Electrophysiological assessment of serotonin and GABA neuron function in the dorsal raphe during the 3rd trimester-equivalent developmental period in mice

Serotonin system development during the 3rd trimester equivalent

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Title Page

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Abstract

Alterations in the development of the serotonin system can have prolonged effects including depression and anxiety disorders later in life. Serotonin axonal projections from the dorsal raphe undergo extensive refinement during the first two weeks of postnatal life in rodents (equivalent to the 3rd trimester of human pregnancy). However, little is known about the functional properties of serotonin and GABA neurons in the dorsal raphe during this critical developmental period. We assessed the functional properties and synaptic connectivity of putative serotoninergic neurons and GABAergic neurons in the dorsal raphe during early (postnatal day (P) P5-P7) and late (P15-P17) stages of the 3rd trimester-equivalent period using electrophysiology. Our studies demonstrate that GABAergic neurons are hyperexcitable at P5-P7 relative to P15-P17. Furthermore, putative serotonin neurons exhibit an increase in both excitatory and GABA\textsubscript{A} receptor-mediated spontaneous postsynaptic currents during this developmental period. Our data suggest that GABAergic neurons and putative serotonin neurons undergo significant electrophysiological changes during neonatal development.
Significance Statement:

During development serotonin plays a major role in neuronal proliferation and differentiation, axonal migration, and synaptogenesis. However, the understanding of the functional development of this neurotransmitter system is limited. We characterized the functional properties of developing GABAergic and serotonergic neurons in the dorsal raphe that release 5-HT into many forebrain regions. Our studies indicate that both GABAergic and serotonergic neurons undergo significant functional maturation during the first two postnatal weeks in mice. Therefore, alterations of the serotonin system induced by stress, drugs, or anti-depressants during this critical period could have deleterious effects within the dorsal raphe as well as other brain regions innervated by serotonergic axons.
Introduction

Serotonin (5-hydroxytryptamine; 5-HT) belongs to the monoamine family of neurotransmitters and is synthesized from tryptophan by tryptophan hydroxylase. The 5-HT system is involved in the control of mood, arousal, and a number of cognitive processes. Alterations in this neurotransmitter system have been linked to several neuropsychiatric conditions, including depression, and anxiety disorders (Nikolaus et al., 2010, Ravindran and Stein, 2010, Lin et al., 2014, Olivier, 2015). Deficits in the interplay between 5-HT and other neurotransmitters, such as dopamine, play a role in the pathophysiology of schizophrenia, attention deficit hyperactivity disorder, and addiction (Kapur and Remington, 1996, Oades et al., 2008, Kirby et al., 2011). The cell bodies of 5-HT neurons are located in the raphe nuclei of the midbrain, pons, and medulla. Within the midbrain, these neurons can be found in the median and dorsal raphe (DR) nuclei and their axons project to many brain regions including the cerebral cortex, thalamus, and hippocampus (Jacobs and Azmitia, 1992). Local inhibitory GABAergic neurons regulate the excitability of DR 5-HT neurons and mediate feed-forward inhibition driven by the prefrontal cortex (Celada et al., 2001). Importantly, this GABAergic modulation has been shown to play a role in the sleep-wake cycle (Gervasoni et al., 2000) and the acquisition of avoidance after social defeat (Challis et al., 2013). Furthermore, alterations in GABAergic signaling in the dorsal raphe play a role in anxiety induced by withdrawal from either cocaine or alcohol (Craige et al., 2015, Lowery-Gionta et al., 2015).

During development, 5-HT is a key modulator of neuron proliferation and differentiation, axon migration, and synaptogenesis (Frederick and Stanwood, 2009). Disruption of 5-HT levels or 5-HT receptor function during development causes emotional and cognitive deficits that last into adulthood. These deficits could be a consequence of abnormalities in neuronal circuitry assembly due to altered brain-derived neurotrophic factor levels (Vitalis et al., 2007, Lo Iacono and Gross, 2008, Migliarini et al., 2013, Witteveen et al., 2013, Donaldson et al., 2014, Suri et al., 2015). Studies with rodents have demonstrated that 5-HT neurons are born approximately on embryonic day 10.
(E10) and begin to produce 5-HT between E12 and E13. 5-HT axons elongate between E13-E16 and begin to reach their targets in the developing forebrain by E18 (Bonnin and Levitt, 2011). In human and non-human primates, these processes have been shown to occur during the 1st and 2nd trimesters of pregnancy (Takahashi et al., 1986, Whitaker-Azmitia, 2001). During the first two weeks of life in rodents (equivalent to the 3rd trimester of human pregnancy) (Workman et al., 2013), 5-HT production dramatically increases and the majority of innervation of the forebrain by 5-HT axons occurs. However, the axonal refinement continues until P21 (Lidov and Molliver, 1982, Lambe et al., 2000). The electrophysiological properties of dorsal raphe 5-HT neurons also change significantly during this period. Between P4 and P12, the membrane potential becomes more negative, the membrane resistance increases, action potential firing in response to current injection decreases, and excitatory and inhibitory synaptic currents begin to be detectable (Rood et al., 2014). These findings indicate that the 5-HT neurotransmitter system is significantly refined during the 3rd trimester-equivalent developmental period, making it particularly vulnerable to a variety of insults, such as exposure to substances of abuse (e.g., ethanol, cocaine), medications, and environmental toxins.

In this study, we investigated the electrophysiological properties of GABAergic neurons in comparison to putative 5-HT neurons in the DR at P5-P7 and P15-P17. Our data show that putative 5-HT neurons (in agreement with previously published data (Rood et al., 2014) and GABA neurons in the DR undergo significant electrophysiological changes during the first two weeks of postnatal life.
Materials and Methods

Animals and Slice preparation

All animal procedures were performed in accordance with the authors’ University Institutional Care and Use Committees. Using the Venus fluorescent protein developed by Dr. Atsushi Miyawaki at RIKEN (Wako, Japan), Dr. Yanagawa’s and colleagues generated the vesicular GABA transporter (VGAT)-Venus mouse line (Wang et al., 2009). We bred VGAT-Venus heterozygous females with wild-type C57BL6 males. Transgenic females were bred with wild-type C57BL6 males. Male mice were removed once pups were born. The VGAT-Venus pups were visually screened within the first 2 postnatal days using fluorescence equipped goggles with an 480/40 nm excitation filter, and a long pass 520 nm emission filter (BLS ltd. Budapest, Hungary). Both male and female pups were used for all experiments. For brain slice preparation, animals were heavily anesthetized with 0.75 g/kg ketamine followed by decapitation. Brain tissue was removed and incubated for 2-4 minutes in oxygenated ice cold cutting solution containing (in mM): KCl, 2; NaH₂PO₄, 1.3; NaHCO₃, 26; MgSO₄, 12; CaCl₂, 0.2; sucrose, 220; glucose, 10; ketamine hydrochloride, 1 μg/mL. Coronal brainstem slices were generated using a vibrating slicer (1000 Plus Vibratome, Leica, Bannockburn, Illinois) at a thickness of 250 μm. Slices were incubated in oxygenated artificial cerebral spinal fluid (ACSF) containing (in mM): NaCl, 125; KCl, 2; NaH₂PO₄, 1.3; NaCO₃, 26; glucose, 10, CaCl₂, 2; MgSO₄, 1 at 35°C for 40 minutes and allowed to recover at room temperature (21-22°C) for at least 30 minutes prior to recording. All chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless specified.

Electrophysiological Recordings

Slices were maintained in ACSF during recording at approximately 32°C. Neurons were morphologically identified by video monitoring of infra-red Differential Interference Contrast Microscopy using an Olympus BX51WI upright microscope (Olympus, Center Valley, PA) equipped with a metal-oxide semiconductor digital camera (Model 01-ROL-BOLT-M-12, Q-Imaging, Surrey,
Canada) with a LUMPlan Fl/IR 40x water immersion lens 0.8 N.A (Olympus). VGAT positive neurons were identified by Venus fluorescence using a mercury bulb, excitation filter ET470/40x, beam splitter T495ipxr, and emission filter ET525/50m (Chroma Technology Corp. Bellows Falls, VT).

Patch pipettes were pulled from thin wall filament-containing borosilicate capillary glass with a Sutter Flaming-Brown P-97 multi-stage puller (Sutter Instruments. Novato, California) resulting in resistances between 2 and 5 MΩ. Electrodes were filled with either K-Gluconate ((in mM): K-Gluconate, 130; NaCl, 5; Na-Phosphocreatine, 10; MgCl₂, 1; HEPES, 10; EGTA, 0.02; MgATP, 2; NaGTP, 0.5; pH 7.3 with KOH) or KCl ((in mM): KCl, 135; MgCl₂, 2; EGTA, 0.5; HEPES, 10; Mg-ATP, 5; Na-GTP, 1; QX-314-Cl, 1; pH 7.25 with KOH) internal solutions. Recordings were obtained with a Multiclamp 700B amplifier and a Digidata 1440A and data were acquired at 10 kHz with pClamp 9 software and filtered at 1 kHz (Molecular Devices Sunnyvale, CA).

To measure current injection-induced action potential firing, the whole-cell patch-clamp configuration was used in the current-clamp mode. No current was injected to the cells to maintain a certain membrane potential. Current injections ranging from -40 pA to 100 pA were injected in 10 pA increments to induce hyperpolarization and depolarization. Action potential properties were measured from the first action potential triggered by the 70 pA current injection to avoid any confounding effects of adaptation. The ΔV was calculated for each action potential and we generated the phase plots by plotting ΔV/Δt versus membrane potential (mV).

Spontaneous excitatory and GABA_A receptor-mediated postsynaptic currents (sEPSCs and GABA_A-PSCs, respectively) were recorded using the whole-cell patch-clamp configuration in the voltage-clamp mode. Excitatory currents were pharmacologically isolated using 10 µM Gabazine (SR 95531) [6-Imino-3-(4-Methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide] Tocris Bioscience, Minneapolis, MN) and inhibitory currents were isolated using 50 µM DL-APV [DL-2-Amino-5-phosphonopentanoic acid] Tocris Bioscience (Minneapolis, MN), 1 mM kynurenic acid
Sigma-Aldrich (St Louis, MO), and 1 μM CPG 54626 [S-(R*,R*)-3-[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid. For each cell the first 50 events were analyzed for each cell type at both ages using Mini-Analysis (Fort Lee, NJ).

Cumulative probability plots and averaged data was generated from each. The cumulative probability plots from all the cells of the same cell type and age were averaged together to generate the cumulative probability plots shown in the figures. The cumulative plots were analyzed using the Kolmogorov-Smirnov test. The averaged data from each cell was combined to generate the bar graphs.

Immunohistochemistry

Animals were anesthetized with isofluorane (Phoenix, Clipper Distribution Company, St Joseph, MO) and perfused with ice-cold phosphate-buffered saline (PBS) containing heparin sodium 1 unit/L followed by ice cold 4% paraformaldehyde in PBS. Brains were removed and immediately submerged in 4% paraformaldehyde and incubated for 48 hours at 4°C. After fixation, tissue was submerged in 30% sucrose in PBS until the tissue sank. Tissue was embedded in Optimal Cutting Temperature compound (Ted Pella, Inc. Redding, CA) and flash frozen in 2-methyl butane cooled in a dry ice ethanol bath. Tissue was stored at -80°C and 16 μm thick coronal sections were cut using a HM505E cryostat (Microm International, Germany). Sections were stained with monoclonal anti-Tryptophan Hydroxylase (1:500) (Sigma-Aldridge cat# T0678) (Calizo et al., 2011, Zhao et al., 2011) primary antibody and goat anti-mouse IgG conjugated to Alexa-555 (1:1000) (Invitrogen, # A21434) secondary antibody. DNA was labeled with NucRed live 647 (Molecular probes, Grand Island, NY) and were mounted using Fluormount-G (Electron Microscopy Sciences, Hatfield, PA). Low magnification images were acquired using an Olympus BX51 upright microscope (Olympus, Center Valley, PA) equipped with a 10x UPlanSApo/0.4 NA objective, mercury lamp, fluorescent filter sets.
and an Olympus DP72 CCD camera. Confocal images were acquired using a Zeiss 510 LSM inverted microscope (Carl Zeiss, Germany) equipped with an argon laser, and an emission filter of 535/30 nm for green fluorescence detection. For red fluorescence images, a 543 nm HeNe laser was used with an emission filter of 605/55 nm. Far-red fluorescence was visualized with a 633 nm HeNe laser with a long pass 560 nm emission filter. A 40x PlanApo/1.0 NA objective was used to collect images.

**Statistical Analysis**

All statistical analyses were performed with Graph Pad Prism 5.0 (San Diego, CA). All data sets were tested for a normal distribution using the D’Agostino & Pearson and tested for outliers using the Rout test with Q=1%. All statistical analyses of pooled data were performed using two-tailed Student’s t-test or Mann-Whitney tests, and the level of significance was considered when \( p < 0.05 \). All statistics are given and data are presented as group means and standard error of the means. Action potential properties were measured using the action potential waveform 2 analysis utility in Mini-Analysis program. Spontaneous EPSCs and GABA\(_{\alpha}\)-sPSCs were also analyzed with Mini-Analysis program (including analysis by Kolmogorov-Smirnov (K-S) test). For details of results of statistical analyses, please see Statistical Table.
Results

Distribution of GABAergic and serotonergic neurons in the dorsal raphe

Serotonergic neurons receive inhibitory inputs from local GABAergic neurons (Liu et al., 2000) that are localized to the lateral portions of the DR (Gocho et al., 2013). Immunohistochemistry and fluorescence microscopy were used to assess the local distribution of serotonergic and GABAergic neurons in the DR during the 3rd trimester-equivalent developmental period. DR sections between -4.24 mm and -4.84 mm from Bregma were obtained from P5-P7 and P15-P17 mice (Figure 1A-D). GABAergic neurons were identified by Venus fluorescence and serotonergic neurons were immunolabeled with anti-tryptophan hydroxylase. The anatomical distribution of both serotonergic and GABAergic neurons was similar to that previously reported (Calizo et al., 2011, Gocho et al., 2013, Cortein et al., 2014, Rood et al., 2014). Serotonergic neurons were localized to the medial and lateral wings of the DR, whereas the GABAergic neurons were localized to the lateral portions of the DR (Figure 1A and C). Higher magnification confocal images show that there was no overlap between the Venus and tryptophan hydroxylase fluorescence at either age (Figure 1B and D).

Serotonergic neurons located within different subfields of the DR have been shown to be electrophysiologically and morphologically distinct (Calizo et al., 2011) especially during this development period (Rood et al., 2014). Our studies focused on GABAergic neurons (i.e., Venus+) and putative 5-HT neurons (i.e., Venus-) located in and around the ventral medial subfield of the DR. Representative recording positions for both Venus+ and Venus- neurons in the DR are shown in Figure 1E and F. Passive membrane properties (membrane capacitance and resistance) were recorded with whole-cell patch-clamp electrophysiology from both Venus+ and Venus- neurons. The membrane capacitance was similar in Venus+ and Venus- neurons (~65 pF) and did not significantly change with age (Figure 1G; Venus+, Mann-Whitney U=67.50, n = 12 neurons from 7 animals, P=0.5828; and Figure 1I; Venus-, t(26)=0.3719, P=0.7130, n=15 neurons from 7 animals). Membrane resistance was also similar at the two ages in both Venus+ and Venus- neurons (Figure
Excitability of Venus+ and Venus- neurons

Active membrane properties of both Venus+ and Venus- neurons were examined using whole-cell current-clamp electrophysiology with no current injected at baseline. The resting membrane potential did not significantly change during this developmental period in Venus- ((P5-P7 = -57.02 ± 5.5 mV vs P15-P17 = -57.01 ± 5.8 mV (t(19)=0.001)) or Venus+ (P5-P7 = -55.87 ± 4.8 mV vs P15-P17 = -60.71 ± 2.7 mV t(17)=0.780)) neurons. Representative traces of events triggered by hyperpolarizing and depolarizing current injections are shown in Figures 2A and B. Input-output plots were generated by graphing the current injections from 0 pA – 100 pA in 10 pA steps versus the action potential frequency for each cell type at P5-7 and P15-17 (Figure 2Ce and Df). Venus+ neurons from the P5-P7 group fired at a higher frequency than those from the P15-P17 group (Figure 2C, two-way ANOVA: interaction F(10,150)=6.685, P<0.0001; current injected F(10,150)=74.14, P<0.0001; age F(1,150)=10.22, P=0.006; Sidak post-hoc test, P<0.05 at 50-100 pA). However, this effect was not observed in Venus- neurons (Figure 2D, two-way ANOVA: interaction F(10,180)=0.2960, P=0.9814; current injected F(910,180)=41.95, P<0.0001; age F(1,18)=0.1495, P=0.7036; Sidak post-hoc test, P>0.05). Venus+ neurons from P5-P7 animals fired at a higher frequency than did those from P15-P17 animals when a current injection of 100 pA was applied (Figure 2Eg (t(15)=2.800, P=0.0135, n=11 neurons from 7 animals), and time to the first action potential was shorter in P5-P7 animals (Figure 2Fh (t(17)=2.508, P=0.0226, n=11 neurons from 7 animals). Neither the firing frequency at 100 pA nor time to the first action potential at 60 pA were significantly different in Venus- neurons from P5-P7 versus P15-P17 mice (Figure 2Gi; t(18)=0.3254, P=0.7486, n=8 neurons from 5 animals; and Figure 2Hj; Mann-Whitney U=38.50, P=0.2578, n=8 neurons from 5 animals). A current injection of -40 pA caused similar membrane potential changes in Venus+ and Venus- neurons.
hyperpolarization regardless of cell type or animal age (Figure 2Ik Venus+; t(13)=1.353, P=0.1991, n=8 neurons from 5 animals; and Figure 2Km Venus-; t(14)=0.7094, P=0.4897, n=8 neurons from 5 animals). Spike firing adaptation is an intrinsic property of some neurons that reduces the frequency of action potentials upon sustained depolarization. To investigate adaptation in our experiments, we divided the instantaneous frequency between the last two action potentials by the instantaneous frequency between the first two action potentials when a current injection of 100 pA was applied. Adaptation was determined to be present if the instantaneous frequency ratio was significantly different from 1. Venus+ neurons overall did not display significant firing rate adaptation (Figure 2Jl; P5-P7 t(10)=2.010, P=0.0722; P15-P17, t(6)=1.504, P=0.1834, n=6 neurons from 4 animals).

Serotonergic neurons are known to display adaptation that is dependent upon the inactivation rate of voltage-gated sodium channels (Milescu et al., 2010). Venus- neurons at both age groups displayed significant adaptation (Figure 2L0; P5-P7; t(9)=11.85, P<0.0001, n=9 neurons from 7 animals; P15-P17; t(7)=4.481, P=0.003, n=7 neurons from 4 animals).

**Action potential characterization**

We analyzed the properties of individual action potentials by examining the first action potential generated by a 70 pA current injection to avoid any confounding effects of adaptation. The first action potential triggered by the 70 pA current injection for each cell was averaged together to generate the traces shown in Figure 3A and B for both Venus+ and Venus- neurons at each age. We generated phase plots by plotting the ΔV/Δt versus the membrane potential (mV). Each line represents individual action potentials from Venus+ or Venus- neurons at either age (Figure 3C-F). Both the depolarizing and repolarizing slopes were measured for each individual cell (Figure 3G-J). Again, using the first action potential triggered by the 70 pA current injection from each cell, we measured the action potential threshold, peak amplitude, duration, and AHP. The action potential
threshold (Figure 3K; Venus+, t(16)=1.004, P=0.3304, n=11 neurons from 7 animals; and Figure 3M; Venus-, t(17)=0.6243, P=0.5407, n=11 neurons from 7 animals) and action potential duration (Figure 3L; Venus+, t(14)=0.4238, P=0.6782, 2 outliers removed; Figure 3N; Venus-, Mann-Witney U=30, P = 0.2341) did not differ between cell types or age. Neither did the peak amplitude (Figure 3O; Venus+, t(16)=1.270, P=0.2224; Figure 3Q; Venus-, t(17)= 0.4051, P=0.6904, n=11 neurons from 7 animals), or AHP (Figure 3P; Venus+, t (16) = 0.1497, P=0.8829; Figure 3R; Venus- t(17)=0.3717, P=0.7147) between ages.

**Spontaneous excitatory post-synaptic currents**

We examined spontaneous excitatory postsynaptic currents (sEPSCs) in Venus+ and Venus- neurons at P5-P7 and P15-P17. Representative traces of sEPSCs from individual cells are shown for Venus+ and Venus- neurons at both ages with the average sEPSCs for those same cells being shown in the right panel (Figure 4A and B). To assess sEPSCs, we generated both cumulative plots from the average of all recordings and bar graphs of pooled data obtained from all the cells. In Venus+ neurons the sEPSC inter-event interval, (Figure 4C; K-S D= 0.06, P>0.9999; t(14)=0.06833, P=0.9465, n=16 neurons from 5 animals), amplitude (Figure 4E; K-S D=0.12, P=0.8643; Mann-Whitney U=22, P=0.5358, n=16 neurons from 5 animals), or rise-time (Figure 4G; K-S D=0.22, P=0.1777; t(13)=0.4816, P=0.6381, 1 outlier removed, n=16 neurons from 5 animals) did not differ between ages. Venus- neurons displayed a significant left shift in the cumulative probability distribution of the inter-event interval but there was no significant difference in the averaged data (Figure 4D; K-S D=0.28, P= 0.0397; t(9)=0.8686, P=0.4076, n=11 neurons from 5 animals). In the Venus- neurons, the cumulative probability distribution of the peak amplitude for the P15-P17 group was left-shifted in comparison to that of the P5-P7 group. Analysis of the averaged data revealed a significant decrease in the sEPSC peak amplitude (Figure 4F; K-S D=0.38, P=0.0015, t(9)=3.176, P=0.0113, n=11 neurons from 5 animals). The sEPSCs rise-time was not...
significantly different between the two ages in the Venus+ or Venus- neurons (Figure 4Hbb; K-S D=0.2203, P=0.114; t(9)=1.639, P=0.1356, n=11 neurons from 5 animals). Average traces of sEPSCs recorded from an individual cell were used calculate EPSC decay using a double exponential function. In Venus+ neurons the decay constants did not significantly change between the two ages (Figure 4Icc; 2-way ANOVA, interaction F(1,26)=1827, P<0.1881; tau=F(1,26)=10.02, P=0.0039; age=F(1,26)=0.9819, P=0.3309, 2 outliers removed, Sidak P>0.05,). In Venus- neurons there was no significant interaction but tau2 was significantly longer in the older animals (Figure 4Jdd; interaction=F(1,17)=3.312, P=0.0864; tau=F(1,17)=4.241, P=0.0551; age=F(1,17)=3.886, P=0.0652; 1 outliers removed, Sidak ,tau1 t(17)=0.1086, P0.9927; tau2 t(17)=2.642, P=0.0339).

**Spontaneous GABA_\textsubscript{A} receptor-mediated post-synaptic currents**

We next examined GABA\textsubscript{A}-PSCs in both cell types. Representative traces from individual cells are shown for both Venus+ (Figure 5A) and Venus- (Figure 5B) at P5-P7 and P15-P17 along with the averaged current trace of that same cell in the right hand panel. Both Venus+ and Venus- show a significant left-ward shift in the older animals. However, analysis of the bar graph did not reveal a significant differences between age groups (Venus+ (Figure 5Cee; K-S D=0.48, P<0.0001; t(16)=1.503, P=0.1522, 1 outlier removed, n=19 neurons from 10 animals); Venus- (Figure 5Dff; K-S D=0.34, P=0.0062; t(18)=1.694, P=0.1075, n=20 neurons from 12 animals)). The cumulative probability distributions of GABA\textsubscript{A}-PSC peak amplitudes were similar in both age groups in Venus+ neurons (Figure 5Egg; K-S D=0.12, P=0.8643; t(17)=0.7429, P=0.4677, n=19 neurons from 10 animals). However, we did not find a significant difference in the cumulative probability plots but analysis of the bar graph revealed a significant decrease in event amplitude in Venus- neurons (Figure 5Fhh; K-S D=0.18, P=0.3927; t(17)=2.208, P=0.0413, 1 outlier removed, n=19 neurons from 12 animals). The GABA\textsubscript{A}-PSCs in Venus+ neurons displayed a faster rise-time in the older animals (Figure 5Gii; K-S D=0.44, P=0.0001; t(17)=2.564, P=0.0201, n=19 neurons from 10 animals).
Conversely, the rise-time was similar in Venus- neurons from both ages (Figure 5Hjj; K-S D=0.1, P=0.9639; Mann-Whitney U=25, P=0.2463, 1 outlier removed, n=20 neurons from 12 animals). The GABA<sub>A</sub>-PSCs recorded from individual cells were averaged and fitted to a double exponential function to calculate the decay time constants for Venus+ and Venus- neurons at both ages. For both Venus+ and Venus- the tau2 time constant was reduced in older animals (Venus+ (Figure 5Ikk; interaction=F(1,29)=3.963, P=0.056; tau=F(1,29)=10.79, P=0.0027; age=F(1,29)=13.44, P=0.001; 3 outliers removed, tau1 t(29)=1.24, P>0.05; tau2 t(29)=3.837, P<0.05); Venus- (Figure 5Jll; interaction F(1,28)=0.9709, P=0.3329; tau F(1,28)=32.04, P<0.0001; age F(1,28)=8.352, P=0.0074, 5 outliers removed, t(18)=2.591, P<0.05); tau1 t(28)=1.435, P>0.05; tau2 t(28)=2.591, P<0.05).
Our studies demonstrate that DR GABAergic neuron excitability decreases as the frequency of GABA\textsubscript{A}-PSCs increases during the 3\textsuperscript{rd} trimester equivalent. At P15-P17, putative 5-HT neurons exhibit an increased frequency of both sEPSCs and GABA\textsubscript{A}-sPSCs. Taken together, these data highlight the important developmental processes that take place in the DR during this period of development.

GABAergic neurons and putative 5-HT neurons co-exist in the DR and undergo distinct changes in excitability during the 3\textsuperscript{rd} trimester equivalent

We found that the vast majority of GABAergic neurons are located in the lateral regions of the DR, whereas 5-HT neurons are located predominantly in the medial region of the DR. However, we also observed scattered Venus+ GABAergic neurons and tryptophan hydroxylase+ 5-HT neurons near midline and lateral areas, respectively. Furthermore, the distribution of these neurons was not different in sections from P5-P7 and P15-P17 mice and we did not detect any neurons that co-expressed VGAT-Venus and tryptophan hydroxylase at these developmental periods. These findings are in agreement with those of Gocho et al. (2013) who found a similar distribution of 5-HT and GABAergic neurons in the DR of adolescent mice expressing green fluorescence protein driven by the glutamate decarboxylase 67 (GAD67) promoter. Our results are also generally consistent with those of Rood et al. (2014) who detected scattered clusters of neurons that stained positive for GAD67 in the lateral portion of the ventral-medial DR of P4 mice, as well as in the vicinity of 5-HT neurons located in the midline. It should be noted that the density of GABAergic neurons in our DR sections from P5-7 VGAT-Venus mice appears to be higher than the density of these neurons in the P4 Pet-1-YFP mice used by Rood et al. (2014). Potential explanations for this difference include that: there are strain differences in the number of DR GABAergic neurons present during the first week of
neonatal development, the number of DR GABAergic neurons significantly increases at P5-7 with respect to P4, or more GABAergic neurons can be detected using mice expressing Venus protein than using antibodies against endogenous GAD67. Regarding the GAD67 immuno-reactivity, it is possible that a significant population of GABAergic neurons may not have been detected at P4 because they express low levels of GAD67.

We compared the functional properties of Venus+ and Venus- neurons in the DR in neonatal mice. Venus+ can be unambiguously defined as GABAergic neurons because expression of this fluorescent protein is driven by VGAT. Venus- neurons likely correspond to 5-HT neurons for their electrophysiological properties are consistent with those previously described using mice expressing YFP under the control of the 5-HT neuron-specific Pet-1 promoter (Pet-1::YFP) (Rood et al., 2014). Specifically, Venus- cells exhibit similar resting membrane potentials, membrane resistance, action potential threshold, action potential duration, AHP, and action potential adaptation (Milescu et al., 2010, Calizo et al., 2011). However, given that we cannot eliminate the possibility that some of the Venus- cells that we recorded from are of other neuronal subtypes (e.g, glutamatergic), we cautiously refer to these as putative 5-HT neurons.

We found a striking difference in the excitability of GABAergic neurons between P5-P7 and P15-P17. Specifically, GABAergic neurons from P5-P7 mice fired action potentials at a lower threshold and at a higher frequency in response to current injections. The hyperexcitability was not due to changes in membrane resistance. Redistribution of voltage-gated sodium channels from the axon hillock to the soma has been shown to reduce the firing threshold in developing cortical and dentate granule neurons (Cummins et al., 1994, Kress et al., 2010). The redistribution of voltage gated sodium channels could explain the firing of APs with smaller current injections and decreased time to the first AP without changing the AP threshold. It is also possible that changes in Iₘ currents may play a role in these developmental changes (Gorter et al., 1995).
In contrast to Venus+ GABAergic neurons, we found that Venus- putative 5-HT neurons fired action potentials in response to current injection with a similar frequency at both ages. Consistent with this, we did not observe any age-dependent differences in membrane resistance or resting membrane potential. These findings are different from those of, Rood et al. (2014) who showed with Pet-1-YFP mice that the excitability of 5-HT neurons in the ventral-medial DR is reduced at P12 and P21 with respect to P4, which may be partially due to a shift to a more hyperpolarized membrane potential at the older ages. A potential explanation for the differences between these studies is that the reduction in putative 5-HT neuron excitability and the membrane potential hyperpolarization take place relatively rapidly, between P4 and P5, reaching a stable level between P5-P7. Alternatively, it is possible that the functional maturation of 5-HT neurons follows a different developmental time course in VGAT-Venus and Pet-1-YFP mice. However, all other parameters we measured at the two ages such as action potential threshold, action potential peak amplitude, AHP amplitude, and action potential duration are in general agreement with those reported by Rood et al. (2014).

Adaptation of action potential frequency is an intrinsic property of 5-HT neurons (Kirby et al., 2003) and is mediated by the inactivation of Na⁺ channels (Milescu et al., 2010) rather than activation of BK/SK channels, Ca²⁺-dependent K⁺ channels, or Kv7/M channels, which participate in this process in neurons from several other brain regions (Madison and Nicoll, 1984, Storm, 1990, Gu et al., 2005, Nigro et al., 2014). Spike-frequency adaptation has been used for the electrophysiological identification of 5-HT neurons and is thought to play a role in signal integration and self-inhibition (Vandermaelen and Aghajanian, 1983). Our data show that putative 5-HT neurons exhibit significant adaptation as early as P5-P7 and that the magnitude of this phenomenon does not change by P15-P17. Overall, Venus+ neurons did not display significant adaptation at the current injections we used. However, in our studies a few GABA neurons at P15-P17 did display significant adaptation. Furthermore, Gocho and colleagues showed that larger current injections in older animals resulted in significant adaptation (Gocho et al., 2013). Adaptation is one of many firing
characteristics of GABA neurons from other brain regions, including the cortex and hippocampus (Ali et al., 1998, Stiefel et al., 2013).

**Changes in synaptic currents in GABAergic neurons and putative 5-HT neurons**

Rood et al. (2014) found that sEPSC frequency gradually increased with age in 5-HT neurons of the ventro-medial and lateral wing regions of the DR of Pet-1-YFP mice. Specifically, sEPSC frequency increased by approximately 5 fold between P4 and P21 in 5-HT neurons of the ventro-medial DR without a significant change in amplitude or decay. This finding could be interpreted to reflect integration of 5-HT DR neurons into the glutamatergic synaptic network. In agreement with the previous study, we detected a developmental change in the frequency and amplitude of sEPSCs in Venus- neurons in the ventro-medial DR. An uncertainty that must be kept in mind is that whole-cell patch-clamp somatic recordings are only able to sample events generated at sites near the location of the recording electrode. Therefore, it is possible that the density of glutamatergic synaptic connections increases with age at distal dendritic sites, which would not be detectible due to dendritic filtering mechanisms. The fact that DR 5-HT neurons undergo significant dendritic outgrowth between P4 and P21 (Rood et al., 2014) would support the hypothesis of dendritic filtering.

In addition to the results with sEPSCs, we detected a significant increase in GABA_A-sPSC frequency associated with a decrease in amplitude in Venus- neurons at P15-P17, with respect to P5-P7. The developmental increase in the frequency of these events can be interpreted to reflect an increase in spontaneous firing of GABAergic neurons, the probability of GABA release, and/or the number of active GABA_A receptor-containing synapses. However, the reductions in the decay time constants in both Venus+ and Venus- neurons suggest changes in the subunit composition (e.g., an increase in α₁ GABA_A receptor subunit expression), clustering, and/or phosphorylation state of postsynaptic receptors. Our results with DR Venus- neurons are in general agreement with those of Rood et al. (2014) who reported 8 to 10-fold increases in IPSC frequency between P4 and P12 in the
ventro-medial and lateral wing DR of Pet-1-YFP mice, with a further increase in the frequency of these events at P60. Increases in sIPSC frequency during the 3rd trimester-equivalent have been detected in other neuronal populations across the brain, including cerebellar granule cells, CA3 pyramidal neurons, neurons in the CA1, and cortical pyramidal neurons (Sebe et al., 2010, Everett et al., 2012, Riebe and Hanse, 2012, Diaz et al., 2014). We refer to these currents as GABA<sub>A</sub>-sPSC and not inhibitory postsynaptic currents because during the early stages of the 3rd trimester equivalent developmental period, GABA<sub>A</sub> receptor activation can induce membrane potential depolarization due to elevated intracellular Cl<sup>-</sup> concentrations in some populations of immature neurons, including those in some brainstem nuclei (Kaila et al., 2014, Witte et al., 2014). Future experiments should determine whether GABA<sub>A</sub> receptor activation exerts excitatory or inhibitory actions in DRN 5-HT neurons in neonatal mice.

**Implications and future directions.**

This study provides additional evidence indicating that putative 5-HT DR neurons undergo significant functional maturation during the 3rd trimester equivalent period. We further demonstrate that this is also the case for GABAergic neurons located in this brain region. Alterations in the 5-HT neurotransmitter system during development have been implicated in a variety of diseases. For instance, studies suggest that prenatal exposure to 5-HT reuptake inhibitors increases the risk of developing autism spectrum disorders (Kinast et al., 2013, Gentile, 2015). Prenatal exposure to alcohol during the rodent equivalent to the 1<sup>st</sup> and 2<sup>nd</sup> trimester of pregnancy in humans has been shown to damage developing 5-HT neurons and their axons (Eriksen and Druse, 2001, Sari and Zhou, 2004, Zhou et al., 2005). Environmental pollutants and prenatal stress have also been shown to affect immature 5-HT neurons (Cory-Slechta et al., 2008, Boix and Cauli, 2012, Glover, 2015). Therefore, future studies should investigate whether these and other insults alter the late
developmental stages of this important neurotransmitter system, potentially leading to psychopathology later in life.
References


Workman AD, Charvet CJ, Clancy B, Darlington RB, Finlay BL (2013) Modeling transformations of 


Zhou FC, Sari Y, Powrozek TA (2005) Fetal alcohol exposure reduces serotonin innervation and 
compromises development of the forebrain along the serotonergic pathway. Alcohol Clin Exp 
Res 29:141-149.
Figure Legends

**Figure 1: Distribution and passive membrane characteristics of Venus+ and Venus- neurons.**

Immunohistochemistry of GABAergic neurons expressing Venus (Green) and 5-HT neurons labeled with mouse anti-tryptophan hydroxylase (Tph) (1:500) and goat anti-mouse IgG-Alexa 555 antibodies (1:1000) (Red). Representative 10x images of the entire DR are shown for P5-P7 and P15-P17 (A and C) (Scale Bar = 100 μm). Higher magnification confocal images of boundary areas between midline and lateral areas taken from separate immunolabeled sections show that majority of neurons either express GABA (Venus = Green) or serotonin (Tph = Red) (nuclei = blue) (B and D) (Scale Bar = 10 μm). Representative locations of recordings from Venus+ and Venus- neurons in sections approximately -4.24 mm (E) and -4.84 mm (F) away from bregma. Membrane capacitance and resistance are shown for Venus+ (G and H) and Venus- (I and J) neurons.

**Figure 2: Excitability of Venus+ and Venus- neurons.** Representative traces of hyperpolarizing and depolarizing potentials are shown for Venus+ (A) and Venus- (B) neurons at both ages elicited by either a -40 pA or 100 pA current injection. Giving sequential current injections and recording the action potential frequency, we generated input/output curves for both Venus+ (C) and Venus- (D) neurons at the indicated age ranges (2-way ANOVA/ Sidak* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001). Excitability was assessed by measuring the action potential firing frequency with 100 pA injected and the time to the first action potential when 60 pA was injected in both Venus+ (E and F) and Venus- (G and H). Hyperpolarization was measured as the negative peak membrane potential evoked by -40 pA injection in Venus+ (I) and Venus- (K) at both ages. Adaptation was measured by dividing the instantaneous frequency of the last two action potentials by the instantaneous frequency of the first two action potentials when 100 pA was injected into Venus+ (J) and Venus- (L) neurons at both ages.
Figure 3: Action potential properties of Venus+ and Venus- neurons. The first action potential that was generated from the 70 pA current injection from each cell was then averaged together to generate the action potential traces. The averaged action potentials traces are shown for Venus+ (A) and Venus- (B) neurons at P5-P7 and P15-P17. We generated the Phase plots by comparing the ΔV/Δt versus the membrane potential (mV) for each Venus+ (C and D) or Venus- (E and F) neuron at both ages. We also measured the depolarizing and repolarizing slopes of the action potentials from Venus+ (G and H) and Venus- (I and J) neurons. The individual action potentials were used to measure the action potentials properties. We used the mini-analysis software AP waveform analysis 2 to measure the action potential threshold (K and M), Duration (L and N), peak amplitude (O and Q), and after hyperpolarization (AHP) (P and R).

Figure 4: Spontaneous excitatory post-synaptic currents (sEPSCs) in Venus+ and Venus- neurons. Representative currents and averaged currents from individual cells are shown for both Venus+ (A) and Venus- (B) neurons. The cumulative probability plots from all the cells were averaged together to generate the cumulative probability plots shown for each parameter. Pooled data are shown the inset bar graph. The inter-event interval (C and D), peak amplitude (E and F), and rise-time (G and H) are shown as a cumulative probability plot and averaged data in the inset bar graphs for both Venus+ and Venus- neurons. The averaged sEPSC for each cell was fitted with a double exponential function to calculate the decay rates for Venus+ (I) and Venus- (J) neurons.

Figure 5: GABA<sub>A</sub> receptor-mediated spontaneous post-synaptic currents (GABA<sub>A</sub>-sPSCs) in Venus+ and Venus- neurons. Representative traces and averaged currents from individual cells are shown for Venus+ (A) and Venus- (B) neurons. The cumulative probability plots from all the cells were averaged together to generate the cumulative probability plots shown for each parameter.
Pooled data are shown in the inset bar graph. The inter-event interval (C and D), peak amplitude (E and F), and rise-time (G and H) are shown as a cumulative probability plot and averaged data in the inset bar graphs for both Venus+ and Venus- neurons. The averaged GABA\textsubscript{A}\textsuperscript{-}PSC for each cell was fitted with a double exponential curve to calculate the decay rates for Venus+ (I) and Venus- (J) neurons.
Figure 1

A 10x

B 40x

C

D

E

F

G

H

I

J

-4.24 mm

-4.84 mm

Venus +

Venus -

P5 - P7

P15 - P17

Capacitance (pF)

Membrane Resistance (MΩ)

P5 - P7

P15 - P17

P5 - P7

P15 - P17

P5 - P7

P15 - P17

P5 - P7

P15 - P17
Figure 4

A) Venus+ sEPSCs

B) Venus- sEPSCs

C)

D)

E)

F)

G)

H)

I)

J)
Figure 5

A  Venus+ GABA_A-PSCs

B  Venus- GABA_A-PSCs

C  Inter-event interval (ms)

D  Inter-event interval (ms)

E  Cumulative Probability

F  Cumulative Probability

G  Amplitude (pA)

H  Amplitude (pA)

I  Time Constant (ms)

J  Time Constant (ms)
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