Rectification of Aberrant Mossy Fiber connections by DCP-LA, Selective Activator of PKC Epsilon: A Possible Target for Later-Life Mood Disorders

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Abstract

Development of a few but relevant connections is crucial for the proper functioning of the early hippocampus. Unnecessary and redundant sprouting can result in excessive activity leading to the anxiety-like behavior later in life. Aberrant connections observed in the female mice lacking 5-HT1A- receptor can be corrected by using a selective activator of PKC epsilon isoform, a downstream molecule and also possibly eventually could be used to rectify anxiety-like phenotype in adulthood.

Key words: PKC Isozymes; Development; 5-HT1A receptor; Mossy Fiber; Hippocampus.

Introduction

The hippocampus in one of the most intensely studied parts of the brain. Over the years researchers have tried to unravel all aspects of hippocampus starting from its anatomy, physiology, biochemistry, its diverse roles in emotion, mood, anxiety, stress, learning, memory, its effect on other regions of the brain as well as its connectivity with other regions of the central nervous system. The hippocampus is surrounded by the entorhinal, parahippocampal and perirhinal cortices and is connected to cortical and sub-cortical parts of the brain. Majority of the hippocampus's neocortical inputs come from the perirhinal and the parahippocampal cortices through the entorhinal cortex and its neocortical outputs project through subiculum, which again project back to the entorhinal cortex [1].

The hippocampus is differentiated along its length, the dorsal part being involved in learning and memory and the ventral part being associated to emotionality. This topological difference is linked to different functions: The ventral hippocampus receives inputs from the rostromedial entorhinal cortex, and provides output projections to the prefrontal cortex, amygdale, and nucleus accumbens. On the other hand, the dorsal hippocampus receives input signals from the lateral and caudomedial entorhinal cortex, and has efferents to the dorsal lateral septum and mammillary complex.

The hippocampus is well known for its tri-synaptic loop (Fig 1). The tri-synaptic loop is the circuit of synaptic transmission in the hippocampus. The first projection comes from the cells of the entorhinal cortex, which make connections with granule cells of the dentate gyrus called the 'Perforant pathway'. The dentate gyrus then synapses with the pyramidal cells of the CA3 region via the 'Mossy fiber pathway'. The CA3 ultimately sends projections to the CA1 pyramidal cells through the 'Schaffer collaterals', which are carried out through the fornix into the deep layers of the entorhinal cortex. However, recent studies have hinted towards other synaptic connections apart from these three already known connections. It is believed that projections from the layer III of the entorhinal cortex make direct connections with the CA1 region.

Hippocampal development has been studied since a long time using 3H-thymidine autoradiography. Joseph Altman and Shirley Bayer have done some pioneering studies related to the development of the hippocampus from the embryonic period all the way to adulthood. Though the hippocampal regions are fully formed by P22, new neurons are added to the granular layer of the dentate gyrus throughout life. Hippocampal development involves multiple steps like cell proliferation, cell maturation, radial migration and cell survival.

The hippocampus has been linked to various mood disorders like anxiety, depression, stress and panic disorder to name a few. Genes and environment play a synchronous role in the development of anxiety, depression, stress and panic disorder to name a few. Genes and environment play a synchronous role in the development of anxiety. Serotonergic signaling via the Serotonin 1A Receptor (5-HT1A- receptor) during the critical postnatal period is important for the developmental programming of anxiety-related behavior [5, 8]. Antidepressants acting through the serotonergic pathway have been shown to increase neurogenesis in the hippocampus and...
attenuate anxiety-like behavior [12]. All these studies have pointed out to the fact that serotonin and its signaling through the 5-HT1A receptor play a crucial role in the development of depression and anxiety-like behavior in adulthood.

Our lab has been long involved in the study of Serotonin and its signaling through the 5-HT1A receptor during early hippocampal development. We have been trying to nail down the signaling cascade, their role and expression in the postnatal developmental process, thereby understanding how malfunctioning of any of these molecules might result in a later-life mood disorder. In the course of our study, we have been able to decipher the different isoforms of Phospho Kinase C (PKC), involved in the 5-HT1A receptor mediated signaling during the two crucial time points P6 (peak of proliferation, PKCe) and P15 (peak of synaptogenesis, PKCa) of postnatal hippocampal development [11]. The involvement of PKCa in synaptogenesis has been further confirmed in in vivo studies [10]. Furthermore, the role of PKCe in 5-HT1A receptor mediated signaling have been demonstrated using P6 hippocampal slice cultures [9]. We have also worked out the pathway in vivo with P6 mice and elucidated the importance of PKCe at this critical time point with rescue studies involving the selective activator of the latter, DCP-LA in 5-HT1A receptor deficient mouse (manuscript submitted). The decreased neuroproliferation has been corrected to near control levels by the use of the selective activator DCP-LA in the 5-HT1A receptor deficient mouse. Our studies have also revealed a female-specific decline in neuroproliferation at P6, P6-born hippocampal neurons and anxiety-like behavior in adulthood (manuscript submitted). This female specific deficiency of neuroproliferation and mature neurons could be responsible for aberrant connectivity in the hippocampus which would consequently lead to anxiety-like behavior later in life.

In the hue of these previous studies we went ahead and studied the connections that the mature granule cells of the dentate gyrus makes with the pyramidal cells of the CA3 region, the mossy fiber pathway. Timm’s sulfide silver method [13] is a sensitive method for demonstrating the histological distribution of laminated pattern of the hippocampus. Timm staining of the mossy fibers is a very reliable method for defining the mossy fibers. The intense staining is due to the presence of a large amount of zinc, and it is associated with neuronal excitability and also with neurotrophic nerve growth factor [4]. The outgrowth of the mossy fibers stars prenatally. The mossy fibers first originate from the granule cells which are located more laterally, followed by the ones located medially. At about postnatal day 24, all the granule cells have mossy fibers projecting to the region inferior [4].

Discussion

Our preliminary studies with the Mossy Fiber connections using Timm staining, revealed a female-specific excessive sprouting in the SO region of the 5-HT1A receptor deficient mice that the WT . These extra connections were retracted in the presence of DCP-LA, selective activator of a downstream signaling molecule PKC epsilon in the 5-HT1A receptor mediated signaling. The granule cells synapsing on the pyramidal cells of the CA3 region in the Mossy fiber pathway is a highly regulated process. In the hilus, mossy fibers are believed to make synaptic connections with basket cells, mossy cells, oviform cells and long-spined multipolar cells, of which the functional consequence of the synapses with the basket cells and the mossy cells are of importance in this study. Evidences have shown that the dendrites of the hilar basket cells are potential targets of the mossy fiber boutons. Thus the mossy fibers also excite the basket cells apart from the exciting the pyramidal cells of the CA3 region. These basket cells in turn form axo-dendritic, axo-somatic and axo-axonic synapses on the granule cells. These latter synapses are inhibitory in nature, demonstrated by the presence of the inhibitory neurotransmitter GABA and its synthesizing enzyme, glutamate decarboxylase. These synaptic connections between the granule cells, and the basket cells form an inhibitory feed-back circuit [4].

The connections that the mossy fibers make with the pyramidal cells are therefore regulated by an intrinsic inhibitory feed-back mechanism, ensuring there is no scope of any redundancy. Aberrancies with this auto-regulatory mechanism might lead to development of unnecessary mossy fiber sprouting (Fig 3). This hypothesis is well supported by some learning studies done with some status epilepticus rats where a negative correlation was observed between the score of mossy fiber sprouting and the latency of learning. Thus fewer mossy fiber terminals aided better learning compared to more number of terminals [3]. In the light of this controlled connectivity of the mossy fibers in learning, mossy...
fiber connectivity might be equally important in the development of anxiety-like behavior and depression in adulthood.

The lack of P6 born neurons in the 5-HT1A receptor deficient female mouse might be responsible for the development of excessive unnecessary connections in place of few but relevant connections required in maintaining normal functioning of the developing hippocampus. Further studies are required to establish a functional relationship between the proper pruning and connectivity to the normal development of the hippocampus and its effects in later-life mood disorders.

(a, b) Compared with the WT females at P18, the KO females showed an extension of MF collaterals in the SO both in dorsal as well as ventral planes. (c) DCP-LA treatment of the KO females at P6, P10, and P14 partially eliminated this aberrant extension of MF collaterals at P18. (d) Punta intensities in the MF collaterals in the circular cursor areas showed in the SO were normalized to the intensities recorded in the cursor areas shown in the SR. (e, f) The X-axis in (Fig. 5e) or f shows cursor distance in inches from the bifurcation of the collaters (shown by a black arrow).
(h) Graphical presentation of the length of the MF collaterals normalized to the total length of the MF fibers up to CA3. (i) Compared with the WT males (i I), the male KO mice did not show any aberrant MF extension (i II).

Materials and Methods

Animals

Mice (C57BL/6) were housed in the College of Staten Island (CSI) Animal Care Facility and handled following a protocol approved by the CSI Institutional Animal Care Committee. Some of the Wild type (WT) (C57BL/6) mice were obtained from Taconic and bred in CSI Animal Care Facility. The 5-HT1A-R (-/-) pups used were generated by crossing 5-HT1A-R (+/-) mice (Het) against the C57BL background obtained from Dr. Lawrence Tecott (Heisler 1998). The WT, Het, and 5-HT1A-R (-/-) mice of the F1 generation were genotyped according our earlier report (Mogha 2012), and the 5-HT1A-R (-/-) mice were paired to obtain pups (F2 generation), which were used in our experiments. Animals were kept in a 12-hour light/dark cycle with ad libitum access to food and water. Mice were housed in the College of Staten Island (CSI) Animal Care Facility and handled following a protocol approved by the CSI Institutional Animal Care Committee.

Treatment of WT and KO Mouse Pups for Timm Staining

C57BL/6 pups were injected intraperitoneally with 3 mg/kg DCP-LA at P6, P10, P14. For basal level comparison of the mossy fiber pathway, WT pups were not injected (serving as WT un-injected controls) and the 5-HT1A-R (-/-) KO pups were injected with vehicle (PBS+DMSO as discussed above) (serving as knockout injected controls). On day 18, the mice were anesthetized and perfused first with Na2S and then with glutaraldehyde and processed for Timm staining as described below. The brains were divided into left and right hemispheres, embedded in Tissue-Tek and sectioned with a cryostat (30 μm thickness). The sections were collected over gelatin-coated slides and allowed to stick for at least 12 hours. The slices were then developed as described the following sections.

Timm Staining[13]

The staining has been performed based on an earlier protocol from Dr. Tammy Ivanco with a few modifications [6]. Each mouse was first perfused with a freshly made solution of 2.9 g Na2S in 100 ml of 0.12 M monobasic sodium phosphate and then with freshly made 6% glutaraldehyde in 0.12 M monobasic sodium phosphate.

Preparing Gelatin-Coated Slides

First the slides were cleaned by placing them in a mixture of 95% ethanol (300 ml) and 8 ml of acetic acid in a Wheaton dish and incubating for 5 min and then dried in an oven.

Type A gelatin from porcine skin (G1890; Sigma, St. Louis, MO) (2.5 g) was dissolved in 500 ml of deionized water by heating to 55 °C with stirring and then cooled to 21 °C. This solution was further supplemented with chromium potassium sulphate (0.25 g) (added with mixing). The slides were placed in this gelatin solution for 5 min. Excess gelatin was removed from the slide rack and then the rack with slides placed in a 40-°C oven and dried overnight. After drying, the slides were stored in slide chambers.

Gum Arabic (GA) solution was made by stirring 50 g of GA in 100 ml of deionized water overnight at room temperature and then strained through a piece of cheesecloth. The solution was divided in 120-ml portions and frozen at -20 °C.

A developer solution was freshly made by mixing 2 M citrate buffer (20 ml), 0.5 M Hydroquinone (60 ml), 1 M silver Nitrate (1 ml) (protected from light), and freshly thawed Gum Arabic solution (120 ml) for each Wheaton dish. (for 8-10 slides).
The sections on slides were placed in glass rack in a Wheaton dish and re-hydrated by treating serially with 100% ethanol for 20 min, 95% ethanol for 10 min, 70% ethanol for 10 min, 50% ethanol for 10 min, and deionized water twice (10 min each). The rehydrated slides with the sections were next developed in a dark room by adding the developer mix and incubating for 45-60 min. The slides with sections were next rinsed by gently passing tap water through the Wheaton dish for 45 min, and then overlaid with a few drops of 90% glycerol and 10% PBS for mounting with coverslips for imaging.

Analysis of the Mossy Fiber Axons

The slides were viewed under microscope (Diaphot Nikon, Tokyo Japan). Images were captured at 2X magnification. Timm staining or Timm’s sulphide silver staining had been used to visualize a variety of metals in brains and other tissues. This method, originally developed by Timm [13], was later modified [2]. The principle of the technique was based on sulphide-precipitation of metals in tissue followed by a physical development. During the later stage, the silver sulphide formed was reduced to silver metal in the presence of reducing agents. Analysis and measurements of Timm(+) fibers in the Stratum Oriens (SO) were performed as described by Ivanco and Greenough, 2002 [7]. For all groups, matching sections from identical planes in the ventral-dorsal regions were selected for densitometric analysis. Densitometric measurements for analyzing the synaptic connections and also measurements of total distance of the connections were performed on images (5 in x 13 in) of 4X magnification in Photoshop. To prepare for analysis, images were first inverted and then converted to greyscale. Cursors (open circles of 0.5 inch diameter) were consecutively placed along the length of the stratum oriens (SO) (to measure the density of Timm granules); and along the stratum radiatum (SR) (to measure the background density). Photoshop’s histogram tool was then used to obtain the mean density within the area of each cursor position. These values were then imported into Excel. For each dorsal section, 7 measurements were obtained from the SO, and 4 from the Stratum Radiatum (SR). For each ventral section, 16 measurements were obtained from the SO, and 8 from the Stratum Radiatum (SR). The relative optical density (R.O.D.) ratio of each cursor position along the SO was calculated by dividing the mean density of that cursor position by the average background density (i.e. the SR cursors) of the same sample. The radial distance in inches (in the 5 in x 13 in image) of each cursor from the bifurcation of the collateral in the hilus (position ZERO) for a section with coordinates (x, y) was calculated by the standard Euclidean distance formula: The R.O.D values were plotted against the radial distances of the cursors to compare the extensions of Timm stained MF fibers in the SO among WT, KO, and DCP-LA-treated KO. The ratio of the length of the Mossy Fiber (MF) collateral was also measured relative to the entire length of the Mossy Fiber using ImageJ. d = (xn - x1)2 + (yn - y1)2.

Genotyping of C57bl/6 5-Ht1a-R (-/-) (Ko) Mice

Mouse pups were anaesthetized as mentioned before with a mixture of ketamine (90 mg/kg body weight) and xylazine (10 mg/kg). 2 mm of tail was removed from each mouse and placed into a 1.5 ml Eppendorf tube and kept on ice until digestion. The mice were ear-tagged for identification. To each tube, 500 μl of SNET buffer was added (20 mM Tris-Cl (pH 8), 5 mM EDTA (pH8), 400 mM NaCl, 1% SDS) and 10 μl of 20 mg/ml i.e. 400 μg/ml Proteinase K (Invitrogen, Cat. # 25530-0-15) was added and then incubated at 50-55 °C overnight. The next day, 500 μl of freshly mixed phenol, chloroform and isoamyl alcohol in the ratio of 25:24:1 was added and incubated for 30 minutes at room temperature. To separate the organic and the aqueous phase, the tubes were centrifuged for 5 minutes in a micro-centrifuge (Eppendorf) at 14,000 rpm after which the upper, aqueous phase was removed from the organic layer and placed in a fresh 1.5 ml tube. The DNA was precipitated by adding 500 μl of 100% isopropanol and centrifuged at 13,200g for 15 minutes at 4 °C. The isopropanol was carefully removed and the DNA pellet was washed twice with 70% ethanol by centrifuging each time at 14,000 rpm for 5 min. The ethanol was removed and the pellet air-dried. The pellet was dissolved in 30 μl of TE (Tris-EDTA) buffer and left overnight on a shaker at 4 °C for proper dissolving. The DNA concentration was measured by NanoVue (GE Healthcare, Life Sciences). For the PCR reaction 50ng of DNA was used. To each 0.2ml tube (USA Scientific, 1402-8100) 2 μl 10x PCR Buffer, 2mM MgSO4, 0.25 mM dNTPs, 500 nM of both forward and reverse primers, 0.1 μl of Platinum Taq Polymerase (Invitrogen, 10966-034). The volume was brought to 20 μl with H2O. The reaction was run in a thermo cycler (Applied Biosystems).

The wild type primers used were:

Htr1a Forward: CTGCTCATGCTGGTCCTCTATG
Htr1a Reverse: TAGGAGGTAGCTCCTGATTCGC

The product size was 323 bp.

The Knock out primers used were

NeoD (forward): CACCTTGCTCCTGCGAGAAA
NeoH (reverse): AGAAGGCATAGAAGGCATAG

The product size was 464 bp.

PCR Conditions maintained were:

1. 94 °C for 3 minutes
2. 94 °C for 30 seconds
3. 65 °C for 30 seconds
4. 68 °C for 30 seconds
5. Step (2-5) 29 more times (total 30cycles).
6. 68 °C for 7 minutes
7. Hold at 4 °C
The PCR product was run for 1 hour at 100 volts on a 1.5% Agarose gel in TBE (Tris-Boric acid-EDTA) buffer. A WT sample, KO sample and No-template sample were also run in the gel as controls. The gel was stained with Ethidium bromide and visualized under UV light.

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