygen species (ROS), accompanied with increased activity of alanine transaminase (ALT), aspartic acid transaminase (AST), glutathione S-transferases (GSTs), and large amount of uric acid excretion. The ROS and ammonia produced injured synapses at metabotropic glutamate receptor (mGlu1) and cerebellum, eliciting neuronal loss, overactivating microglia and reactive astrocytes, inducing apoptosis of Bergmann glia and thereof the PC degeneration (H&E  $\times$ 40). mGlu1: metabotropic glutamate receptor 1.

‘Hepatic Encephalopathy’ has traditionally been considered a reversible disorder. However, recent studies suggested that repeated episodes of hepatic encephalopathy cause persistent impairment leading to neuronal loss (Inkielewicz-Stępniak, 2011; García-Lezana et al., 2017).

According to the ‘Hepatic Encephalopathy’ Hypothesis (García-Lezana et al., 2017), post Fluoxetine treatment, the generated ammonia induced reversible motor impairment, and in cerebellum, stereology showed a reduction in Purkinje cell population in portacaval anastomosis (García-Lezana et al., 2017), a modulation of neurodegeneration-related genes and the presence of apoptosis in Bergmann glia and mimic clinical course of episodic hepatic encephalopathy with ammonia as the precipitant factor were observed, demonstrating the existence of neuronal loss in cerebellum (García-Lezana et al., 2017). Further, the persistence of over-activated microglia and reactive astrocytes could participate in the apoptosis of Bergmann glia and therefore Purkinje cell degeneration (García-Lezana et al., 2017).

On the other hand, chronic Fluoxetine treatment increases daytime plasma melatonin, such circadian rhythm disturbances can occur (Reiersen et al., 2009). The pinealocytes in pineal gland is relevant to circadian rhythms as it secretes the hormone melatonin following

activation of the cyclic adenosine monophosphate (cAMP) signaling cascade and of arylalkylamine N-acetyltransferase (AA-NAT), the rate-limiting enzyme for its synthesis (Reiersen et al., 2009). Melatonin exerts a wide spectrum of activities, which include regulating circadian rhythms and sleep (Agez et al., 2009; Antonioli et al., 2012), promoting and modulating neurogenesis and immune system (Sotthibundhu et al., 2010; Maldonado et al., 2007), defending inflammation, and regulating metabolism (Sanchez-Hidalgo et al., 2007; Antonioli et al., 2012).

Based on these results, we propose that the action mechanism associated with Fluoxetine regarding the reduction of Purkinje cells (Fig. 1) may involve three relevant pathways: 1) the ‘‘Hepatic Encephalopathy’’ (García-Lezana et al., 2017); 2) damaging the synaptic receptor mGlu1 (Hoxha et al., 2018), and 3) disturbing the circadian rhythm by increasing day time plasma melatonin (Reiersen et al., 2009). The overall result is shown in Figure 2.

## Conclusion

Despite that a number of studies have shown beneficial effects of Fluoxetine, many adverse effect of Fluoxetine are emerging. Recently, the side effects emerging more and

more include sexual dysfunction, weight gain, and sleep disturbance, the most troubling adverse events seen during long-term SSRI therapy. In this study, we report the impairment of locomotor activities with poorer motor performance of day-1 chicks by Fluoxetine therapy. Histological examination (H&E stain) showed severe reduction in cell number associated with abnormally swollen and dispersed Purkinje cells after treated with Fluoxetine. The action mechanism of Fluoxetine in reducing the Purkinje cell population may be associated with the 'Hepatic Encephalopathy', the damaging of synaptic receptor mGlu1, and the disturbance of the circadian rhythm by increasing day time plasma melatonin.

Currently, the selective serotonin reuptake inhibitors (SSRIs) are often prescribed as antidepressant for treating pregnant women. Fluoxetine affects the timing of developmental stages in embryos, in fetus it induces expression and secretion of several proteins in a dose-dependent manner. For these reasons, and in line with current guidelines, the lowest possible dose of SSRI should be used in pregnant women who need to continue treatment. Conclusively, the structure and function of the motor system are affected by early Fluoxetine exposure, and it is remarkable that the long-term effects of early exposure to high dose SSRI-type antidepressants should be concerned.

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