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# Serotonin-Dopamine Interactions in *Pet*-1 Knockout Mice

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## ABSTRACT

Serotonin (5-HT) has been implicated in playing a role in the release of dopamine (DA) in the prefrontal cortex of the brain. Interactions between these two neurochemicals are believed to play a role in the regulation of brain functions such as memory and emotion. *Pet*-1 knockout (KO) mice are transgenic mice lacking a transcription factor that specifically regulates the development of brain 5-HT systems. As a consequence, most forebrain 5-HT neurons are not present in these mice. They will be used in this study to assess the effect of 5-HT depletion on the release of DA. The variable that will be measured is DA release in the prefrontal cortex in *Pet*-1 KO mice and wild-type controls. DA levels will be compared both before and after exposure to a mild psychological stressor. It is hypothesized that basal and stress-induced DA release will be diminished in KO mice. *In vivo* microdialysis will be used to collect extracellular DA in the mouse prefrontal cortex. In this technique, a microdialysis probe, which is perfused with a physiological solution and consists of a semipermeable membrane across which DA and 5-HT can pass, will be implanted into the mouse brain during surgery. The next day, the microdialysis probe will be perfused at a rate of 1.5 μl/min and dialysate samples will be collected every 20 minutes. The stressor (gentle handling) will be applied for 20 min once DA levels are stable. High Performance Liquid Chromatography (HPLC) with electrochemical detection will be used to measure the amount of DA in the dialysate samples. In HPLC, the fluids collected from the probes will be forced through a column (stationary phase) by introduction into the mobile phase. The neuro-

chemicals can be differentiated by different times at which they will interact with the stationary phase as they travel through the column. DA will be oxidized at a carbon surface within the electrochemical detector; the resultant current flow will be quantified and will be proportional to the amount of DA in the samples. After the experiment is completed, brains will be analyzed histologically to confirm placement in the prefrontal cortex. The data (pg DA/20 μl) will be expressed as a percentage of the last 3 baseline samples. It is predicted that the levels of DA observed in the prefrontal cortex will be higher in the wild-type mice than in the *Pet*-1 KO mice. This result would indicate that physiologically released 5-HT normally acts to stimulate the release of DA. As a result, the regulation of DA release by the actions of the 5-HT system will be more thoroughly understood, and pharmacotherapy will be advanced in the treatment of mood and cognitive disorders.

## INTRODUCTION

Two major neurochemicals that regulate the behavior and emotions of animals are dopamine (DA) and serotonin (5-HT). An insufficient amount of serotonin in the brain has been associated with many mood disorders, including depression, and a variety of anxiety disorders (Nestler et al., 2001). Abnormalities in dopaminergic transmission have been implicated in illnesses such as schizophrenia and Parkinson's disease (Alex & Pehek, 2006). Furthermore, interactions between DA and 5-HT may play a role in the etiology/treatment of these disorders.

There are three main pathways that ultimately serve to release DA in the brain (Wolf et al., 1987). The mesolimbic pathway begins in the ventral tegmental area (VTA) of the midbrain and extends to the limbic system, including the nucleus accumbens. These neurons play a major role in memory, emotional responses, and rewards. The nigrostriatal pathway projects from the substantia nigra to the striatum, and regulates voluntary motor activity. The mesocortical pathway begins in the VTA and terminates in the prefrontal cortex. DA released at the terminus of this pathway regulates cognitive processes such as attention and memory (Alex & Pehek, 2006).

Serotonergic neurons arise in the raphe nuclei of the brain. These neurons project to many forebrain sites including the limbic system, striatum, and prefrontal cortex (Alex & Pehek, 2006). The cell bodies and terminal regions of the DA pathways are innervated by 5-HT neurons; studies have proven the existence of direct synapses between 5-HT terminals and DA cells in the midbrain (Herve et al., 1987; Nedergaard et al., 1988), suggesting a possible effect of 5-HT on DA neurons and DA production (Alex & Pehek, 2006).

One particular class of 5-HT receptors, known as 5-HT2A receptors, has been implicated in the regulation of the action of corticotegmental projection neurons which regulate the activity of DA neurons (Pehek et al., 2006). Thus, 5-HT2A receptors are believed to stimulate DA release in all three of the aforementioned DA pathways. In particular, DA release in the mesocortical system was shown to be increased upon stimulation of the cortical 5-HT2A receptors. These receptors have also been suggested to play a modulating role in DA release triggered by causes such as physiological stress (Devaud et al., 1992; Pehek, 1996; Pehek & Bi, 1997; Shi et al., 1995; Gobert & Millan, 1999; Pehek et al., 2001; De Deurwaerdere & Spampinato, 1999; Schmidt

& Fadayel, 1996; Zhang et al., 2000). 5-HT2C receptors have also been suggested to have an effect on DA activity; their localization in the brain (on DA receptors in the VTA) would suggest this role (Bubar et al., 2005; Ji et al., 2006). Evidence indicates that 5-HT2C receptors inhibit DA release in the striatum, nucleus accumbens, and prefrontal cortex of the brain (Alex & Pehek, 2006). The presence of dopamine and serotonin in the prefrontal cortex is particularly significant, because structures in this area of the brain have a profound influence on control of emotions, memory, and the response to rewards. Alterations in the levels of DA and 5-HT may be the cause of mood disorders such as schizophrenia, depression, and panic and anxiety disorders (Nestler et al., 2001).

The *Pet*-1 gene is a gene that has been found to be required for generation of normal serotonin neurons in the brain; the mice that lack this gene lack 80% of the normal amount of 5-HT neurons, and the neurons that are present in the adult mouse are generally defective. The *Pet*-1 gene is imperative for normal levels of anxiety and aggression. *Pet*-1 KO mice are deficient in neuronal tissue content of 5-HT as a result of the lack of 5-HT neurons in the forebrain. In mice that do not express the *Pet*-1 gene, most of the serotonin neurons do not differentiate, and the ones that do differentiate do not express the gene adequately for normal 5-HT production. Thus their behavior is significantly more impulsive, reflecting the lack of control over anger and anxiety due to the lack of 5-HT neurons (Hendricks et al., 2003).

While the tissue content of 5-HT is lower in *Pet* -1 KO mice, no studies have measured the actual extracellular release of 5-HT. Additionally, the lack of forebrain 5-HT in these mice may alter the amount of DA release. The present study examined the effects of neuronal 5-HT depletion (characteristic of *Pet*-1 KO mice) on extracellular release of DA and 5-HT by microdialysis in Pet-1 KO and wild type (WT) mice. Since the release of 5-HT in the brain has previously been shown to stimulate the release of DA, we hypothesized that the DA levels observed will be higher in the wild-type mice than in the KO mice, due to the higher amount of 5-HT present in the WT mice.

## METHODS

***Animals.*** The mice were PET heterozygote (+/-), KO (-/-), and WT (+/+) littermates generated by and obtained from Dr. Evan Deneris, Dept. of Neurosciences, Case Western Reserve University. Male and female mice were housed separately, with four mice per cage. There was a 12/12 hour light/dark cycle, and food and water were available ad libitum. All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals.

***Surgery.*** Animals were first anesthetized via isoflurane (0.1 to 0.5% balance air to $O_2$). This anesthesia was employed throughout each surgery, and the level of anesthesia would be adjusted as needed. Using a stereotaxic instrument, a microdialysis probe was implanted into the prefrontal cortex of the mouse. Bregma was first located, and then the probe was implanted $\pm$0.300 mm laterally, 2.300 mm anteriorly and 3.500 mm ventrally (Paxinos & Franklin, 2004).

***Microdialysis.*** *In vivo* microdialysis was employed using the implanted probe, in order to analyze the extracellular fluids (particularly the amounts of DA, 5-HT, and relevant metabolites) in the brain of the

mouse. The probes were of a concentric flow design with a 2.0 mm active dialyzing surface membrane (Carnegie-Medicin (CMA-7) MW cut-off = 6,000 Daltons, outer diameter=0.24 mm). The probe was connected to both inflow and outflow tubing. Through the inflow tubing, it was perfused with artificial cerebrospinal fluid (aCSF) that was devoid of the compounds that would ultimately be analyzed. In microdialysis, the compounds of interest diffuse from the extracellular fluid of the brain, through the dialysis membrane, into the aCSF pumped through the probe. Through the outflow tubing, the solution being perfused, along with the collected compounds from the brain, travel so that they can be collected (Shippenberg & Thompson, 1997). aCSF was prepared by adding 1.2 mM $CaCl_2$ and 5.0 mM glucose to Dulbecco's solution and was pumped through the probe using a Harvard infusion pump at a rate of 1.5 μL/min. Dialysate samples were collected from the mouse every 20 minutes. Baseline samples were recorded until the values (as shown by high performance liquid chromatography) of the neurochemicals/metabolites in the samples were stable over the course of 3 samples.

***High Performance Liquid Chromatography (HPLC) with electrochemical detection.*** The physiological fluids collected from the mouse were analyzed using reverse phase HPLC. In HPLC, the compounds injected into the apparatus travel through a column which contains both a stationary and a mobile phase. HPLC is dependent upon the compounds being distributed differently between the stationary and mobile phases within the column of the machine. The compound injected into the apparatus is essentially dissolved in the mobile phase, and it moves through the column with the mobile phase (composed of 32 mM anhydrous citric acid, 54 mM sodium acetate trihydrate, 0.074 mM EDTA, 50 mg/L octylsulfonic acid, and 3% methanol; pH=4.2). However, different compounds interact to different extents with the stationary phase of the column, which is responsible for the differing elution times (times taken for different compounds to reach the detector). The compounds which interact the least with the stationary phase will exhibit the shortest elution times (Holman, 1993). The HPLC system consisted of a Schimadzu LC-ADvp solvent delivery system and a Bioanalytical Systems (BAS) LC-4C electrochemical detector equipped with a glassy carbon electrode. This electrode was maintained at a potential of +0.6 V relative to a reference electrode. Compounds were oxidized at the surface of this electrode and the current (electron flow) generated was converted to peaks. The respective height of each peak was proportional to the amount of the compound that it represented.

In this study, after standards containing DA and 5-HT as well as the metabolites 5-HIAA, HVA, and DOPAC were injected, the equipment was calibrated for the experiment based on the elution times and peak heights of the components of those standards. The samples of physiological fluids from the mouse (which contain the same compounds present in the standards) were then be injected onto a 2 x 100 mm Phenomenex (Torrance, CA) column (Ultracarb™, 3μm particle size, ODS 20). 20 μl of each sample were injected into the column. In cases of less than 20 μl of the sample being available, the amount present was injected, and an extrapolation was performed to estimate the amount of each neurochemical/metabolite in a normal 20 μl sample.

***Histology.*** After the experiments are run, the mouse was euthanized via an injection of 0.3 mL of sodium pentobarbital. After this, the brain was removed, and the brain was sectioned with the aid of cryostat to ensure that the probe was placed properly during surgery. Slides were then stained with cresyl violet dye in order to clearly visualize the probe track. Data was discarded from mice with incorrect probe placements.

***Experimental design.*** This study was divided into two experiments using the PET mice.

Experiment 1: Heterozygotes were used to determine whether DA, 5-HT, and metabolites could be successfully measured via *in vivo* microdialysis in the prefrontal cortex (PFC) of mice.

Experiment 2: The wild-type and knockout mice were compared following two different were compared following two different experimental treatments. In the control condition, the baseline data was collected, and then subsequent samples were collected in the same manner, without changing the environment of the mouse in any way. In the other treatment, the mouse was subjected to a mild stressor during the course of the experiment. During the second sample collected for the data, the mouse was stroked for the entire 20 minutes, as well as exposed to light (the cover normally placed on the cage was removed during this time).

## RESULTS

***Experiment 1.*** 4 PET heterozygotes (+/-) were used. DA, 5-HT, and the associated metabolites (DOPAC, HVA, and 5-HIAA) were shown to be measurable by *in vivo* microdialysis in the extracellular fluid of the PFC. Levels were relatively stable across time except for an unexpected increase in DA at time 20 (see Figure 1).

***Experiment 2.*** Levels of DA, 5-HT, and metabolites were compared between WT and KO mice. The KO mice (n=3; average basal level 0.52 $\pm$ 0.16 pg/20 µl) did not appear to have a lower amount of DA than the WT mice (n=3; average basal level 0.47 $\pm$ 0.15 pg/20 µl). The levels of DA in each group over time are shown in Figure 2. The KO mice (n=3; average basal level 0.55 $\pm$ 0.08 pg/20 µl) also do not appear to have a

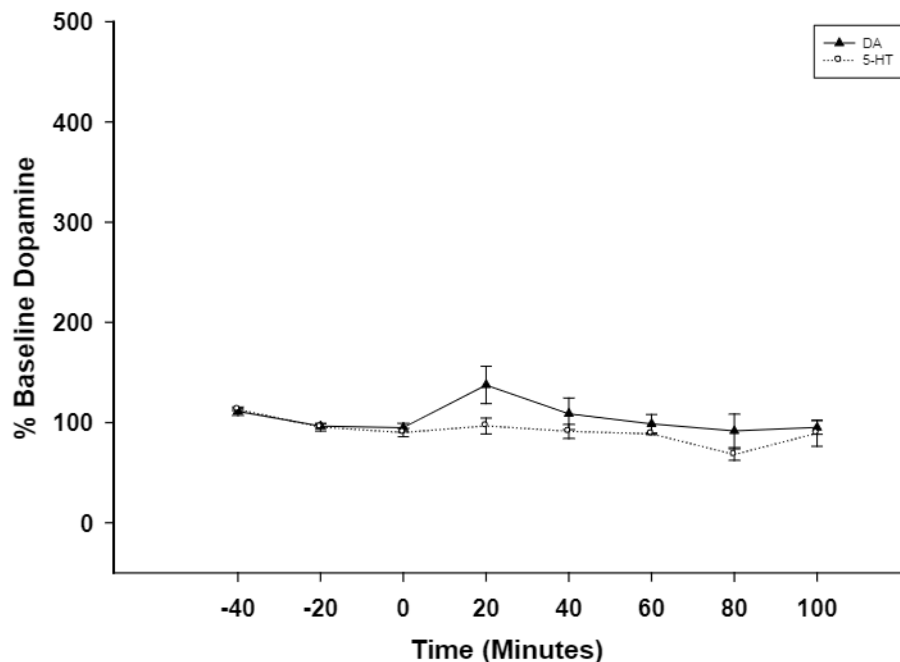


Figure 1. DA and 5-HT levels in PET +/- mice as taken from HPLC with electrochemical detection. The levels of DA and 5-HT are consistent with one another.

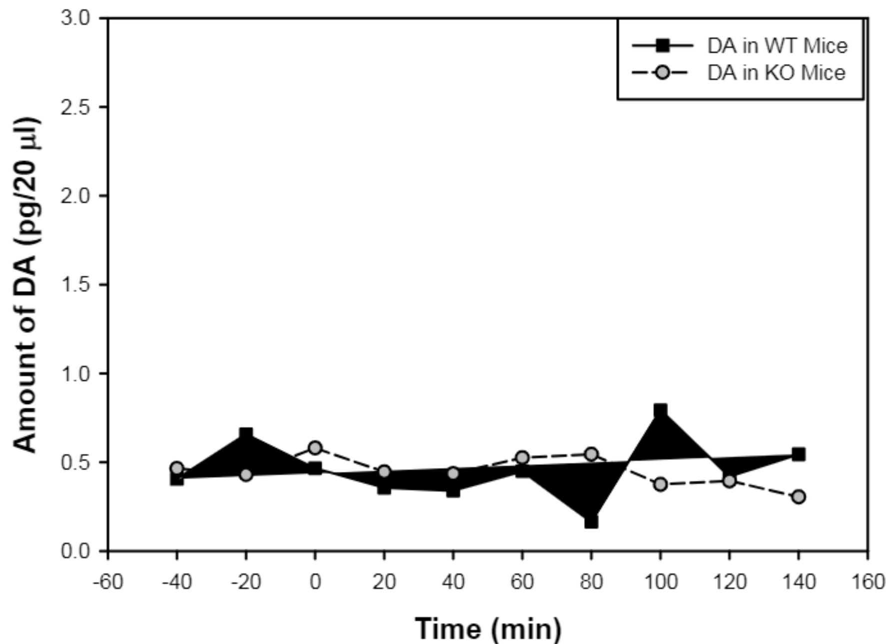


Figure 2. DA levels in WT and KO mice. The KO mice (preliminary data; n=3) do not appear to produce lower levels of DA than do the WT mice (preliminary data; n=3).

lower amount of 5-HT than the WT mice (n=3; average basal level 0.37 $\pm$ 0.12 pg/20 μl). The levels of 5-HT in each group over time are shown in Figure 3. The stress applied during the experiment did not appear to have an effect.

Though the differences between the groups in terms of DA and 5-HT appear to be insignificant at this point, there are differences in metabolite levels. The most significant difference is the amount of extracellular 5-HIAA; the KO mice (n=3; average basal level 11.03 $\pm$ 2.42 pg/5 μl) appear to produce significantly less 5-HIAA than do the WT mice (n=2; average basal level 66.80 $\pm$ 27.81 pg/5 μl) (see Figure 4). KO mice (n=3; average basal level 32.46 $\pm$ 5.56 pg/5 μl) appear to produce somewhat less DOPAC than do WT mice (n=2; average basal level 50.99 $\pm$ 17.93 pg/5 μl). Also, the KO mice (n=3; average basal level 142.54 $\pm$ 26.55 pg/5 μl) appear to produce significantly more HVA than do the WT mice (n=3; average basal level 90.58 $\pm$ 27.45 pg/5 μl).

## DISCUSSION

The present results verified that *in vivo* microdialysis could be used to measure neurochemicals and metabolites in the mouse PFC. Interestingly, even though the levels of DA and 5-HT did not differ significantly between the two groups as expected, the levels of metabolites were different. The *Pet*-1 KO mice produced less extracellular 5-HIAA and DOPAC, and more HVA, than did the WT mice. The difference in 5-HIAA was the most noticeable and significant difference. Because of the small sample sizes thus far, more testing is needed to verify the significance of these trends.

Previous studies measuring neurochemicals in the brain have primarily used rats; microdialysis has been used to obtain results from the PFC of the rat (e.g. Pehek et al., 2006). Though extracellular neurochemicals had been quantified in other regions of the mouse brain, such as the nucleus accumbens core and striatum (Thomas et al., 2007), quantification in the PFC is difficult because of the relatively sparse innervation of this region of the brain with 5-HT and DA neurons.

While different studies have been done to elucidate the function of the *Pet*-1 gene (Hendricks et al., 2003), as well as the details of 5-HT neuron development (Gaspar, 2004; Stankovski et al., 2007), the role of

the *Pet*-1 gene in regulating neurotransmitter release has not been studied. While 5-HT tissue content is known to be depleted in *Pet*-1 KO mice (Hendricks et al., 2003), this is the first study to measure *extracellular* release. The results indicate that extracellular 5-HT levels are maintained despite a profound loss of 5-HT neurons in the knockout mouse.

While previous findings would suggest that there would be a lower amount of 5-HT measured in KO mice than in WT mice, the data from this study at this point do not support this assertion. The DA and 5-HT levels would be expected to be significantly lower in the KO mice than in the WT mice, but the amounts of these neurochemicals do not appear to be lower. However, the metabolites DOPAC, HVA, and 5-HIAA appear to be significantly different between the two groups. These results, though preliminary, indicate that the effect of the *Pet*-1 gene is exercised chiefly on the metabolites rather than on the extracellular levels of DA and 5-HT.

One major limitation to this study is the possibility that the fluids collected from the brain during microdialysis are not truly representative of the neurochemicals and metabolites being released by neurons, since there are other cells in the brain that release these chemicals. However, previous experiments in rats have shown that dialysate levels of DA and 5-HT are $Ca^{2+}$-dependent and increase in the presence of high $K^+$ (e.g. Moghaddam & Bunney, 1990), indicating that the measured release is truly neuronal. It is likely that dialysate DA and 5-HT are of neuronal origin in the mouse as well, although this should be tested in the future. Another limitation is that the deletion of the *Pet*-1 gene during development may have produced effects other than 5-HT neuronal loss that would confound interpretation of the results.

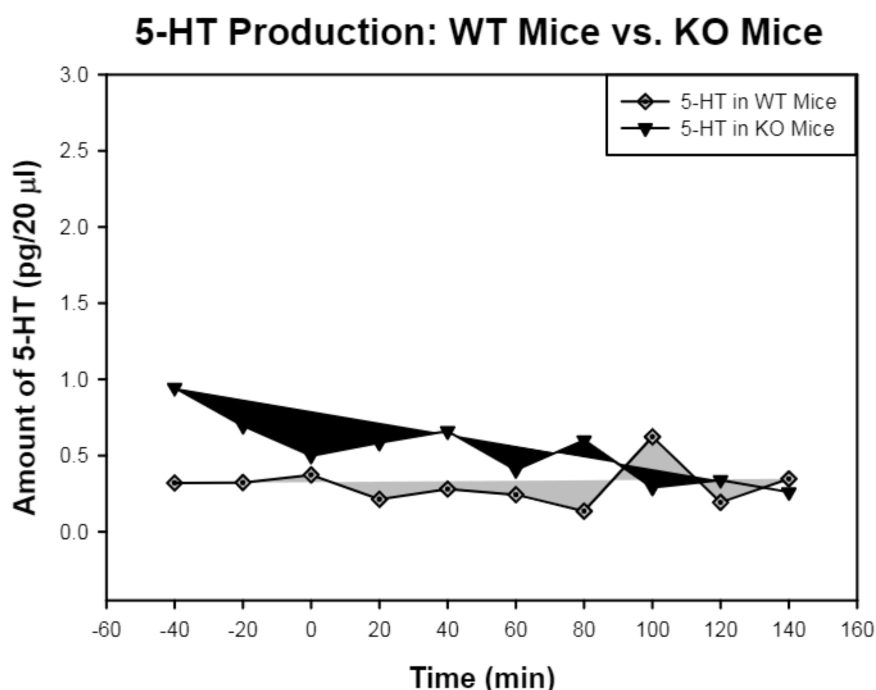


Figure 3. 5-HT levels in WT and KO mice. The KO mice (preliminary data; n=3) do not appear to produce lower levels of 5-HT than do the WT mice (preliminary data; n=3).

The difference in metabolites, particularly 5-HIAA, introduces a new possible direction to explore with these mice. It is likely that this effect is due to the depletion of 5-HT neurons in the *Pet*-1 KO genotype. The absence of the *Pet*-1 gene in the KO mice could cause a higher percentage of the 5-HT to be released from the neurons, which may explain the similar amount of 5-HT found in WT and KO mice. The gene may also alter the metabolism of 5-HT. Monoamine oxidase (MAO) is the enzyme that oxidizes 5-HT to

form 5-HIAA. It is possible that MAO may be inhibited in the knockout mice in order to preserve extracellular 5-HT concentrations. Reuptake inhibition is also a possibility. The preservation of extracellular 5-HT may be a mechanism present in order to keep the 5-HT levels as close to normal as possible, due to the inherent drive for homeostasis within the animal. Though the mechanism by which the number of 5-HT neurons would affect the amount of extracellular 5-HIAA is not currently known, it introduces the possibility of new implications that can elucidate factors that affect the release, metabolism, and reuptake of 5-HT and thus aid in providing new genetic and pharmacological treatments for mood disorders that are affected by the amounts of DA and 5-HT in the brain.

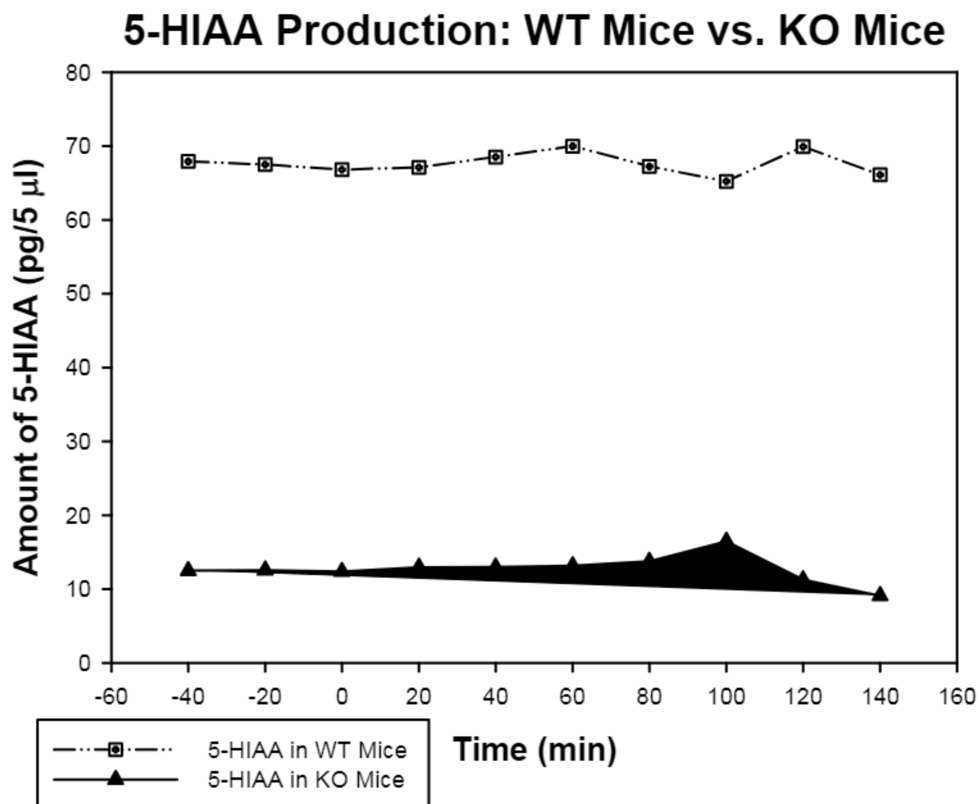


Figure 4. 5-HIAA levels in WT and KO mice. The KO mice (preliminary data; n=3) appear to produce significantly lower levels of 5-HIAA than do the WT mice (preliminary data; n=2).

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