Combined behavioral studies and *in vivo* imaging of inflammatory response and expression of mGlu5 receptors in schnurri-2 knockout mice

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Abstract

Schnurri-2 (Shn-2) knockout (KO) mice have been proposed as a preclinical neuroinflammatory schizophrenia model. We used behavioral studies and imaging markers that can be readily translated to human populations to explore brain effects of inflammation. Shn-2 KO mice and their littermate control mice were imaged with two novel PET ligands; an inflammation marker [^{11}C]PBR28 and the mGluR5 ligand [^{18}F]FPEB. Locomotor activity was measured using open field exploration with saline, methamphetamine or amphetamine challenge. A significantly increased accumulation of [^{11}C]PBR28 was found in the cortex, striatum, hippocampus and olfactory bulb of Shn-2 KO mice. Increased mGluR5 binding was also observed in the cortex and hippocampus of the Shn-2 KO mice. Open field locomotor testing revealed a large increase in novelty-induced hyperlocomotion in Shn-2 KO mice with abnormal (decreased) responses to either methamphetamine or amphetamine. These data provide additional support to demonstrate that the Shn-2 KO mouse model exhibits several behavioral and pathological markers resembling human schizophrenia making it an attractive translational model for the disease.

Keywords: Schnurri-2 (Shn-2) knockout (KO) mice, PET, mGluR5, inflammation, schizophrenia

1. Introduction

Schizophrenia is a disease with a complex constellation of brain dysfunctions and symptomatology suggesting that a number of different neurotransmitter and pathways may contribute to its etiology. Infection and inflammatory responses have been shown to play a role in pathophysiology of schizophrenia [15]. It has been reported that drug naïve-first episode schizophrenia patients have high levels of cytokines such as serum interleukin (IL)1 β when compared with healthy controls [23].

Generating animal models for schizophrenia has been a crucial part of research to develop specific antipsychotic drugs with reduced side effects [3]. However, developing a reliable animal model has been challenging because of the complex nature of schizophrenia. An increasing number of genetic manipulation models have been proposed with identification of schizophrenia candidate genes. Schnurri-2 (Shn-2) was originally identified as a nuclear factor- κ B (NF- κ B) binding protein that binds to NF- κ B to suppress NF- κ B-dependent gene expression including TNF α and IL-1 β and the knockout (KO) of this gene induces chronic neuroinflammation [24, 25]. Shn-2 KO mice also show behavioral abnormalities that resemble those of schizophrenia such as hyperactivity and impaired prepulse inhibition (PPI) [24, 25]. In addition Shn-2 KO mouse brain showed similar changes in parvalbumin and GAD67 levels that are comparable to postmortem brains from schizophrenia patients [25]. Based on comprehensive behavioral testing and postmortem brain analysis Shn-2 KO mice have been proposed as a good candidate for a preclinical model of schizophrenia [25]. To enlarge this evidence we conducted *in vivo* PET imaging studies of inflammatory response using [11 C]PBR28, a novel ligand for translocator protein (TSPO) and expression of mGluR5 using [18 F]FPEB in Shn-2 KO and the control mice. TSPO is a mitochondrial outer membrane protein and its expression increases in activated glial cells. TSPO is widely used as an inflammatory marker and considered as a potential therapeutic target. Selective modulation of mGluR5 in preclinical models of schizophrenia has demonstrated its potential for therapeutic approaches. In addition recent studies have demonstrated that mGluR5 can be associated with disrupted sensorimotor gating response that is reminiscent to deficits observed in

schizophrenia [4]. It has been also published that activation of mGluR5 reduces activated microglia associated inflammation and restores the resting state [5]. We postulated that mGluR5 expression would be altered in the Shn-2 KO mice since these mice show chronic neuroinflammation and such measures have yet to be made in schizophrenia. We also performed an open field exploration test with methamphetamine (meth) and amphetamine (amph) to measure locomotor activity under the influence of a dopaminergic challenge.

2. Methods

2.1. Animals

Shn-2 KO mice and their wild type control littermates were obtained by breeding heterozygotes with a c57BL/6J background and those with a BALS/cA background. Shn-2 KO (n=8) and control mice (n=8) were transported from Japan to Charles River Laboratories (Wilmington, MA, USA) to quarantine for 6 weeks before transferring to the MGH. The original ear tags were lost so genotyping was performed after completion of all experiments. Two Shn-2 KO mice died before PET and three Shn-2 KO mouse died after completion of PET studies. The higher mortality of these mice compared to the controls may be due to their chronic inflammation. Each cage housed two to five mice with 12/12h light/dark cycle with food and water *ad libitum*. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the MGH and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.2. PET ligand and imaging

PET imaging studies were conducted in six Shn-2 KO and eight control mice. The animals were anesthetized using isoflurane (1.0-1.5% with oxygen flow of 1-1.5 L/min) and a tail vein was catheterized for radiotracer injection. Animal physiology was monitored using a system included in the imaging device (Triumph®-II, TriFoil Imaging). The field of view of the PET scanner is 7.8 cm with a center resolution of 1.2 mm, and the resolution of the CT scanner is 50 μ m. Radioactivities of

[¹¹C]PBR28 (250-350 μ Ci) followed two hours later by [¹⁸F]FPEB (150-200 μ Ci), were injected through the tail vein. After each radiotracer injection dynamic volumetric data were acquired for 60 min. PET studies were followed by CT to obtain anatomical maps and data for attenuation correction. The 4D PET images (Matrix size 200x200x128x27 with voxel spacing 0.5x0.5x0.6mm³, 27 frames) were reconstructed after corrections for decay, uniformity, scatter and attenuation using the maximum likelihood fitting with 30 iterations. CT images (Matrix size 256x256x224 with voxel spacing 0.34x0.34x0.34 mm³) were reconstructed by a modified Feldkamp algorithm. The mice brain-atlas template from LONI (Laboratory of Neuro Imaging) was applied for segmentations of regions of interests (ROIs). The CT image was registered to the LONI template by a rigid (linear) transformation and the corresponding PET image was transformed to the template. The ROIs selected for analysis were whole brain, olfactory bulb, cortex, striatum, hippocampus, hypothalamus and cerebellum. The data analysis was performed using PMOD3.2 (PMOD, Zurich, Switzerland) and binding potential (BP_{ND}) was determined for mGluR5 using the cerebellum data as an input function. Inflammatory response was calculated as standard uptake values (SUV; (%ID/g) x weight) since cerebellum can have inflammation so the data cannot be used for input function.

2.3. Open field test

Extensive locomotor tests were performed in Japan using a meth challenge in a different set of animals than were used for PET imaging and open field test in the US. In Japan mice were habituated in an open field apparatus (Accuscan Instruments, Columbus, OH) for 60 min before the i.p. administration of meth (0.3mg/kg, 1mg/kg, 3mg/kg, n=10-12/group). Data were collected for 120 min after meth injection and total distance traveled before and after meth injection was measured. The mice which had PET imaging were tested with amph 3 months after the imaging (n=3 for Shn-2 KO mice, n=8 for control group). Locomotor activity after amph challenge was measured in a chamber (Noldus Information Technology, The Netherlands) with a white floor. Mice were habituated for 5 min one day before the test. Data were recorded for baseline (20 min), amph injection (1mg/kg, i.p.) and post

injection (60 min). The video recordings were analyzed with the EthoVision XT, 9.0 (Noldus Information Technology, The Netherlands).

2.4. Statistics

For statistical analysis of the PET data left and right hemispheres were determined separately and then averaged for cortex, hippocampus and striatum. The values were also determined for olfactory bulb, hypothalamus and whole brain. The two groups (Shn-2 KO vs. control) were compared using a simple one way ANOVA (brain region vs. group). Comparisons for multiple corrections were determined by calculating which group survived a Benjamini-Hochberg test using a false discovery rate of 10%[2]. For the behavioral data repeated measures of ANOVA (locomotion vs. time) were used. The differences of degrees of freedom in the series of scans were observed due to the loss of animals resulting a smaller group numbers.

3. Results

All the experimental work and data analysis were performed in the same way in each mouse without their genetic information. After genotyping the data was categorized into two groups to investigate the group specific features.

3.1. Inflammation in Shn-2 Mouse Brain

The accumulation of [^{11}C]PBR28 was significantly increased in the striatum (76.3%), hippocampus (57.3%), cortex (61.4%), olfactory bulb (37.4%), cerebellum (66.3%) and whole brain (37.4%) but not in hypothalamus (7.4%) of the Shn-2 KO mice (n=5 for Shn-2 KO group, n=8 for control group) (Figs.1 and 2A).

3.2. mGluR5 Expression in Shn-2 Mouse Brain

Binding potential of [^{18}F]FPEB was significantly increased in cortex (31.4%) and hippocampus (10.5%) in Shn-2 KO mice. But the whole brain (14.4%), striatum (5.9%), hypothalamus (18.3%) and olfactory bulb (-7.1%) were not significantly different from the control mice (n=6 for Shn-2 KO group, n=8 for control group) (Figs. 1 and 2B).

3.3. Locomotor Abnormalities in the Shn-2 Mice

The Shn-2 KO and control mice showed significantly different open field activity both before and after meth and amph challenge. Open field activity was measured in two different groups of mice (Japan with meth and MGH with amph). Meth injections at three different doses were each paired with a saline control. When placed in the box the Shn-2 KO mice showed increased novelty-induced locomotion compared to the controls (Fig. 3A). The data were fit to a sigmoid logistic function with four parameters (initial novelty locomotion, $t_{1/2}$ (time to reach half maximum), the slope and the baseline locomotion value). The initial novelty-induced locomotion was 567.0 ± 11.7 for the Shn-2 KO vs. 189.1 ± 4.8 cm for controls ($p < 0.001$ using the R-factor ratio test [8] for comparison of the fits). The slope and the baseline locomotion values were not significantly different between the two groups. There were also significant differences in the response to meth in the two groups of animals as shown in Fig. 3B and 3C. It can be seen that meth induces a dose dependent increase in locomotion in the control mice. However there is no dose effect between 1.0 and 3.0 mg/kg in the Shn-2 KO mice. Further, they do not show as large difference compared to the pre-meth as the WT mice. There was a similar increase in initial locomotor activity before amph in the Shn-2 KO mice. After amph there was a large increase in locomotor activity in the WT mice but not in the Shn-2 KO mice similar to meth. The data were compared as a function of time as Pre (first 60 min), Post (first 60 min after meth or amph) and Post2 (60-120 min after meth). The differences between the Pre-condition for the Shn-2 KO and control mice were similar to that seen in Fig. 3A, with a very significant effect sizes (Cohen's d) at 0.3, 1.0 and 3.0 mg/kg meth respectively of 1.33 ± 0.7 , 1.45 ± 0.48 and 1.45 ± 0.51 (p for all < 0.01). The differences between the Pre-condition for the Shn-2 KO and WT mice was similar to that seen in Fig.

3A, with a very significant effect sizes (Cohen's d) at 0.3, 1.0 and 3.0 mg/kg meth respectively of 1.33 ± 0.7 , 1.45 ± 0.48 and 1.45 ± 0.51 (p for all < 0.01) and an effect size of 1.96 ± 0.89 ($p < 0.05$) for amph. Differences between Pre and Post and Post2 in the Shn-2 KO and WT animals were significant at most doses showing that the effects of amph were dependent upon the genotype of the mice (Fig. 3E).

4. Discussion

Translocator protein (TSPO) is mostly expressed in the activated microglia and linked to the inflammatory responses that occur after ischemic brain injury and some neurodegenerative diseases [21]. It is present in low levels in normal brain parenchyma but it is upregulated locally in response to damage [22]. Therefore TSPO is a potential marker for neuroinflammatory processes and a radiolabeled ligand such as [^{11}C]PBR28 is already used in multiple sclerosis and Alzheimer's disease in humans [11].

Shn-2 KO mice exhibit mild chronic neuroinflammation evidenced by increased inflammation markers in postmortem brain [25]. We measured the extent of neuroinflammation in Shn-2 KO mice using [^{11}C]PBR28 PET imaging. Previous studies done using (*R*)-[^{11}C]PK11195, which is another ligand that binds to TSPO, found increased binding in whole brain gray matter and particularly in the hippocampus of schizophrenia patients [6]. However more recent studies using [^{18}F]FEPPA did not find significant difference in neuroinflammation between treated schizophrenia patients and the control group [10]. Takao et al. reported inflammatory-like phenomena only in the hippocampus but it is a challenging task to perform immunohistochemistry on the whole brain [25]. However we found here significantly increased uptake of [^{11}C]PBR28 in most brain regions of Shn-2 KO mice that indicates that inflammatory processes are global rather than localized like in a rat model of ischemic stroke [26]. Our study showed the possibility of detecting activated microglia in the whole brain *in vivo* using PET. Another interesting finding to arise from the [^{11}C]PBR28 studies in Shn-2 KO mice was the significantly increased inflammation observed in the olfactory bulb. Impaired olfactory function is present in individuals with schizophrenia and the first degree family members[16]. A recent study showed the

involvement of the olfactory bulb in PPI regulation by blockage of olfactory sensory input using lidocaine/MK801 injection [19]. Thus the decreased PPI shown in Shn-2 KO mice [25] may be related to the inflammation in the olfactory bulb.

mGluR5 is expressed in several brain regions and preclinical studies support agonism or positive allosteric modulation of mGluR5 as a potential therapeutic target for schizophrenia via links between this receptor and the NMDA glutamate receptor [12]. Also emerging evidence suggests that aberrant mGluR5 function may lead to mental disorders such as schizophrenia [14]. The expression of mGluR5 in schizophrenia patients has been studied for more than a decade but mostly on postmortem brains. Recently our laboratory developed [^{18}F]FPEB, a specific PET radiotracer for mGluR5 and it has been tested in humans for safety and tolerance [27]. We used [^{18}F]FPEB to investigate mGluR5 expression in Shn-2 KO mice and found significantly increased [^{18}F]FPEB binding in the cortex and hippocampus. Mild chronic inflammation in Shn-2 KO mice may increase the expression of mGluR5 to prevent further damages that are associated with activated microglia or astrocytes. Excitotoxic ibotenic acid induced lesion in neonatal ventral hippocampus in rats led to significant and persistent astrogliosis and microglial activation and this response was accompanied by an increase in mGluR5 expression [7]. Also inflammation induced by Freund's adjuvant (CFA) significantly increased the number of plasma membrane associated mGluR5 immunogold-labeled particles in the neurons of spinal cord in rats [20]. There are studies reporting that the activation of mGluR5 can reduce microglial associated inflammation in spinal cord injury [5]. The advent of new PET ligands to study mGluR5 will improve our ability to image this receptor *in vivo*. Studies of mGluR5 using PET in humans will generally lead to better quantification than in mice since the partial volume effects caused by the small size of the mouse brain compared to the resolution of the imaging device (about 1.2 mm) will likely lead to lower and noisier uptake values. However, since this drawback affects all our data similarly it does not change the overall conclusions.

Lipina et al. [13] reported that mGluR5 KO mice show baseline hyperactivity and impaired PPI that are also observed in Shn-2 KO mice. Thus it is certain that mGluR5 expression can play a role in

the regulation of locomotor behavior. The observation of novelty-induced hyperlocomotion in the Shn2-KO mice is consistent with other rodent models of schizophrenia. For instance, chronic PCP administration leads to a hyperlocomotive phenotype that is thought to mimic some of the positive symptoms of schizophrenia [18]. Takagi et al. previously reported that Shn-2 KO mice showed spontaneous hyperactivity during the open field testing and acclimatization contributes to partial suppression of anxiety like behavior [24]. The novelty-induced hyperlocomotion was noted consistently in all the Shn-2 KO animals studied. The response of the animals to both meth and amph was smaller than that observed in the control mice (Fig. 3). however it is difficult to say how much of this was related to the increased basal hyperlocomotion, thus, making the differences between the Shn-2 KO and control mice seem smaller. The locomotor activity seen after injection of psychostimulants was not as profoundly different between the WT and Shn-2 KO mice as the pre-drug contrasts. Increased locomotor activity is often associated with elevated dopaminergic tone and baseline dopamine release is increased in drug-naïve patients with schizophrenia [1]. Thus spontaneous hyperactivity of Shn-2 KO mice may be due to the increased extracellular dopamine levels in these mice. However our [¹¹C]raclopride study did not show a significant difference in dopamine D2 receptor binding potential between two groups (not published). Typically indirect dopamine agonists like amph and methylphenidate, when administered to schizophrenic patients increase symptoms such as psychosis and disorganized thought [9]. However, the response to other effects of amph such as insomnia and anorexia has been measured to be blunted in some schizophrenic subjects compared to controls [17]. Thus, the lower response that we see suggests that this behavioral outcome may better reflect these other amph properties and not so much the florid psychotic symptoms seen in schizophrenics with amph administration.

Much effort has been made to generate an adequate rodent model of schizophrenia and it is a daunting task to satisfy all the complexities of schizophrenia with a single model. The *in vivo* markers used in this study i.e. inflammation and mGluR5, combined with the extensive prior molecular and

behavioral data [25, 26], make this one of the stronger candidates yet developed for a pre-clinical schizophrenia model.

Conclusions

Shn-2 gene knock-out induced inflammation that was observed in cortex, striatum, hippocampus, olfactory bulb, and cerebellum. Expression of mGluR5 was increased in the cortex and hippocampus. Shn-2 KO mice showed significant basal hyperactivity what was diminished after amphetamine challenge. Overall our results agree with other literatures that explored various preclinical models of schizophrenia and some aspects of schizophrenia patients. Our results support that Shn-2 KO mice can be used as a potential model for schizophrenia.

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Figure Legends

Figure 1: Binding of [^{11}C]PBR28 and [^{18}F]FPEB to Shn-2 KO and their littermate control mice show significant difference between two groups.

Figure 2: A) Bar graph of binding of [^{11}C]PBR28 to Shn-2 KO (n=5) and the control mice (n=8). Shn-2 KO mice show significant increases in several brain regions compared to the control mice. Cb:

***p<0.001, Cortex, Striatum, Hipp, and wholeBrain:**p<0.01, Olf: *p<0.05.B) Bar graph of binding of

[¹⁸F]FPEB to Shn-2 KO (n=6) and control mice (n=8). There are significant increases in Cortex (***p<0.001) and Hipp (*p<0.05). Other brain regions show no significant changes. Abbreviations: Olf (olfactory bulb), Hipp (hippocampus), Hypo (hypothalamus) and Cb (cerebellum).

Figure 3: Open field locomotor behavior. Total distance traveled is in cm. A) Novelty-induced locomotion in the Shn-2 KO mice. These data were averaged from the three saline data sets collected with (n=10-12 animals/group/data set). The data were fitted to a sigmoidal logistic function showing significant differences in novelty locomotion (567.0 ± 11.7 for the Shn2-KO vs. 189.1 ± 4.8 cm for controls ($p < 0.001$). B) Effects of i.p. meth at the specified doses on locomotor activity in control mice. There is a large increase in locomotor activity induced by the higher doses of meth (n=9-12 per dose). C) Effects of meth on locomotor activity in the Shn-2 KO mice (n=11-12). D) Effects of 1 mg/kg amph on locomotor activity in control (n=8) and Shn-2 KO (n=3) mice. E) Effect sizes (Cohen's d) for the differences between the control and Shn2 KO mice in the mean distance traveled comparing the differences of the pre-drug period (Pre) and the 60 min post-drug (Post), and the 60-120 min after drug (Post2). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 1

Figure 2

Figure 3