A single Dose of 5-MeO-DMT Stimulates Adult Neurogenesis in Mouse Dentate Gyrus

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Resumo

A zona subgranular (SGZ) do giro denteado (DG) é uma das poucas regiões do cérebro em que a neurogênese é mantida na fase adulta. Acredita-se que os neurônios recém-nascidos nesta região codifiquem informações temporais sobre memórias contextuais parcialmente sobrepostas. Sabe-se que diversas moléculas são capazes de interferir com o ciclo de produção destes neurônios. A 5-metoxi-N, N-dimetiltriptamina (5-MeO-DMT) é um composto natural capaz de induzir um poderoso estado psicodélico. Recentemente, foi observado que os análogos de N,N-Dimetiltriptamina (DMT) podem ser usados no tratamento de transtornos de humor.

Devido à forte ligação entre alterações na neurogênese e transtornos do humor, testamos se a 5-MeO-DMT é capaz de aumentar a neurogênese no DG in vivo. No presente trabalho mostramos que uma única injeção intracerebroventricular de 5-MeO-DMT aumenta a proliferação celular no DG de camundongos, como evidenciado pela marcação por 5-bromo-2’-desoxiuridina (BrdU).

Além disso, utilizando um animal transgênico que expressa Cre-recombinase dependente de tamoxifeno sob controle de promotor de doublecortin (DCX) para marcar as novas células granulares do DG, observamos que estas desenvolvem morfologia dendrítica mais complexa após a 5-MeO-DMT. Além disso, as novas células granulares apresentam potenciais de hiperpolarização (AHP) mais longos e um menor limiar de disparo quando comparado ao tratamento com 5-MeO-DMT. Nossos resultados mostram que o 5-MeO-DMT afeta a neurogênese e este efeito pode contribuir para as propriedades antidepressivas conhecidas dos compostos derivados da DMT.

**Palavras Chaves:** 5-MeO-DMT, DMT, Neurogênese, Depressão, DCX
Abstract

The subgranular zone (SGZ) of dentate gyrus (DG) is one of the few brain regions in which neurogenesis is maintained throughout adulthood. It is believed that newborn neurons in this region encode temporal information about partially overlapping contextual memories. The 5-Methoxy-N,N-dimethyltryptamine (5-MeO-DMT) is a naturally occurring compound capable of inducing powerful psychedelic states. Recently, it has been suggested that N,N-Dimethyltryptamine (DMT) analogues can be used in the treatment of mood disorders. Due to the strong link between altered neurogenesis and mood disorders, we tested whether 5-MeO-DMT is capable of increasing DG neurogenesis in vivo. We show that a single intracerebroventricular injection of 5-MeO-DMT increases cell proliferation in the DG, as evinced by 5-Bromo-2'-deoxyuridine (BrdU) staining. Moreover, using a transgenic mouse that expresses tamoxifen-dependent Cre recombinase under doublecortin (DCX) promoter control, we found that newborn DG granule cells have more complex dendritic morphology after 5-MeO-DMT. Moreover, newborn granule cells display longer afterhyperpolarization potentials (AHP) and lower action potential threshold when compared to 5-MeO-DMT treated. Our findings show that 5-MeO-DMT affects neurogenesis and this effect may contribute to the known antidepressant properties of DMT-derived compounds.

Keywords: 5-MeO-DMT, Neurogenesis, Depression, DCX,
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Introduction

The neurogenesis was thought to not occur in the mammalian adult brain until the 60’s when a group of scientists found that new neurons are added to the songbird’s brain throughout life with a decaying capability thru aging. Until the 90’s scientist though that this would not occur to mammals, as the evidence was scarce, and this sounds like a “dogma” to the scientific community. After controversial times, neurogenesis was known to occur at least in two different regions of adult mammalian brain, the subventricular zone in the olfactory bulb, and in the subgranular zone of the dentate gyrus. The later being producing only one kind of neuron, the dentate gyrus granule cell, these cells are though to encode the temporal information of a engram decoded, and the young granule cells would help to integrate already established engrams with new ones, in a process called Pattern separation. The young granule cells hold a elevate excitability, and remarkably different membrane properties, which may help them to integrate a existing circuit. The 5-Meo-DMT is non-selective serotonin agonist, acting in many receptors besides the 5-ht2c a 5-ht 2A, his main targets, prolific studies has shown a possible positive effect of ayahuasca to treat remissive depression in humans, in this study we aim to looking forward to understand the molecular and cellular mechanisms of action of this enhancement, establishing comparison between classical antidepressant which may act increasing adult neurogenesis in the dentate gyrus and the yet enigmatic 5-Meo-DMT. Our results shown that a single dose of the 5-MeO-DMT compound, delivery i.c.v to adult mice act as classical antidepressants when chronically administered, since the proliferation levels right after the injections are higher to experimental groups, together with electrophysiological and morphological changes toward fully mature granule cell. These results indicate that a single dose
of 5-meO-DMT not only is capable of increasing proliferation levels, but also to increase neuronal plasticity, readily increasing the number of new neurons available to form new engrams in an adult mice dentate gyrus.

5-metoxi-N,N-dimetiltriptamine

The 5-MeO-DMT is the active ingredient of the psychedelic beverage *ayahuasca*, a millenarian decoction used by indigenous tribes to induce a powerful hallucinogen state (Araújo, Carvalho, Bastos, Guedes de Pinho, & Carvalho, 2015). That although the scientific interest in this substance began only in the last three decades, it has been used by popular culture to treat various physical and mental illnesses for centuries (Frecska, Bokor, & Winkelman, 2016b).

Psychoactive tryptamines are a class of molecules that act as serotonin agonists in the vertebrate brain (Jacob & Presti, 2005). Among the most common found in nature are: (I) N,N-Dimethyltryptamine (DMT) and the 5-methoxy-N,N-dimethyltryptamine (5-MeO-DMT), that can be found together in a great variety of plants in South America, always accompanied by a diversity of chemical analogues; (II) bufotenine found in the Colorado river frog (*Incilius alvarius*); (III) psilocybin and psilocin found in a variety of mushrooms from the gender Psilocybe, native of Andean regions in the South American continent; and (IV) the Lysergic acid amide (LSA), found in a variety of seeds such as the Argyrea nervosa, besides other plants of the genus Ipomoea that are originally endemic from the Indian subcontinent, but nowadays are worldwide spread (Araújo et al., 2015; Geyer, Nichols, & Vollenweider, 2010; Greene, 2013). Additionally, there are synthetic psychoactive tryptamines like: (V) the Lysergic acid diethylamide (LSD) (Hofmann, 1981), and (VI) The 25I-NBOMe (Zuba, Sekula, & Buczek, 2013).
5-MeO-DMT, or 5-OMe-DMT, is a serotonin (5-HT) agonists that act in a non-selective manner in those receptors (Szabo, Kovacs, Frecska, & Rajnavolgyi, 2014). 5-MeO-DMT hold higher affinity to the 5-HT\textsubscript{2A} receptor, and it is predominantly associated with the psychedelic effects, but not sufficiently to explain it, 5-HT\textsubscript{1A} and 5-HT\textsubscript{2C} might be also involved, as observed by a discriminative study using serotonin agonists and diverse triptamines (Glennon, Rosecrans, Young, & Gaines, 1979), although 5-MeO-DMT interact with many other class of receptors, e.g. ionotropic and metabotropic glutamate receptors, dopamine, acetylcholine and trace amine-associated receptor (TAAR) (Carbonaro & Gatch, 2016) and in the last decade DMT analogues was demonstrated to act also via sigma-1 receptors, the effects on sigma-1 still been debated (Koornneef et al., 2009).

There is a number of studies regarding the non-selective serotonin action of tryptamines, focusing on the psychedelic effects of this compounds (Frecska et al., 2016b). Also, it has been shown that mammals can synthetize psychedelic tryptamines, such as bufotenine and DMT, in their pulmonary alveolar cells using tryptophan as a precursor, in a similar pathway in which serotonin is synthesized in these animals brains (Axelrod, 1961). More recent evidence point out to another synthesis pathway by the pineal gland (S. A. Barker, Borjigin, Lomnicka, & Strassman, 2013). In the last decade, it was observed that the tryptamines Diisopropyltryptamine (DIPT) and 5-methoxy-N-methyl-N-isopropyltryptamine (5-MeO-MIPT) hold higher binding-to-uptake ratios, when compared to serotonin. This occur in at least two different classes of transporters responsible for the transporter-mediated reuptake: the serotonin transporter (SERT), and the vesicular monoamine transporter 2 (VMAT2), which indicates that DMT analogues can be more used locally as neurotransmitters than previously thought (Cozzi et al., 2009). Many authors associate high bloodstream levels of psychedelic tryptamines in psychiatric conditions
Entheogenic substances, *i.e.* substances capable to induce powerful non-ordinary conscious states, are difficult to study in humans because of their subjective effects, since this studies rely in self-reports of the subject under experiments, and the person is strongly prone to bias via the placebo effect (Benedetti, Mayberg, Wager, Stohler, & Zubieta, 2005). For a proper characterization of the effect of entheogenic substances, it is necessary to apply discrimination studies, which consists of giving to the subjects two samples, a control one and the study component. To look for behavioral cues displayed by the subjects and ignore the subjective effects (Colpaert, 1999), in an experimental study, for example, rats were trained to discriminate between two samples, one containing 5-MeO-DMT and one containing a sham solution. With this approach, animals were successful in discriminating 5-MeO-DMT more than 70% of the time. On the other hand, when the drug were administrated together with serotonin antagonists receptors 5-HT$_{2A}$ and 5-HT$_{2C}$, the success ratio falls to chance, which indicates that the psychedelic effects associated with this drug is mainly due to the action on those two receptors. These results are similar for tryptamines 5-MeO-MIPT, DMT, mescaline and LSD (Appel & Callahan, 1989; Cunningham & Appel, 1987; Glennon et al., 1979; Glennon, Titeler, & McKenney, 1984).

Regarding toxicological effects, the psychedelic tryptamines are less toxic than methamphetamines, with far less adverse side effects, except the blood pressure increase presented only when administered at high doses via intravenous injections (Shen, Jiang, Winter, & Yu, 2010). The chronic or acute ingestion of *ayahuasca* does not seem to have any negative effect to mental or physical health (Barbosa, Mizumoto, Bogenschutz, & Strassman, 2012),
though in the tea form it can provoke strong nausea and vomit, which can be harmful to gastrointestinal system. These effects are strongly associated to others nonpsychoactive components, such as the tannins compounds, present in the gross preparation. Those are very alkaline substances, and can be found concentrated in leaves, twigs and bark of plants to restrain herbivory (Hagerman, 2002) – therefore, the negative gastrointestinal effects can be prevented by not ingesting these compounds, purifying the brew before consumption. Despite the negative effects mentioned, an increasing body of publications has been produced showing many positive effects of the continued use of *ayahuasca*, for example: the reduction of aggressive behavior (Frecska, 2008), immunotherapeutic effect (Szabo, 2015), reducing symptoms of depression (Sanches et al., 2016), treating psychosis and reducing chronic inflammatory process (Frecska, Bokor, & Winkelman, 2016a) and even cancer treatment (Schenberg, 2013).

**Hippocampus**

The hippocampus is delimited into 3 sub regions, morphologically and functionally different, the dentate gyrus (DG), cornus ammonis 1 (CA1) and cornus ammonis 3 (CA3) (Figure 1). The distinct delimited layer organization of the cellular types in hippocampus made this structure suitable for cellular studies aimed for characterize different types of excitatory neurons and even more to interneurons (Kandel, Schwartz, Jessell, Siegelbaum, & Hudspeth, 2000). The glutamatergic neurons of CA1 and CA3 are collectively called pyramidal cells recalling their cell body shape, while the excitatory cells of DG are called granule cells recalling their elliptical cell body (Kullmann, 2011). The majority of glutamatergic connections in the hippocampus are unidirectional (Amaral, Scharfman, & Lavenex, 2007; Andersen, Morris, Amaral, Bliss, & O’ Keefe, 2009). The resulting circuit of this three structures are called trisynaptic loop, very suitable for paired recordings, the classical long term potentiation (LTP)
and long term depression (LTD) but also other types of potentiation or depression in activity, which are the electrochemical components necessary for declarative memory encoding (Amaral et al., 2007; Andersen et al., 2009; Kandel et al., 2000). The well delimited cell population, the delimited laminar organization, together with an increased plasticity associated to his trisynaptic loop, made the hippocampus the focus of many cellular and electrophysiological studies.

**Figure 1: Horizontal hippocampal section.** Nucleic acids were colored with Nissl stain showing the well delimited cellular organization of the hippocampus (modified from George Paxinos, 2012).

Due to its functional complexity, there are significant variations of hippocampus morphology and functional organization among species (Strange, Witter, Lein, & Moser, 2014). The hippocampus functional subdivision, in a simplified manner, is subdivided into: (I) dorsal hippocampus (posterior in primates), historically associated to spatial memory and spatial navigation (Ainge, Van Der Meer, Langston, & Wood, 2007; Cells, Cells, Moser, Rowland, & Moser, 2015; Nakazawa et al., 2002; Tsien, Huerta, & Tonegawa, 1996); and (II) ventral hippocampus (anterior in primates), which is linked to anxiety behavior, stress and emotional
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learning and conditioning (Fanselow & Dong, 2010; Likhtik, Stujenske, Topiwala, Harris, & Gordon, 2014; Strange et al., 2014; Weeden, Roberts, Kamm, & Kesner, 2015). Furthermore, there is some authors which consider the existence of a third region, the intermediate axis, though this vision is emergent and not adopted by most of the scientific community. Although the entire hippocampus is necessary to the encoding of new declarative memories, with subsets of roles for each sub region in the entire process (Shapiro & Olton, 1994), neurogenesis was found only in the dentate gyrus, specifically in the SGZ as will be further detailed. For this reason, we are going to focus on DG from now on.

The DG has a U shape, and receive most of its projections from the entorhinal cortex, through the perforant pathway (PP). The majority of cortical projections make the first synaptic contact directly with the DG granule cells, being the first synapse of the hippocampus trisynaptic loop, which acts as the main target for sensory information relayed through entorhinal cortex. The second step from the hippocampus trisynaptic loop is made between the DG granule cells and the pyramidal cells from CA3, and the projections are collectively called mossy fibers (MF) (Duvernoy, Cattin, & Risold, 2013). The DG cellular organization is laminar, containing three layers: (I) the middle layer, where is located the cell body of granule neurons and some interneurons; (II) the upper layer, where the dendrites of granule cells and axon terminals from PP are located, together with some interneurons; (III) and the lower layer, called hilus (also called polymorphic layer), where there is only a few interneurons, although numerous enigmatic glutamatergic mossy cells (Scharfman, 2016) and mossy fibers projections (Andersen et al., 2009).

The DG local circuit is comprised by granule cells receiving, through its proximal dendrites, projections from the medial perforant path (MPP), and the distal dendrites receiving
projections from the lateral perforant path (LPP). The majority of DG axon terminals end in CA3 pyramidal cells, although there are some local projections to the hilus. Feedback inhibitory circuits are mostly comprised by two classes of interneurons: basket cells and somatostatin positive interneurons (SOM+). Granule cells projections can also target glutamatergic mossy cell, which project back to granule cell layer (GCL). These 3 neuronal subtypes located in the hilus target GCL differentially, and the synapse contact are: basket cell → granule cells soma; SOM+ → granule cells medial dendrites; mossy cells → granule cells proximal dendrites. The granule cells connections to pyramidal cells are exclusively unilateral and targeting the soma and some interneurons in the CA3 (Figure 2) (Amaral et al., 2007).

Among the distal modulatory projections represented in the Figure 2 are: the cholinergic from the medial septum; the GABAergic from the supramammilars nucleus (SUM); the noradrenergic from the locus coeruleus; the dopaminergic from the ventral tegmental area (VTA); and the serotoninergic from the raphe nucleus. The acetylcholine (ACh) delivered from cholinergic projections target mostly inhibitory neurons within the hippocampus, and has been described that its ablation can disrupt the hippocampal theta rhythm (Colgin, 2016). The distal inhibitory neurons coming from the SUM form a plexus around the soma of granule cells (Maglóczky, Acsády, & Freund, 1994). The noradrenergic fibers (NA) target the hilus and also, in less extent, the granule cells (Oleskevich, Descaries, & Lacaille, 1989). The dopaminergic projections (DA), ends in the hilus, without any clear target unlike the cholinergic ones (Ntamati & Lüscher, 2016). Serotoninergic projections (SER) have two major targets: (I) approximately 30% form synaptic buttons directly with interneurons (SOM−, Calb2−, Calb−, NPY+) in their distal dendrites or perisomal regions, the same interneurons which are target from granule cells; (II) the
other 70% of the raphe nucleus projections ends in the hilus without a specific cell as target (Daszuta et al., 1991; Oleskevich, Descarries, Watkins, Séguéla, & Daszuta, 1991).
The pattern separation is the behavioral output of DG granule cells encoding function in memory formation. Everyone has experienced some sensory cues, as a smell, or a sound, which is capable of evoke a memory of something specific, or even an entire day. This process is a simplification of what pattern recognition means to the brain circuitry, being this an ability accounted to the hippocampus (Yassa & Stark, 2011). It has been decades since the hypothesis of this hippocampus capability was developed, and since then, subpopulations are known to be directly responsible for fragments of the entire process (Squire, 2009). Two different events are essential to pattern recognition: (I) pattern separation and (II) pattern completion (Figure 3).

Evidence of these two process has also emerged from computational studies modeling a single cell, local circuitry simulations and models comprising an entire network (McClelland, McNaughton, & O’Reilly, 1995; McNaughton & Morris, 1987; Norman & O’Reilly, 2003; O’Reilly & Norman, 2002; Rolls, 2007, 2013; Shapiro & Olton, 1994; Yassa & Stark, 2011).

DG granule cells connect directly to upper dendrites from CA3 pyramidal cells through dense unidirectional fibers called the mossy fibers (MF). Projections from layer 2 entorhinal cortex arrive in all 3 sub regions of the hippocampus via PP. The strong DG granule cells connections to CA3 lead Rolls to theorize that: DG → CA3 projections (via MF) suits as a
reinforcement to create new representations in CA3, thus allowing new engrams to be formed, through pattern separation. On the other hand, the less numerous EC→CA3 projections (transmitted via PP) are facilitators to “remember” established engrams from sensory inputs, through pattern completion, strongly reducing DG granule cells participation for that engram (Rolls, 2007, 2013). While this idea may sound like speculation, an increasing body of publications corroborates this general idea (James B. Aimone, Deng, & Gage, 2010b; Burghardt, Fenton, & Dranovsky, 2011; Lassalle, Bataille, & Halley, 2000; I. Lee & Kesner, 2004; Yassa & Stark, 2011).
Below the granular zone, there is a thin layer before the hilus where is located SGZ, one of the few regions of the brain where adult neurogenesis can happen in mammals (Altman & Das, 1965). The production of new neurons is pointed as necessary to the maintenance of healthy hippocampal function (Saxe et al., 2006). In mice, electrophysiology assays associated to mRNA analysis for activity-regulated cytoskeleton-associated protein (Arc) showed that, the number of activated neurons are less than expected to subgroups of granule cells representing different experimental contexts in pattern separation tests, nonetheless is proportional to the number of new neurons produced. This information lead the authors to conclude that mature granule cells (mGC), the granule cells that have already completed the maturation process, are excluded from the new information storage (Alme et al., 2010; Ramirez-Amaya, Marrone, Gage, Worley, & Barnes, 2006). An alternative hypothesis is that mGC are able to encode new information about an old environment, and the adult born granule cells (abGC) would be responsible for the new or partially new contexts, whereas following 3 months after their maturation they have increased potentiation capabilities when compared to mGC (J. B. Aimone et al., 2014; James B. Aimone et al., 2010b; S. Wang, Scott, & Wojtowicz, 2000).
Granule cells of the DG possess different properties along the maturation process (James B. Aimone, Deng, & Gage, 2010a; Toni, Toni, & Schinder, 2015), though is an overall assumption that immature granule cells exhibit a more excitable profile, with associated passive and active properties summarized in Table 1. Newly generated granule cells positive for polysialic acid neural cell adhesion molecule (PSA-NCAM+), a glycoprotein associated to neurite outgrowth (Seki, 2002), possess membrane properties prone to plasticity at their glutamatergic input synapses. Young granule cells hold a higher input resistance and expression of low threshold Ca\(^{2+}\) influx channels (T-type Ca\(^{2+}\) channels), which promotes LTP, facilitating action potential (AP) generation by the yet sparse glutamatergic pre-synaptic inputs. Thus, rising the Ca\(^{2+}\) and Na\(^{+}\) concentrations may efficiently induce LTP by generating both increase in newborn granule cell cytoplasmic Ca\(^{2+}\) and maximal relief of Mg\(^{2+}\) block of NMDA-type glutamate receptors (Schmidt-Hieber, Jonas, & Bischofberger, 2004).

Table 1

**Passive and active properties between granule cells at different developmental stages.**

<table>
<thead>
<tr>
<th>Passive membrane properties</th>
<th>Adult born Granule cell (abGC)</th>
<th>Mature Granule cell (mGC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input Resistance (Ω)</td>
<td>4.5 +/- 1.9 GΩ</td>
<td>232 +/- 78 MΩ</td>
</tr>
<tr>
<td>Baseline (mV)</td>
<td>-75.3 +/- 2.0</td>
<td>-80.8 +/- 0.9</td>
</tr>
<tr>
<td>Firing pattern</td>
<td>Single Fire</td>
<td>Higher than 30Hz</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Action Potential Properties</th>
<th>Adult born Granule cell (abGC)</th>
<th>Mature Granule cell (mGC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+) Peak Amplitude (mV)</td>
<td>115 +/- 2</td>
<td>140 +/- 2</td>
</tr>
<tr>
<td>AP Threshold after 100ms pulse (pA)</td>
<td>34 +/- 9</td>
<td>141 +/- 12</td>
</tr>
<tr>
<td>Membrane Time constant (\tau_m) (ms)</td>
<td>123 +/- 10</td>
<td>54 +/- 4</td>
</tr>
</tbody>
</table>

*For Comparison purpose, PSA-NCAM+ granule cells (AbGC, young granule cells) exhibit a characteristic electrophysiological behavior, when compared to PSA NCAM- granule cells (mGC, full growth granule cells) (Schmidt-Hieber et al., 2004)*

Considering the previous cited findings, it is likely that to promote neuron integration in a given hippocampal circuit (Shors et al., 2001), it is necessary that the young granule cell
establish a facilitated contact with an already mature, and connected neuron. As Donald O. Hebb postulated, “neurons that fire together wire together” (Hebb, 1949), so, sensory elements (e.g. present in a enriched environment) are capable of induce hippocampal LTP (Gould, Beylin, Tanapat, Reeves, & Shors, 1999) and consequently will increase the survivability of newborn granule cells by “wiring” them to a working circuit (Pattern separation). Once newborn granule cells reach maturity (which occur 28 days after being born) (J. B. Aimone et al., 2014) they display a three month window in which they exhibit major LTP capacity (Lopez-Rojas & Kreutz, 2016), becoming indistinguishable from older granule cells (Laplagne et al., 2006). In addition, their passive and active membrane properties, morphology and biochemical profile do not differ from other adult granule cells (Esposito et al., 2005).

**Adult Neurogenesis**

Adult neurogenesis occur in all vertebrate taxa (J. M. Barker, Boonstra, & Wojtowicz, 2011). In fact, only the mammals and birds seem to hold a limited neurogenic capacity in the adulthood (Drew, Fusi, & Hen, 2013; LaDage, Roth, Fox, & Pravosudov, 2010). New neurons are continually generated in the mammalian brain (Lledo, Alonso, & M, 2006), though this process is limited to two areas: the subventricular zone (SVZ), which produces excitatory and inhibitory neurons, and the subgranular zone (SGZ) in the DG, which produces only excitatory neurons (granule cells). Newborn SVZ cells migrate to olfactory bulb (Kaplan & Hinds, 1977), and this neurogenesis process is higher for mammals that rely on smell as main sense, like murine animals, when compared to primates for example (Bordiuk, Smith, Morin, & Semënov, 2014; C Zhao, Deng, & Gage, 2008). There are many differences between the neurogenic process in those regions, and because of this, I will focus in the newborn granule cells in the SGZ.
Neurogenesis in the dentate gyrus. Many theories emerged in the last decades to explain the role of SGZ newborn neurons in the adulthood, the so-called adult born granule cells abGC. Many scholars support the idea that the neurons born in the developmental stages of the brain, the mGC, would ‘retire’ at some stage, and then be replaced by abGC (Alme et al., 2010). Some authors also point out other hypothesis that is not completely different from the previous, but indeed complementary, hypothesizing that newborn neurons can act as pattern integrators of temporary adjacent events, enhancing the pattern separation (James B. Aimone et al., 2010a).

Apart of their function, an increasing body of studies suggests a strong relationship between the proliferation and survivability of those newborn neurons with some pathologic conditions (Lledo et al., 2006; Chunmei Zhao, Deng, & Gage, 2008), leading many to believe that the neurogenesis process is not only important for academic purposes, but also for clinical ones.

Granule cells development is composed by different stages, until the maturation process is completed. In the first stage, the pluripotent cells radial glial like cells (RGL), derived from ectodermic embryonic stem cells (C. Zhao, 2006) give origin to morphological intermediate stages distinct from their progenitors, the D-Cells. The following process is the D-cells to become immature neurons, as shown by many undifferentiated embryonic stem cell markers, as doublecortin (DCX), neuroectodermal stem cell marker (Nestin) , the common antigen for neuron hexaribonucleotide binding protein-3 (known as NeuN) and sex determining region Y-box 2 (SoX2) (Canales, 2016). The DCX marker is used in this study to tag newborn neurons, therefore it will be explained with more details in the next topic. The experimental use of thymidine analogues as the 5-Bromo-2’-deoxyuridine (BrdU) to mark newborn cells, show that most of these cells die before participate of any neural circuit (Dayer, Ford, Cleaver, Yassaee, & Cameron, 2003). Thus, the integration of newborn cells is just a subset of the dynamic
To address some of the intrinsic factors relevant for this study, it is worth to mention that the activation of 5-HT$_{1A}$ receptors consequential from selective serotonin reuptake inhibitors (SSRI) action, as fluoxetine (a classical antidepressant), increase cell proliferation levels directly (Malberg, Eisch, Nestler, & Duman, 2000). The projections from the dorsal raphe nucleus, the source of all 5-HT in the central nervous system, seems to release serotonin directly to SGZ, not targeting any cell specifically (Oleskevich et al., 1991). This raises the idea that these projections may directly influence the neurogenesis process, acting not as a neurotransmitter, but as a chemical agent (Kosofsky & Molliver, 1987). As extrinsic factors, an enriched environment, dietary restrictions and voluntary exercise can dramatically increase neurogenesis (Duman, Nakagawa, & Malberg, 2001; J. Lee, Duan, Long, Ingram, & Mattson, 2000; Nurk et al., 2008; Oh et al., 2010; Olah et al., 2009; van Praag, Kempermann, & Gage, 1999). Rodents with higher neurogenesis levels tend to score better in pattern recognition cognitive tests (J. M. Barker et al., 2011). Together, these discoveries support the general idea that neurogenesis can be responsible for the phenotypic changes induced by selective pressure in the course of evolution in mammals, although more evidence is necessary to clarify this hypothesis (Drew et al., 2013).

Just as some factors may contribute positively, there are also factors that act in a reverse manner, reducing neurogenesis, such as: ionizing radiation, chronic stress and sleep deprivation (Guzmán-Marín et al., 2003; Peissner, Kocher, Treuer, & Gillardon, 1999; Pham, Nacher, Hof, & McEwen, 2003). Some factors can act in both ways, such as the hormones ghrelin, oxytocin, estrogen and glucocorticoids in a concentration dependent manner (Cameron & Gould, 1994; Gould, Tanapat, & McEwen, 1997; Johansson et al., 2008; Leuner, Caponiti, & Gould, 2012;
Tanapat, Hastings, Reeves, & Gould, 1999). The consequent cell death, if the cell not integrate any circuit, occur due to proapoptotic signals and absence of neurotrophins (Linnarsson, Willson, & Ernfors, 2000).

As mentioned before, many proteins are expressed during cell development that can be used as neuronal fate biochemical markers. The DCX protein, a marker of neuronal commitment, is a microtubule associated protein expressed in migrating neurons, at both the embryonic and adult stages. In adulthood, DCX expression in brain tissue is restricted to SVZ and SGZ. In the SGZ, during a transient period that starts early after proliferation phase at the end of the cell-cycle, reaching its peak at day 7 and slowly decaying until the day 21 after cell division (Figure 4) (Brown et al., 2003; Christie & Cameron, 2006; Lledo et al., 2006; J. Zhang et al., 2010). The DCX protein targeting is widely accepted as a neuronal fate marker and for this reason a transgenic CreER\(^{T2}\) mice model has been reported (J. Zhang et al., 2010), reviewed and used by several laboratories (Garrett et al., 2015; Imayoshi, Sakamoto, & Kageyama, 2011; Wu, Sahay, Duman, & Hen, 2015; L. Zhang, Hernández, Estrada, & Luján, 2014). Double transgenic mice DCX-CreER\(^{T2}/tdTOM^{lox+/lox}\) (see session 1.4 for more details) were used in this study to target newborn neurons produced while the 5-MeO-DMT was in the brain.
Figure 4: Time course of doublecortin (DCX) expression in newly generated cells of the adult dentate gyrus. Changes in colabeling of bromodeoxyuridine (BrdU) with DCX and nuclear neuronal marker NeuN in the dentate gyrus granule cell layer over a period of 180 days after injecting BrdU into 2-month-old rats. The time course depicts the percentage of BrdU+ cells colabeling for DCX only (green diamonds), NeuN only (red squares), or both (yellow circles). The data are presented as the percentages of BrdU+ cells for each time point after BrdU injection (Modified from Brown et al., 2003)
The neurogenic hypothesis for depression. The theory regarding the role of neurogenesis in depression emerged early in the third millennium, when many researchers started to look over the function of new neuronal cells growing in the adult brain. As the researchers continues to investigate, many found that stress and anxiety negatively affect neurogenesis in the hippocampi of many species (Jacobs, van Praag, & Gage, 2000). For this matter, laboratories started to test the widely used antidepressants fluoxetine and imipramine (both Serotonin Reuptake Transporter inhibitors) to clarify if they were able to: increase neurogenesis (Santarelli et al., 2003); recover synaptic plasticity (J.-W. Wang, David, Monckton, Battaglia, & Hen, 2008); increase cell growth (Encinas, Vaahtokari, & Enikolopov, 2006; Komlósi et al., 2012). The antidepressant improvement in diagnosed depressed patients starts only one month after the beginning of the treatment – exactly the time that SGZ newborn neurons take to be mature and fully integrated into hippocampal circuit (Bordiuk et al., 2014; Canales, 2016; Palhano-Fontes et al., 2017). Many behavioral experiments had been performed in animal models of depression, in which the recovery of the normal neurogenic process, due to antidepressant treatment or any another neurogenic enhancer factor, is able to reduce depression symptoms (Duman et al., 2001; Hill, Sahay, & Hen, 2015; Lledo et al., 2006; Noto et al., 2016; Perera et al., 2007; Sahay & Hen, 2007).

A common test to measure anxiety is the novelty suppressed feeding behavior (NSFB), where experimental animals are put in a context completely new, in this test, an appealing food is placed the middle of the arena, thus anxious animals will forage less when compared to control. Depressed animals are prone to score worse, and antidepressants or other anxiolytic drugs are capable of increase animal score through reducing anxiety levels. Although the reduction of neurogenesis per se in rodent models is not enough to reduce the scores in both NSFB test or
pattern recognition directed tests (Perera et al., 2007; Santarelli et al., 2003), when neurogenesis is ablated by focused x-rays treatment and the antidepressant intake continues, depressed animals still score equally in both tests (Santarelli et al., 2003; Alexandre Surget et al., 2008). These results argue in favor that the reduction of newborn neurons cannot be responsible for the symptoms of depression, though neurogenesis increase can somehow recover the subject from that state, as discussed in many reviews in the last decade (J. B. Aimone et al., 2014; Lau, Lee, & So, 2013; Lledo et al., 2006; Miller & Hen, 2015; Numan, 2015; Petrik, Lagace, & Eisch, 2012).

The clinical interest in this present study relies in the strong correlation between antidepressants and neurogenesis (Duman et al., 2001; Noto et al., 2016; Perera et al., 2007; Sahay & Hen, 2007). The DG newborn neurons take 28 days to reach the adult phase, where they cannot be distinguished from another granule neurons (Canales, 2016). The increased excitability exhibited by new granule cells produced in the DG, qualifies these neurons as the necessary substrate to the imipramine and fluoxetine action, both SSRI agents, observed in patients and murine models of depression (Sahay & Hen, 2007). This argument is also valid to non-serotonin agents, as the synthetic cannabinoid HU210 (Jiang et al., 2005). The effect of antidepressants in the neurogenesis is probably dependent of genetic background, since there is evidence that in BALB/c mice, a highly anxious mice lineage, the ablation of neurogenesis is not sufficient to remove the effects of antidepressant and anxiolytics as was for C57/BL6J mice and rats (Holick, Lee, Hen, & Dulawa, 2008). The same is also true for others neurogenesis enhancer factors, e.g. enriched environment or exercise, for Balb/c mice. Taken together these findings is clear that antidepressants can act through neurogenesis dependent pathways and neurogenesis independent pathways as revised elsewhere (Hanson, Owens, & Nemeroff, 2011).
**Transgenic animals**

Cre+ animals are a widely used tools in biological science since this description approximately 35 years ago in a group effort from Sternberg, Hamilton and Abremski (Hamilton & Abremski, 1984; Sternberg, 1981; Sternberg & Hamilton, 1981). This technique allow the experimenter to visualize differentially spatial gene expression within a cell population through cross breeding the transgenic lineages Cre+ x reportlox/+lox+. The Cre-lox system is based in the activity of the Cre recombinase enzyme (Causes Recombination) with a topoisomerase-like catalysis mechanism, that allows specific recombination action. which recognizes a palindrome sequence of nucleotides collectively called loxP site, the palindrome sequence is a mirrored sequence normally 13 bases long that flank a gene of interest and form a “target” in the DNA for Cre, the loxP. When the DNA is open Cre forms a “clip” conformation and the Cre enzyme recognize this clip as their cleavage site removing with the LoxP anything between them (Van Duyne, 2015).

The enzyme CreERT2 is a mutated version of Cre build in the late 90’s that consist of a normal Cre recombinase fused to a modified estrogen receptor that can only access the nucleus when bound to a metabolite of tamoxifen (OHT,4-hydroxytamoxifen, which correspond to T2) which is not naturally available to mammalian cells. When the tamoxifen is administered by the experimenter, and partially metabolized by host cells, the Cre recombinase become finally complete and allowed by cell machinery to transit from the cytoplasm to nucleus, where the enzyme can find the loxP sequence. This allows control of the specific time when the recombination will occur (Feil, Valtcheva, & Feil, 2009).

In 2010 a group of researcher in the institute of developmental genetics of Helmholtz Association of German Research Centers, developed a genetic tool to mark newborn neurons
after the proliferation phase, a transgenic mice was reported expressing CreER\textsuperscript{T2} under the dominion of a DCX gene (see section 1.2 for more detailed information about DCX gene) (J. Zhang et al., 2010), this protein is only expressed in the brain at the adult phase, in migrating neurons between 1 and 21 days after proliferation (Brown et al., 2003; J. Zhang et al., 2010) the researchers tested the animal for different dosages of tamoxifen, and established one injection of 100µg/g (0.1mg/kg) for 3 days is enough to activate Cre activity (J. Zhang et al., 2010) reducing the toxic effect of tamoxifen.

Reporter animals as the GFP\textsuperscript{loxp/loxp} or the tdTomato\textsuperscript{loxp/loxp} consist in animals with a gene encoding a fluorescent protein, under the domain of ubiquitous promoter as the Rosa26 (Soriano, 1999), the expression of this protein is blocked by a stop sequence for example, or the sequence of the gene is inverted. Once the Cre enzyme found the loxP sequence the gene encoding the fluorescent protein can be normally transcript and then the cell will be continually express it (Sauer, 1998).

In this study neil3-KO\textsuperscript{−/−} animals were used. These animals are a knock out (KO) model for the Nei endonuclease VIII-like 3 enzyme (Neil3), the Neil3 is a glycosylase from the family Neil responsible for the first step in the DNA repair for single base excision for oxidative lesion, the most common type of lesion in eukaryotes (Liu, Doublié, & Wallace, 2013). These animals were produced by our collaborator Prof. Magnar Björas at Oslo University Hospital in Norway. Homozygous KO animals express a phenotype in which reactive neurogenesis (recruitment of new neurons that follow stress or neuronal damage for ischemia for example) is lacking (Regnell et al., 2012). Was observed in our laboratory through patch clamp experiments, and subsequent reconstruction of neuronal morphology using the bioitin-streptavidin reaction (Dundas, Demont, & Park, 2013) that these animals have granule cells with less diverse dendritic trees.
and different passive and active membrane properties (Soares, 2016, Data not published), what make them excellent model to investigate the effect of 5-MeO-DMT to intrinsic reduced neurogenesis context.

**Methods and Materials**

**Animals and ethic statement**

Adult C57BL6J, neil3 and DCX-CreERT²:: tdTom^{lox/lox} transgenic (R. N. Leão, Mikulovic, Leão, & Munguba, 2013; J. Zhang et al., 2010) mice from both sex aged between 55-70 days were used in this study. Animals were housed under a 12h light/12 hours dark cycle. Food and water *ad libitum*. Neil3-KO mice was generated at the Oslo University Hospital by substituting DNA-binding 15 domain H2TH exons 3–5, harboring the sequence encoding by a neomycin-resistance cassette (Sejersted et al., 2011). DNA segments were amplified by PCR, linearized by the NotI enzyme and electroporated into 129/SvJ ES cells. A positive clone was identified by PCR and the ES cell containing it was injected into C57BL6J blastocysts (Regnell et al., 2012). Genotyping of neil3-KO mice was performed with the following primers: Wild-type forward: CTTGTTTTCCACCACAATCTG, neil3-KO forward: GCCTCTGTTCACATACACTTCAT and WT/KO reverse: GTGGGCTGAAATTACACAAACAAT. The conventional PCR was performed in a Thermocycler Vapo-protect from Eppendorf machine using 2 μL of DNA in 18 μL solution (2.5 μL 10 x Buffer (s/ MgCl2), 2.5 μL MgCl2 (50mM), 0.5 μL dNTPs (10mM), 0.5 μL Primer WT-FW, 0.5 μL Primer KO, 1.0 μL Primer WT-RV, 10.32 μL H2O DNAse e RNAse free, 0.18 μL Taq polymerase from Sigma) in a 35 cycles program (94°C - 2 min; 35x 94°C - 10 sec, 62°C - 20 sec, 72°C - 30 sec; 72°C - 5 min). To assess the PCR products (Wild Type product: 280 bp and Neil-3 KO product: 160 bp) the products were electrophoresed on 1% agarose gel and 0.01% of gel red Nucleic Acid Stain 10,000x in water (1/10,000) from
BIOTIUM. All experiments were performed according to the guidelines established by National Council for the Control of Animal Experimentation (CONCEA) and approved by the local animal care institution from the Federal University of Rio Grande do Norte (Protocols 041/2014 and 015.004/2017 annexed in the end of this document).

5-MeO-DMT Treatment

Animals anesthetized with isoflurane (3-5% L/min for induction and 1-3% L/min for maintenance) (Gargiulo et al., 2012) received a single intracerebroventricular (i.c.v) injection of 1µL 5-MeO-DMT solution (100µg 5-MeO-DMT in 10% DMSO/90% saline) prepared fresh (Commissaris & Davis, 1982; Galvao et al., 2014) control groups received 1µL of 10% DMSO in saline (stereotaxic coordinates: 0.3 mm AP, 1.0 mm ML and 2.8 mm DV) (DeVos & Miller, 2013).

BrdU labeling, tamoxifen treatment, cryopreservation and slicing

After 5-MeO-DMT or saline i.c.v. injections, animals (under anesthesia) received 50 mg/kg of BrdU (Sigma) in saline intraperitoneally (i.p.). For proliferation assays mice were sacrificed (120mg/kg ketamine mixed 8/mg/kg xylazine vehicle saline) and perfused with 7.3 pH 10mM PBS followed by paraformaldehyde (PFA) 4% 12 hours after BrdU injection. To induce recombination in DCX-CreER^{T2}::tdTom^{lox/lox} animals were treated 100µg/g/day of tamoxifen i.p. three days after i.c.v. injections. These animals were either perfused for histology or anesthetized and had the brains removed for patch clamp experiments (see below). For histology experiments, brains from PFA perfused animals were removed and postfixed in 4% PFA overnight. Brains were then washed in PB 0.1M (pH=7.4) for 10 minutes then immersed in graded sucrose solutions (10/20/30%) for cryopreservation, then snap frozen by immersion into -80 °C isopropyl alcohol and stored in -80 °C freezer for posterior cryosectioning. The brains were cut in
horizontal sections of 40µm thickness using a cryostat (Thermo Microm HM 550 Cryostat®), slices spaced by 200µm were placed in polarized glass slides (Starfrost®Plus) until the whole hippocampus were sampled (2.68 mm in accordance with George Paxinos 2012) then conducted to immunohistochemistry.

**BrdU Immunohistochemistry**

Hippocampal slices were washed with PBS (pH=7.4) for 10 minutes at room temperature (RT), then placed for 30 minutes into HCl 2N at 37 °C, washed again in PBS, transferred to borate buffer (pH=8.0) at RT for 20 minutes, then washed in PBS and incubated overnight in primary antibody solution: 10% normal goat serum (NGS) (Sigma), 1:500 Rat IgG anti-BrdU (Abcam) and 0.3% X-100 triton in PBS solution (Sigma). Slices were then washed in PBS for 10 minutes and incubated for two hours in secondary antibody solution: 10% NGS, 1:1000 rabbit IgG anti-rat rabbit F(ab')2 Anti-Rat IgG H&L conjugated with Alexa Fluor® 488 (Abcam) and 0.3% triton in PBS. Slices were subsequently washed with PBS solution and incubated in 1:2000 Hoechst 33425 (ThermoFisher) in PBS 10mM (nuclei staining), washed in PBS and mounted on N-propyl gallate solution mounting medium. Hippocampal slices were imaged using an epifluorescence upright microscope (ZEISS) with Stereoinvestigator software (MBF Bioscience), BrdU+ cells were manually counted in both hippocampi by an experimenter blinded for groups.

**Analysis of BrdU+ cells**

After microscopy, images were processed by a personal MATLAB code, to count the total number of cells and calculate the distances between them. We computed this data to generate a graph where each cell was considered a node. If the distance between two cells was less than 25 µm, an edge was created between them, grouping closer cells in the same cluster. We analyzed
Slice preparation, electrophysiology and dendritic morphology analysis

DCX-CreERT2::tdTomlox/lox animals were anesthetized with ketamine hydrochloride (100mg/kg) and xylazine hydrochloride (8mg/kg) and intracardially perfused with RT standard cerebrospinal fluid (aCSF) (in mM: NaCl 124; KCl, 2.5; NaH2PO4, 1.2; NaHCO3, 24; glucose, 12.5; CaCl2, 2; MgCl2, 2, 305-315 mOsm). Animals were then decapitated and had their brains removed and then transferred to a vibratome chamber containing ice-cold aCSF, slices with 300µm thickness were collected in the vibratome (VT1200 Leica) and transferred to a custom designed 3d printed incubation chamber containing recover NMDG solution (in mM NMDG, 92; KCl, 2.5; NaH2PO4, 1.25; NaHCO3, 30; HEPES, 20; glucose, 25; thiourea, 2; sodium-ascorbate, 5; sodium-pyruvate, 3; CaCl2·4H2O, 0.5; 10MgSO4·7H2O, 10; pH controlled to 7.3–7.4 with 2N HCl solution) at 36 ºC for 15 minutes, and then again returned to aCSF for at least 1 hour at RT prior to recordings, all solutions were continually bubbled with carbogen 95% O2 and 5% CO2 (White-Martins) (Ting, Daigle, Chen, & Guoping, 2014). For whole-cell patch clamp recordings the tissue was transferred to a chamber filled with Standard aCSF in a Microscope (ZEISS). Micropipettes were filled with K-gluconate solution (in mM, K-Gluconate, 145; HEPES, 10; EGTA, 1; Mg-ATP, 2; Na2-GTP, 0.3; MgCl2, 2; pH 7.3, 290–300 mOsm) granule cells tdTom+ were identified by fluorescence (543 excitation/580 emission). Current-clamp recordings were obtained using an axopatch amplifier 200B (Molecular Devices) in whole-cell configuration using the winWCP Strathclyde Electrophysiology Software. Two protocols were used in current clamp: 100ms-long current steps with 50 pA increment ranging from -100 pA to 400 pA and a ramp ranging from -50 pA to 200pA in 1500 ms. Data was analyzed using
winWCP in conjunction with Matlab. Some hippocampal sections were fixed after recordings, and used for Sholl analysis (Langhammer et al., 2010). To analyze dendritic morphology, slices from DCX-CreERT2::tdTomlox/lox mice, obtained to electrophysiological recordings as described above, were kept overnight in paraformaldehyde 4% overnight and 40x-amplification pictures were taken using confocal microscopy (Zeiss) to analyze the DCX-CreERT2::tdTomlox/lox neurons. All the cells in which the dendritic process were visible and not overlapped by other DCX-CreERT2::tdTomlox/lox positive cell were selected, then were blind selected to experimental groups before analysis. Morphometry was performed using the Image-J plug-in Simple Neurite Tracer, extracting the number of branches and performing Sholl analysis (Ferreira et al., 2014).

Statistical analysis

All data was tested for normality with D’Agostino & Pearson omnibus normality test, comparisons between groups were made with unpaired t-test. Data is presented as mean ± Standard Error Mean (SEM). Complementary, two-way ANOVA was performed to comparison between groups in proliferation assay and Sholl analysis of dendritic tree morphology, comparing each 10µm-section away from soma from both treatments in a increase radius until 300µm far from soma, statistical difference with p<0.05 was reported differentially for each 10µm section.

Results

In order to check whether a single 100 µg dose of 5-MeO-DMT increases cell proliferation in the adult DG as other serotonin 5-HT1A agonists can (Encinas et al., 2006), we labeled cells in S phase of cell cycle with BrdU (Taupin, 2007) (Figure 5A). Following the i.c.v injection animals received i.p. injections of 50mg/kg BrdU and wait in the animal house 12 hours until perfusion, were their brains were removed for analysis We found that 5-MeO-DMT treated
animals showed a greater number of BrdU+ cells compared to saline injected controls for both genotypes (WT/saline: 8.021±0.758 cells, n=48 sections/3 mice; WT/5-MeO-DMT: 24.08±1.629 cells, n=48 sections/3 mice; neil3-KO/Saline: 12.73±0.8239 cells, n =48 sections/3 mice; neil3-KO/5-Meo-DMT 50.65± 2.206 cells, n=48 sections/3 mice; p=0.0001, two-way ANOVA, Figure 5B). We next checked for differences in clustering (cells within 25µm range from each other were considered a cluster) of BrdU+ cells in control and 5-MeO-DMT-injected mice. Clustered cells suggest that they originate from a single progenitor. The total number of clusters in 5-MeO-DMT treated animals was greater than saline for both genotypes (WT/Saline: 5.233±0.4833 clusters; WT/5-MeO-DMT: 12.07±1.155 clusters; neil3-KO/Saline: 8.604±1.851 clusters; neil3-KO/5-MeO-DMT 23.92±3.547 clusters; n=3 mice for each group; p value: WT/Saline vs WT/5-MeO-DMT p=0.049, neil3/Saline vs neil3-KO/5-MeO-DMT p = 0.0001, WT/5-MeO-DMT vs neil3-KO/5-MeO-DMT p=0.0003, two-way ANOVA, n= 3 mice per group, Figure 5C). However, we found no difference in the number of cells per cluster between control and 5-MeO-DMT-treated mice in both genotypes (WT/Saline: 1.518±0.2071 cells/cluster; WT/Saline: 1.937±0.1815 cells/cluster; neil3-KO/Saline: 1.521±0.2182 cells/cluster; neil3-KO/5-MeO-DMT: 1.968±0.02743 cells/cluster; n=3 mice for each group, t test, Figure 5D). This data suggests that a greater number of progenitor cells, are being recruited by 5-MeO-DMT, in healthy and pathological model of neurogenesis.
Figure 5: Single dose of 5-MeO-DMT increases cell proliferation within the DG of adult mice in WT and neil3-KO. A. Photomicrography showing representative hippocampal sections (BrdU+ cells in green and Hoechst 33342 – blue), upper right is a closer look to BrdU+ cell showing colabeling with Hoechst. B. Average number of BrdU+ cells. C. Mean number of clusters in each group. D. Mean number of cells per clusters; * p = 0.0026, ** p = 0.0055, *** p = 0.0001.
Next, we test if 5-MeO-DMT can modify electrophysiological properties of immature DG granule cells, we performed whole cell patch clamp onto DCX-CreERT2::tdTOMlox/lox granule cells. In these experiments, DCX-CreERT2::tdTOMlox/lox mice were perfused 21 days after i.c.v injections to study passive and active membrane properties (Figure 7A). Passive membrane and AP properties in response to a 500ms-long 100pA current step are shown in
Table 2. Example membrane potential responses for a tdTomato+ cell from saline- and 5-MeO-DMT-injected mouse (Figure 7B and 7C). Cells from 5-MeO-DMT-treated animals exhibited higher action potential (AP) threshold (Saline: -38.25 ± 2.03mV n=8 cells/3 animals; 5-MeO-DMT: -29.60±2.51 mV, n=11cells/3 animals, p=0.022, t test, Figure 7D). These cells also displayed a shorter after hyperpolarization (AHP) potential duration (Saline: 53.33ms±12.04 ms n=9 cells/3 mice; 5-MeO-DMT: 12.40ms±1.302ms, n=8 cells/3 mice, p=0.006, t test, Figure 7E). AP threshold was defined as the voltage in which the rate of rise reaches a value superior to 20mv/ms. We then applied current ramps (-50pA to 200pA in 1.5s) in order to elucidate differences in fast activated currents between the two experimental groups. Example membrane potential responses to the current ramp is shown in Figure 8A. Newborn granule cells from 5-MeO-DMT-treated mice showed a greater linear dependency between injected current and AP instantaneous frequency (Figure 8A and 8B). The slope from the linear regression ramp current (in pA) vs. AP instantaneous frequency was equal to 0.11±0.01 Hz/pA for controls and 0.17±0.03 Hz/pA for 5-MeO-DMT group (n=8 cells/3animals and n=6 cells/3animals, respectively, p=0.0036, t test, Figure 8C). This data suggests that young granule cells from 5-MeO-DMT-injected mice show a higher degree of maturation than cells from control animals, as reflected by their spiking profile.
Passive and active membrane properties extracted from tdTomato+ (DCX-Cre::tdTOMlox/lox) granule cells across treatments.

<table>
<thead>
<tr>
<th>Passive membrane properties</th>
<th>Salina Treated</th>
<th>5-Meo-DMT Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input Resistance (MΩ)</td>
<td>389.6 ± 70.06 N=9</td>
<td>476 ± 32.55 N=11</td>
</tr>
<tr>
<td>Baseline (mV)</td>
<td>-67.02 ± 3.340 N=9</td>
<td>-70.74 ± 2.969 N=11</td>
</tr>
<tr>
<td>ISI (ms) *</td>
<td>0.0571 ± 0.01204 N=4</td>
<td>0.04587 ± 0.005734 N=6</td>
</tr>
<tr>
<td>Number of Spikes *</td>
<td>13.70 ± 4.386 N=4</td>
<td>18.97 ± 2.470 N=6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Action Potential Properties</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(+) Peak Amplitude (mV)</td>
<td>91.11 ± 3.971 N=9</td>
<td>87.15 ± 3.663 N=11</td>
</tr>
<tr>
<td>Rise time (ms) **</td>
<td>12.90 ± 6.359 N=9</td>
<td>19.26 ± 2.099 N=10</td>
</tr>
<tr>
<td>Latency (ms) ***</td>
<td>128.5 ± 15.93 N=9</td>
<td>166.4 ± 22.49 N=11</td>
</tr>
<tr>
<td>Rate of Rise (mv/ms) #</td>
<td>86.92 ± 7.910 N=9</td>
<td>80.99 ± 7.937 N=11</td>
</tr>
<tr>
<td>Ap Threshold (mv) ##</td>
<td>2.711 ± 14.83 N=9</td>
<td>-31.87 ± 3.010 N=11</td>
</tr>
<tr>
<td>AP Half Width (ms)</td>
<td>0.5689 ± 0.07323 N=9</td>
<td>0.5218 ± 0.04441 N=11</td>
</tr>
<tr>
<td>AP AHP Amplitude (mv)</td>
<td>-10.73 ± 2.317 N=8</td>
<td>-6.324 ± 0.8275 N=11</td>
</tr>
<tr>
<td>AP AHP Duration (ms)</td>
<td>53.33 ± 12.04 N=9</td>
<td>12.40 ± 1.302 N=8</td>
</tr>
</tbody>
</table>

* Interval interspikes and no. of spikes calculated from ramp protocol (example show in Figure 3) all other variables were extracted from the first AP of the Current steps protocol (as depicted in Figure 2);
** Rise time stated as the time taken for the signal to go from 5% to 90% of its peak;
*** Latency defined as the time between the beginning of the protocol and the 5% of the first peak (all protocols have 100ms delay);
# Rate of Rise, defined as the maximum rate of change during the rising phase of the signal;
## AP threshold defined as the voltage point where the rate of rise reach a value superior to 20mV/ms.
Figure 7: 5-Meo-DMT injection alters AHP duration and AP threshold in immature hippocampus granule cells. A. Animals received a 100µg-dose of 5-MeO-DMT, followed by 100µg/g of tamoxifen i.p diluted in sesame oil 3 days after, daily for 3 days to allow cre recombination. Experiments were performed on day 21. B. Photomicrography of a recorded DCX-CreER<sup>T2</sup>::<i>tdTom</i><sub>lox/lox</sub> cells from control and 5-MeO-DMT-treated mouse. C. Membrane potential changes in response to current steps, the black line denotes the trace in which the first AP was elicited, red dotted line denote AP threshold for that step. D. Mean AP threshold. E. Mean AHP duration. * p = 0.0216, ** p = 0.0062.
**Figure 8:** Young granule cells in 5-MeO-DMT-treated mice show a greater capacity for high frequency firing. A. Membrane potential recording in response to a current ramp. B. Linear regressions (ramp current vs. instantaneous AP frequency). C. Average slopes (ramp current vs. instantaneous AP frequency relationship). **p = 0.0036.

We then tested if 5-MeO-DMT also alters maturation of newborn granule cells (tdTomato+ neurons, **Figure 9A**). We first traced cells using an ImageJ plugin (see methods, **Figure 9B**) to later perform morphological analysis of dendrites. Number of branches in dendrite tree between treatment groups were different (saline: 5.000±0.5774 branch tips, n=3 cells/2 animals; 5-MeO-DMT 10.14±1.056 branch tips, n=7 cells/3 animals, p = 0.0167, **Figure 9C**).

We then tested the same cells for dendritic complexity relative to cell nucleus, and the experimental group shows a higher number of intersections in the 40-80 µm range of distance from soma when compared to saline (Saline intersect values: 40µm, 4.7±0.49; 50µm, 5.0±0.51; 60µm, 5.5±0.43; 70µm, 5.7±0.42; 80µm, 5.7±0.33; 5-MeO-DMT: 40µm, 1.7±0.33; 50µm,
2.3±0.88; 60µm, 2.7±0.33; 70µm, 2.7±0.33; 80µm, 3.0±0.58, (Figure 9D). Taken together, these results suggest that 5-MeO-DMT accelerates dendritic maturation.

**Figure 9:** Single dose of 5-MeO-DMT increases dendritic complexity in young DG granule cells. A. Sample image showing a tdTomato+ (from DCX-CreER<sup>T2</sup>::tdTom<sup>Lox/Lox</sup> mice) granule cell with visible dendritic processes. B. Vectoral reconstruction of tdTomato+ granule cell. C. Mean number of branch tips of granule cells across treatments. D. Sholl analysis comparing the dendritic complexity between two treatments with increasing radial distance from soma. * p=0.002.
Discussion

In this work we showed that a single dose of 5-MeO-DMT increases proliferation in the dentate gyrus and accelerates the maturation of newborn granule cells. We first used BrdU staining to show that 5-MeO-DMT application increase cell proliferation in the DG, in neil3-KO mice, a model with reduced reactive neurogenesis, and in wild type, healthy, animals. These data suggest that 5-MeO-DMT can increase normal neurogenesis process at least, on proliferation levels. Next, to identify newborn neurons we use an inducible Cre recombinase line under the control of neuronal fate marker DCX, crossed with a fluorescent reporter tdTomato. Dendritic trees of newborn neurons from 5-MeO-DMT-treated DCX-CreER$^{T2}$::tdTOM$^{lox/lox}$ mice were significantly more complex (with more branches and a higher number of intersections), when compared to saline-treated DCX-CreER$^{T2}$::tdTOM$^{lox/lox}$ mice. AP threshold was lower and AHP potential was longer in newborn cells from control group when compared to 5-MeO-DMT treated animals.

Increased proliferation after 5-MeO-DMT injection does not indicate that the cell have neuronal commitment (Canales, 2016). It has been described that serotonin increase RGL proliferation in the fetal and adult DG (Brezun & Daszuta, 2000a), however, it does not seem to have neurotrophins factors as mediators (Brezun & Daszuta, 2000a). Complementarily, our results, suggest that 5-MeO-DMT not only has a positive effect on proliferation, but also on the maturation of granule cells. Hence, our results imply that the positive effect of 5-MeO-DMT in adult neurogenesis differs from that of serotonin alone (Song et al., 2016).

The neil3 enzyme has a role in developing neurons, mainly in progenitor areas for both adult and embryonic neurogenesis (Hildrestrand et al., 2009). In adult mice, neil3 is highly expressed in the hipocampal formation,(Allen Institute for Brain Science, http://www.alleninstitute.org/
experiments nº: 68443381), reinforcing the idea that neil3 play a major role in cell proliferation. Previous studies have shown that reactive neurogenesis, the ability of neuronal proliferation following a damage in adult mice, is affected by neil3 deletion with reduced repair activity and proliferative capacity (Hildrestrand et al., 2009; Regnell et al., 2012). In our lab, earlier experiments found that most membrane properties of granule cells in neil3-KO mice are normal except from the membrane response to hyperpolarization currents and afterhyperpolarization currents, even though cell age was not controlled (unpublished work Soares, 2016). In free running voltage clamp mode, excitatory post synaptic currents between WT and neil3-KO mice were similar in amplitude but showed a slightly faster decay in cells from neil3-KO mice. This result could indicate a different balance between AMPA and NMDA receptors in neil3-KO mice granule cells, but further investigation is necessary. Morphological analysis of neurons filled with biocytin were reconstructed post hoc, showed no gross difference in dendritic morphology between dentate gyrus neurons of WT and neil3-KO (Soares, 2016). Thus as neil3-KO granule cells showed divergent properties in above cited results, in the present study we chose to characterize the effect of 5-MeO-DMT on cell maturation, regarding the effect of neil3 ablation, since in the proliferation assay, the 5-MeO-DMT increase proliferation levels for both genotypes, and in post hoc analysis showed a highly divergent morphology (figure 9), this effects would be covert by association of neil3-KO Genotype to 5-MeO-DMT treatment.

Our current-clamp recordings indicate that young neurons from 5-MeO-DMT-treated mice have a faster maturation than cells from control animals. Mature granule cells show a higher AP threshold, shorter AHP and are able to fire in higher frequencies (Schmidt-Hieber et al., 2004). These differences in maturation were also found in the morphology of dendritic trees. Dendritic complexity is a major indicative of cell maturation (Ohira & Miyakawa, 2011; Schmidt-Hieber et
al., 2004). Cells from animals submitted to a single 5-MeO-DMT injection showed dendrites with more branches and intersections. Interestingly, chronic antidepressant therapy also accelerates the maturation of dendrites (Ohira & Miyakawa, 2011). Future studies should address how tryptamine analogs affect the temporal expression of voltage-dependent currents. Our preliminary results indicate, for example, that the hyperpolarizing-activated current $I_h$ (K. E. Leão, Leão, & Walmsley, 2011) is larger in novel granule cells of 5-MeO-DMT-injected animals, when compared to saline. Also, it will be interesting to examine changes in Cl$^-$ reversal potential, as granule cells show a depolarized potential until adolescence (Chiang et al., 2012).

The long AHP in abGC seems to be elicited by a single spike, in contrast to cultured pyramidal neurons and mGC in dentate gyrus (Mateos-Aparicio, Murphy, & Storm, 2014) where they are elicited after a train of spikes. Slow current afterhyperpolarization ($sI_{AHP}$) with ~600ms duration or longer, occur due to voltage independent efflux of K$^+$ through a slow Ca$^{2+}$-activated K$^+$ channel, from the Ca$^{2+}$-activated potassium channels ($K_{Ca}$) family. The $K_{Ca}$ family is composed of 3 major groups separated by their conductance: the big conductance $K_{Ca}$ (BK) around 100-300pS; the intermediate conductance $K_{Ca}$ (IK) around 25-100 pS; and the small conductance $K_{Ca}$ (SK) around 2-25pS. The observed $sI_{AHP}$ in our saline treated group indicates a young granule cell compared to our 5-MeO-DMT treated (Figure 7). It was recently shown for a simulated abGC, that paradoxically a faster and longer AHP can increase gain in abGC, enhancing the activation of T-type Ca$^{2+}$ channels in dendrites (Schmidt-Hieber et al., 2004), boosting the amplitude of a rebound depolarization in the soma close to axon hillock, so this amplitude determines the activation of a subsequent persistent influx of Na$^+$ in the axon initial segment, limiting the interspike interval (Jaffe & Brenner, 2018) in this way reducing instantaneous frequency as observed in our Figure 8.
For cultured hippocampal pyramidal neurons, the observed $sI_{AHP}$ was described as insensitive to apamin and tetraethylammonium (TEA), both slow conductance $Ca^{2+}$ activated potassium channels (SK $K_{Ca}$) blockers, since even with the blockers the $K^+$ efflux through SK $K_{Ca}$, the AHP remains. Although the $sI_{AHP}$ is sensitive to noradrenaline, muscarine and completely inhibited by $Cd^{2+}$, those 3 agents together decrease the intracellular level of $Ca^{2+}$ (Shah & Haylett, 2000), this results lead Vergara et al (1998) to conclude that the subtype SK1 (encoded by the gene KCNN1) are the only one $K_{Ca}$ described, until now, with matched pharmacokinetics. The KCNN1 has been described in CNS with the highest levels of expression in the hippocampus granule cells (Allen Institute for Brain Science, http://www.alleninstitute.org/ experiments nº: 69860427). Hence, we hypothesize that young granule cells have predominantly expression of SK1 channels, making them able to prolong the undershoot phase of AP without the need of longer spike trains (Vergara et al., 1998). It may work as a method to remove cell hyperexcitability which may lead to apoptosis since the abGC hold a smaller AP threshold (Figure 7) and can fire more frequently then mGC, even with a smaller frequency, as evinced by Arc activation elsewhere (James B. Aimone et al., 2010b). When deprived of depolarization, cultured granule cells enter in apoptosis with a decreasing in $Ca^{2+}$, on the other hand, this effect is rescued by the increase in cAMP via forskolin (Galli et al., 1995). Recently computational studies suggest that a fast AHP (fAHP) are capable of paradoxically increase cell gain and cell firing (Jaffe & Brenner, 2018)

Therefore, we believe that young abGC presents a predominantly expression of SK1 channels from the family of $K_{Ca}$ channels, which when later in development will be complemented with BK and IK $K_{Ca}$ channels to produce the effect observed in mGC which is similar to our 5-MeO-DMT treated group with 21 days after proliferation. In addition, the abGC
from saline treated group have a non-developed sarcoplasmic reticulum (SR), consequently relying in the influx of Ca\(^{2+}\) to bind on SK1 and produce the observed longer AHP, the Ca\(^{2+}\) might diffuse faster to abGC due to their smaller size. This idea is reinforced by the increased expression of T-Type Ca\(^{2+}\) channels, which has a major influence on the increase of Ca\(^{2+}\) influx (Schmidt-Hieber et al., 2004). To test this hypothesis, future experiments should use immunohistochemistry to stain the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) or ryanodine receptor 3 (encoded by the gene RyR3), co-labeling with DCX-CreERT2::tdTOM\(^{lox/lox}\) to characterize the presence and size of young abGC SR at 21 days. Besides immunostaining, with perforated whole-cell patch clamp, we shall record if sI\(_{AHP}\) present in young abGC with 21 days, is inhibited by Cd\(^{2+}\), a Ca\(^{2+}\) voltage gated pore channel blocker, preventing Ca\(^{2+}\) influx, complementary to application of micromolar concentrations of ryanodine to remove intracellular release of Ca\(^{2+}\). Considering that young abGC hold a mature and efficient SR, the expected result of these proposed experiments was that with Cd\(^{2+}\) and caffeine (an indirect agonist of ryanodine receptors) the sI\(_{AHP}\) would occur and the cell would behave as in washout conditions, but in the presence of ryanodine and Cd\(^{2+}\) the sI\(_{AHP}\) will be completely abolished.

It should be noted that the majority of electrophysiological studies regarding the maturation of granule cells in the DG rely on a wide time window. As an example, the expression of PSA-NCAM, this protein is expressed by newborn neurons during all the maturation phase, \textit{i.e.} 28 days after proliferation phase (Brezun & Daszuta, 2000b; Seki, 2002), also in presynaptic neurons after induction of LTP, acting as a surface glycoprotein, which guide neurite outgrowth adhering new cell projections to extracellular matrix (Cremer et al., 2000). However, cells just after division, are very different from cells with 2 or 3 weeks, and markers as PSA-NCAM cannot be a biomarker for differentiating granule cells in the intermediate process. If we want to
account the effect of an agent in a smaller time window, to see if a drug can act accelerating the normal maturation or retarding the synaptic plasticity process of maturation, through morphology development or active and passive membrane properties changes, the experimenter should rely in a marker with a narrower expression time window.

Dorsal raphe nucleus profusely targets the SGZ (Kosofsky & Molliver, 1987), although a previous study showed that lower serotonin levels in the brain can increase neurogenesis (Song et al., 2016). Additionally, serotonin agonists and serotonin uptake inhibitors seem to increase neurogenesis (Ohira & Miyakawa, 2011; A Surget et al., 2011). Hence, specific 5HT receptors might be involved in neurogenesis modulation. 5-HT1A, 5-HT2A and 5-HT2C, 5-MeO-DMT targets, are all expressed in the DG (Allen Institute for Brain Science, http://www.alleninstitute.org/ experiments nº: 79394355, 81671344 and 71393424 respectively). Therefore, 5-MeO-DMT may have distinct effects in neurogenesis when compared to serotonin.

Aside from an increasing volume of experimental evidence shows that neurogenesis occurs in the adult mammalian hippocampus, and its function has been a hot topic in neuroscience (J. B. Aimone et al., 2014; J. M. Barker et al., 2011; Canales, 2016; Drew et al., 2013; Duman et al., 2001; Spalding et al., 2013). There are also some controversial experimental findings, pointing a fast decay in neurogenesis through human growth during the first post-natal year, becoming undetectable around 10-13 years age (Sorrells et al., 2018). It is necessary to point out that this study was made with post-mortem human brain tissue, from patients hospitalized in psychiatric hospitals, many of the subjects had experienced brain inflammation, a condition already known to drastically reduce neurogenesis (Kohman & Rhodes, 2013). Moreover, many patients developed depression due to the way of life in hospitals, also known to strongly reduce neurogenesis (Sahay & Hen, 2007).
In conclusion, we show here that a single dose of 5-MeO-DMT can increase cell proliferation and accelerate the maturation of newborn neurons in the DG. To our knowledge, this work was the first to demonstrate a direct effect of a naturally occurring psychoactive compound in adult neurogenesis. New lines of investigation have suggested that serotonergic hallucinogens can significantly improve depression and anxiety symptoms (Reiche et al., 2018). Thus, the effect of 5-MeO-DMT in modulating neurogenesis can shed light on the mechanism behind the beneficial effects of hallucinogenic compounds in mood disorders.

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5-MEO-DMT Stimulates Adult Neurogenesis

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5-MEO-DMT STIMULATES ADULT NEUROGENESIS


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CERTIFICADO

Natal (RN), 06 de junho de 2017.

Certificamos que a proposta intitulada “Investigando os efeitos da 5-Metoxi-N, N-dimetiltriptamina na neurogênese do giro dentado de camundongos NEIL3-KO adultos”, PARECER n° 015.004/2017, sob a responsabilidade de Richardson Naves Leão, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica encontra-se de acordo com os preceitos da Lei n.º 11.734, de 8 de outubro de 2008, do Decreto n.º 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi aprovado, após adequações, pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS da Universidade Federal do Rio Grande do Norte – CEUA/UFRN.

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