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Title: Cytokine Receptor IL-33: ST-2 signaling developmentally regulates susceptibility to seizures, synaptic plasticity and microglial phagocytic activity

Declaration of Academic Integrity

The research team hereby confirm that the present paper is based on results obtained under the guidance of the instructor, and that in all cases material from the work of others (in books, articles, essays, dissertations, and on the internet) in acknowledged, and quotations and paraphrases are clearly indicated. No material other than that listed has been used. I have read and understood the organization's regulations and procedures concerning plagiarism.

Date: September 7, 2019

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Abstract

Microglia are capable of diverse functions to accommodate the changing needs of the central nervous system (CNS) and have emerged as central players in CNS development, homeostasis, inflammation, and disease. Despite recent advances in understanding microglial function during homeostasis and disease, a comprehensive understanding of immune signaling cues that regulate microglial activity and neurodevelopment is lacking. Here, we found that mice lacking immune cytokine receptor for Interleukin 33 (IL-33) called ST2 results in increased susceptibility to epilepsy-like seizures in mice. Moreover, long term potentiation induction is impaired in the brains of ST2-deficient mice. We further demonstrated that IL-33 administration could enhance the phagocytic activity of microglia. Our work identifies a key role for IL-33 dependent transcriptional circuits in regulating microglial activity and function during early brain development with implications for neurodevelopmental and neuropsychiatric disorders.

Key words: Neurodevelopment, Cytokine, IL-33, microglia, synaptic pruning, seizure, electrophysiology, synaptic plasticity, synaptosome

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Introduction

Neurodevelopment and diseases

Brain composition consists predominantly of neurons and glia（**Figure 1**). The neuron is often considered as the basic working unit of the brain that transmits information to other cells, such as muscle cells and gland cells. Within each neuron, there exists the soma, the neuronal membrane, the cytoskeleton, axons, and dendrites. The soma contains organelles similar to ones of other body cells. Functioning as a barrier between extracellular fluids and the cytoplasm, the protein-studded membrane regulates substances traveling in and out of the neuron. The cytoskeleton is composed of microtubules, microfilaments, and neurofilaments. Together, these elements provide structure and formulate distinct shapes for the neuron. Dendrites extend from the soma. The point where the axon contacts the next neuron is called the synapse. Dendrites are covered with thousands of synapses, and they have many receptors on their membranes to detect neurotransmitters. Neurons communicate using chemical and electrical signals through dendrites and axons. As mentioned previously, neurons first receive a signal through its dendrites. As the electrical signal reaches the terminal end of the neuron, it approaches a synaptic cleft, or rather a space or a gap between the original neuron and the adjacent neuron. Before communication occurs with the next neuron, the electrical signal is converted into chemical signals, represented as neurotransmitters. Aside from neurons, glial cells similarly serve a significant in the nervous system. Three particular types of glia define the brain: namely astrocytes, oligodendrocyte, and microglia. Glial cells fill significant roles in neurodevelopment, synaptic communication, and synaptic plasticity through the monitoring and altering of central nervous system structure and function[1]. In particular, microglia, the brain resident macrophages derived from yolk sac progenitors (rather than neural stem cells), significant in bridging the immune system with the nervous system, and play critical roles in throughout the neurodevelopment processes.

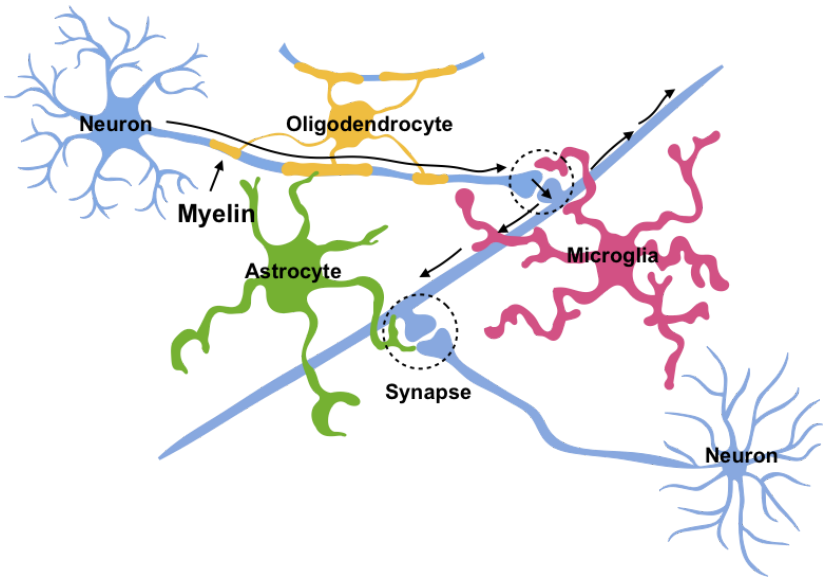


Figure 1: *Cellular composition of central nervous system.*

The formation of the various aforementioned elements of the brain during development is achieved by a series of tightly regulated developmental processes. Occurring in multiple steps, this process sets the stage for the essential functions of the nervous system. The first two stages are neurogenesis and neuronal migration. Neurogenesis is the process where neural progenitor cells develop into various kinds of specified neurons. Neuronal migration is the movement of the newly-developed neurons to their specific regions in the brain. Next is synaptogenesis, where synapses form between neurons. Similarly, Gliogenesis follows, and this process witnesses the formation of glial cells. Finally, synaptic pruning completes the overall process. This step removes unnecessary synapses in the brain. In particular, the steps of synaptogenesis and synaptic pruning defines the following explorations of diseases credited to dysregulated neurodevelopment. (**Figure 2**).

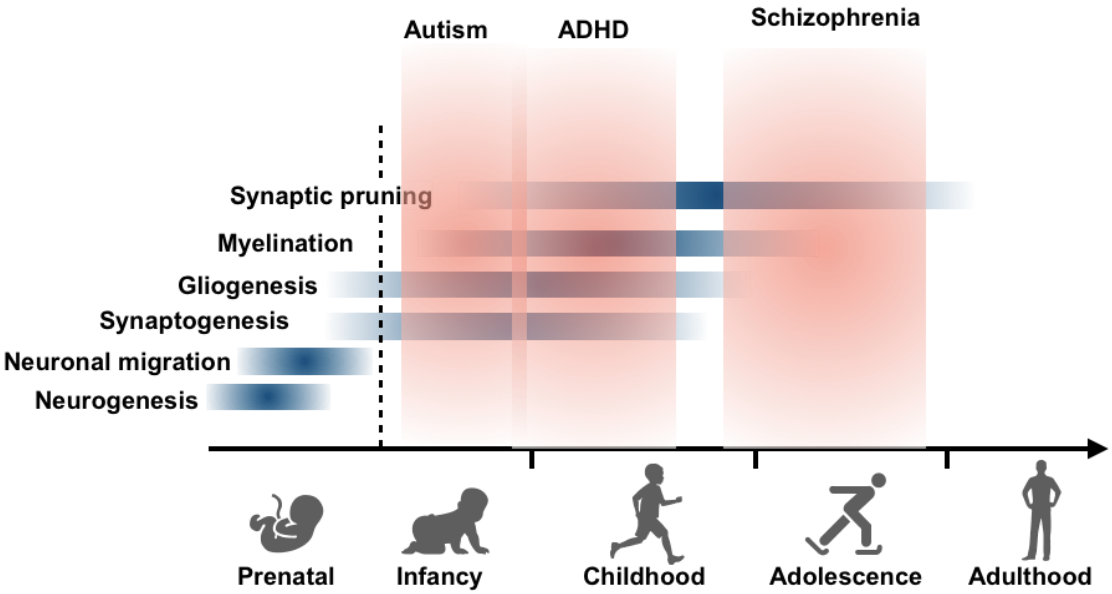


Figure 2: *Temporal profile of neurodevelopmental sequences in relation to the age of onset of mental disorders. Abbreviation: ADHD is attention deficit hyperactivity disorder.*

Immune mechanisms in neurodevelopmental and neuropsychiatric disorders

While most evidence indicates that a genetic component plays an important role in the etiology of neurodevelopmental and neuropsychiatric disorders, a number of studies suggest that immunological dysfunctions may also participate in the pathophysiology of these disorders. In particular inflammatory insults that occur *in utero* as well as during infancy and early childhood, when the brain is highly vulnerable, have the potential to cause long-lasting damage that would persist and contribute to impaired functional brain connectivity and behavioral deficits[2]. For example, maternal inflammation (MIA, activation of the innate and adaptive immune system during pregnancy due to infection) has been associated with several neuropathologies in the progeny, particularly with Autism spectrum Disorders[3]. In addition, microbiota residing in the GI tract could also modulate the inflammatory responses and behavioral abnormalities associated with neurodevelopmental disorders in early life[4].

Microglia and synaptic pruning

Microglia, the predominant immune cells residing in the brain, play a significant role in mediating the crosstalk between the central nervous and immune systems during development, homeostasis, and inflammation. In particular, microglia are both the main source and a target for inflammation in the CNS. In addition to their fundamental immunological roles as macrophages in detecting damages and pathogens, microglia actively participate in complex neurodevelopmental programs, such as brain wiring, synapse development, and pruning. It has been demonstrated that microglia constantly interact with and strip synapses from dendrites during brain development (**Figure 3**). Microglia-mediated synapse pruning is necessary for proper neuronal circuitry maturation and function[5]. Thereby these cells are essential in establishing and maintaining the circuitry of the brain for appropriate physiological functions. Dysregulated microglial activity and phagocytic function have been implicated in neurodevelopmental and neuropsychiatric disorders.

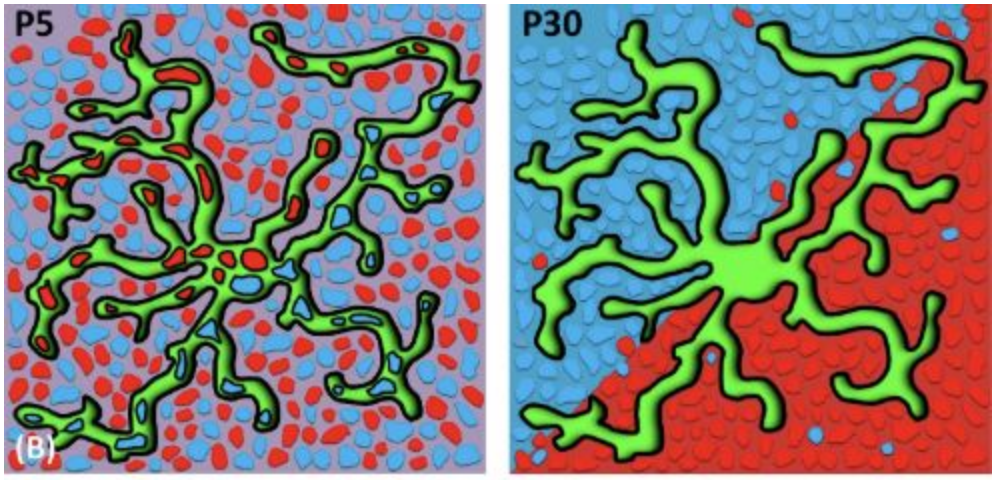


Figure 3: *Microglial-Mediated Synaptic Pruning (adapted fromWu et al 2015[6]). Shown is microglial engulfment of synaptic material observed at postnatal day 5 (P5) in mouse visual cortex. By P30, microglial phagocytic activity is limited. Green: microglia. Red and blue: axonal terminals.*

Methods

Brain slice electrophysiology

1) Euthanize the mice with isoflurane.
2) Remove the mice brains quickly. Then, submerge the brain in ice-cold oxygenated sucrose-replaced artificial cerebrospinal fluid (ACSF) cutting solution. This solution includes 206 mM sucrose, 26 mM NaHCO3, 10 mM D-glucose, 2 mM MgSO4, 2 mM KCl, 1.25 mM NaH2PO4, 1 mM CaCl2, 1 mM MgCl2, pH 7.4, 315 mOsm.
3) Using a vibro-slicer, cut transverse slices of 350um thickness from the middle portion of each hippocampus.

4) Incubate the brain slices in ACSF which contains 124mM NaCl, 2mM KCl, 2 mM MgSO4, 1.25mM NaH2PO4, 2.5mM CaCl2, 26mM NaHCO3, 10mM D-glucose, pH 7.4, 310 mOsm. Allocate 90 minutes for the slices to recover before recording.
5) Transfer a single slice to the recording chamber, and submerge beneath continuously perfusing ACSF. ACSF is saturated with 5% O2 and 5% CO2.
6) Incubate the slice for 20 minutes in the recording chamber before stimulation under room temperature.
7) Place a bipolar stimulating electrode (FHC Inc., Bowdoin, ME) in the Schaffer collaterals to administer conditioning and test stimuli.
8) Place two borosilicate glass recording electrodes filled with ACSF in stratum radiatum (apical dendrites) and stratum oriens (basal dendrites) of CA1, 200~300 μm from the stimulating electrode.
9) Induce fEPSP in the CA1 region by test stimuli at 0.05 Hz with an intensity that elicited a fEPSP amplitude 40-50% of maximum.
10) Record test responses for 30-60 minutes prior to beginning the experiment to ensure response stability.
11) To induce LTD, 300 pulses are delivered at 1 Hz. Field potentials are amplified 100x with an Axon Instruments 200B amplifier. Then, they are digitized using Digidata 1322A. Data are sampled at 10 kHz and filtered at 2 kHz. Next, traces are acquired by pClamp 9.2 and analyzed by the Clampfit 9.2 program.

Microglia isolation

1) Euthanize mice by CO2 asphyxiation, followed by transcardial perfusion with 10ml ice-cold PBS.
2) Open the skull and reveal the brain by lifting the skull bone.
3) Place the brain in 5ml D-PBS containing 1mM HEPES and 0.5% glucose.
4) Prepare the papain dissociation mix (LK003150, Worthington Biomedical Corporation):
    - Add 5ml of EBSS to a papain vial and place the resulting solution in a 37°C water bath for ten minutes or until the papain is completely dissolved and the solution appears clear.
    - Add 500 μls of EBSS to a DNase vial and mix gently. Add 250ul of DNase solution to the papain solution.
5) Add 1ml papain-DNase dissociation mix into 1.5ml Eppendorf tube and place the brain in the mix. Cut the brain into small pieces using scissors.
6) Incubate the dissociation mix at 37°C for 20 minutes.
7) Triturate the mixture with a 1ml pipette. Then, allow remaining pieces of undissociated tissue to settle to the bottom of the tube.
8) Remove the cloudy cell suspension carefully. Then, place suspension in a 15ml conical tube and centrifuge at 400g for 5 minutes at 4°C. Discard the supernatant and resuspend the cell pellet with albumin-ovomucoid inhibitor solution immediately. Again, centrifuge at 400g for 5 minutes at 4°C.
9) Discard the supernatant and resuspend the pelleted cells in 36% Percoll solution (GE Healthcare). Centrifuge at 500g for 5 minutes at 4°C.

10) Aspirate the supernatant including the myelin layer (loose layer on the top) carefully, leaving only the pelleted mixed brain cells.
11) Wash the cell pellet with 200ul D-PBS and transfer into 96 well plate. Spin at 400g for 5 minutes at 4°C.
12) Resuspend the cell pellet in 200ul blocking buffer (2% FCS and 10% mouse IgG in D-PBS), incubate at 4°C for 15 minutes.
13) Spin for 5 minutes at 4 °C and 400 × g and remove supernatant.
14) Resuspend the cell pellet in 50μL blocking buffer containing α-CD11b, α-CD45 antibodies, and viability dye. Incubate at 4°C for 15 minutes.
15) Wash the cells with 150 μL D-PBS, and spin at 400g for 5 minutes at 4°C.
16) Resuspend the cell pellet with 300 μL FACS buffer (D-PBS containing 2% BSA), and sort microglia ($CD45^{int}CD11b^{int}$) on a BD Aria sorter.

Synaptosome extraction and labeling

1) Dissect out mice brains, and place them into 10 volumes of ice-cold homogenization buffer (0.32 M sucrose, 10 mM HEPES pH 7.4, 2 mM EDTA, protease inhibitors, phosphatase inhibitors).
2) Homogenize mixture using 10-15 strokes of a motor-driven glass-Teflon homogenizer.
3) Spin at 1000 x g for 15 minutes to remove pelleted nuclear fraction (P1).
4) Take supernatant (S1) and spin at ~10,000 x g for 15 minutes at 4°C to yield crude synaptosome pellet (P2).
5) Resuspend the crude synaptosome (P2) in cold homogenization buffer. Centrifuge at 10,000g for another 15 minutes at 4°C to yield washed synaptosome pellet (P2').
6) Resuspend P2' in homogenization buffer. Then, layer the resuspended membranes on top of a discontinuous gradient comprised of 0.8 to 1.0 to 1.2 M sucrose.
7) Centrifuge at 30,000 rpm for 1.5h in an SW41Ti rotor (Beckman Coulter Corporation, Palo Alto, CA, USA)
8) Recover synaptic plasma membranes in the layer between 1.0 and 1.2 M sucrose.
9) Dilute to 0.32 M sucrose by adding 2.5 volumes of 4 mM HEPES pH 7.4.
10) Spin at 10,000 x g for 30 minutes to obtain the synaptosome pellet (S).
11) Resuspend S in 1ml PBS, and add 100ul 1M sodium bicarbonate. Pipet up and down several times to mix thoroughly.
12) Add the calculated amount of pHrodo iFL labeling solution and mix thoroughly.
13) Incubate the reaction mixture for 2 hours at room temperature.
14) Wash the labeled synaptosome by 4 rounds of washing with ice-cold PBS.

Synaptosome engulfment

For the *ex vivo* synaptosome engulfment assay, freshly sorted microglia from various stimulation conditions were incubated with 5μl of pHrodo Red-conjugated synaptosomes for 2 hours. Then microglia were enzymatically detached and analyzed on a BD LSR analyzer.

Results

Adult ST2$^{-/-}$ mice exhibit handling-induced seizures

When performing routine animal husbandry, we noticed that aged, breeding ST2$^{-/-}$ mice exhibited seizure-like behavior after handling. The brief severe generalized tonic-clonic seizures in ST2 deficient mice are usually 1 minute in duration. Seizures begin with limb clonus, which is the involuntary muscle contractions, and tonic hindlimb extension. Afterwards, rapid breathing and short periods of immobility follow. (**Figure 4**). After the episodes, mice may recover fast and regain normal mobility.

Figure 4: *handling induced seizures in homozygous ST2 deficient mice. Frames from multiple videos recording of handling induced seizures in 4-8-month-old ST2 KO mice with posture immobility.*

We monitored ST2 KO mice and wild type controls for up to 7 months to determine the onset age (when ST2 KO mice start to display handling induced seizures) and the penetrance (the percentage of ST2 KO mice that display handling induced seizures) of seizures. We found that ST2 KO mice have later onset of non-lethal seizures (60%, $n = 5$) seizures that begin between the ages of 3-6-month-old (**Figure 5**).

Figure 5A: Observed Seizures in Postnatal 295 KO Mice

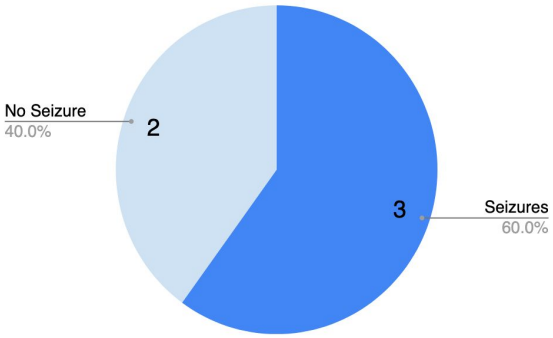
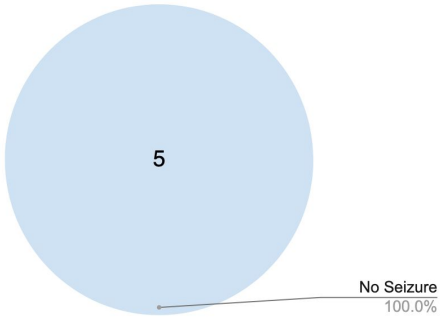
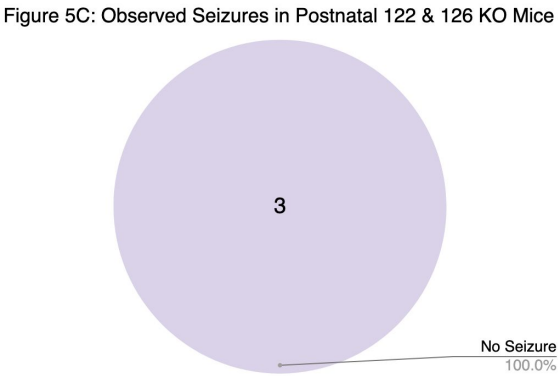


Figure 5B: Observed Seizures in Postnatal 295 Wildtype Mice





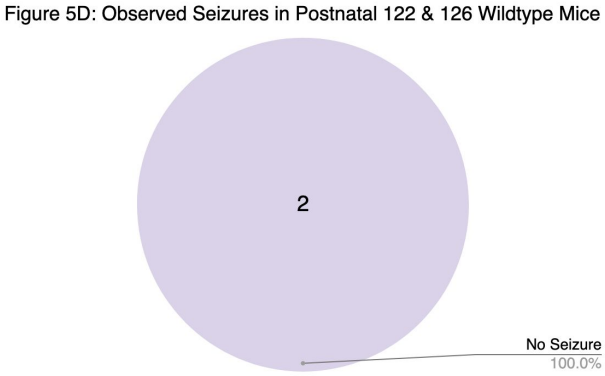


Figure 5: *ST2 deficient mice are susceptible to handling induced seizures. Pie charts detail seizure and non-seizure ration at postnatal 295 day (A and B) and postnatal 122 & 126 day (C and D) in ST2 KO and wildtype mice.*

ST2$^{-/-}$ mice exhibit long term potentiation (LTP) impairment

Given the handling induced seizures observed in ST2 deficient mice, we hypothesized that the neuronal firing and/or synaptic function in ST2 KO brains may be altered. It has been reported that repeated electroconvulsive seizures could "saturate" synapses and decrease the capacity for synaptic plasticity[7], we then performed brain slice electrophysiology to assess synaptic plasticity in ST2 KO brains. Long term synaptic potentiation or LTP reflects the ability of synaptic enhancement followed by high-frequency electrical stimulation and has been widely used as a sensitive and readily quantifiable electrophysiological correlate of synaptic plasticity. We assessed LTP induction by burst stimulation of Schaffer collaterals in area CA1 and recording of fEPSP in the stratum radiatum of CA1. We found that hippocampal LTP was dramatically reduced in ST2 KO brains (**Figure 6**), suggesting that synaptic plasticity in ST2 KO brains is impaired.

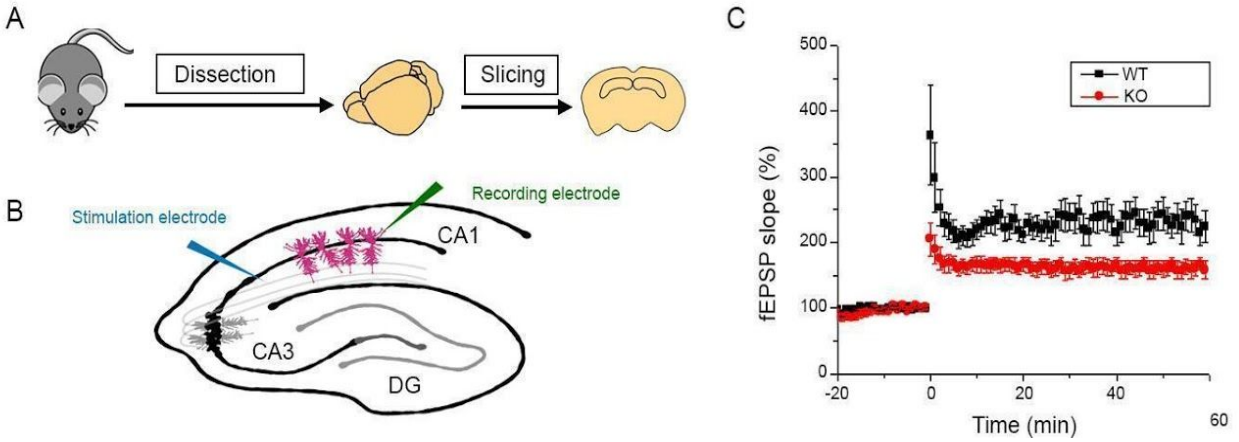
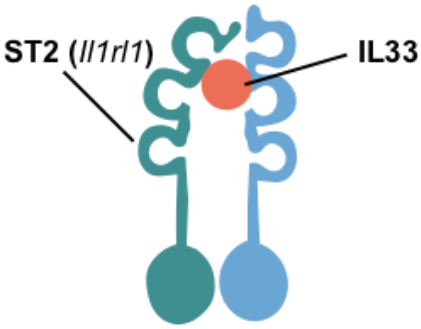


Figure 6: *LTP impairment in ST2 KO mice. (A)Experimental procedures (B) Diagram of field recordings fEPSPs in CA1 regions of hippocampus. (C) Average traces of fEPSP data showing that HFS evoked LTP of fEPSPs ST2KO brains was reduced compared to wild type controls.*

IL-33/ST2 promotes synapse engulfment by microglia



ST2, a member of the interleukin-1 receptor family, was originally discovered as an orphan receptor that participates in many inflammatory processes. Later on, IL-33 was identified as the functional ligand for ST2[8] (**Figure 7**). We and others found that in developing brain, IL-33 is predominantly produced by astrocytes and mature oligodendrocytes while ST2 (aka. *Il1rl1*) is exclusively expressed by microglia[9], suggesting that IL-33 might regulate microglial function.

Figure 7: *Illustration of ST2 as a receptor for the cytokine IL-33.*

To determine if administration of IL-33 could enhance the phagocytic activity of microglia, we developed an *ex vivo* engulfment assay: microglia were isolated from postnatal brain, and incubated with pHrodoRed labeled synaptosome for 2 hours. The total amount of pHrodoRed dye is used as a measurement of engulfment capacity of microglia (**Figure 8**).

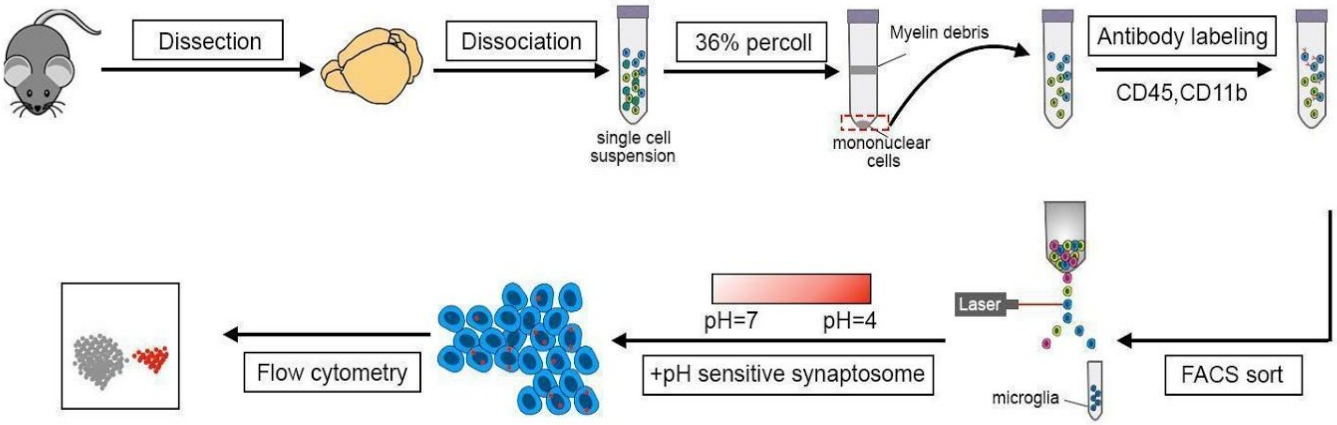


Figure 8: *Schematic of ex vivo synaptosome engulfment assays.*

As introduced earlier, the brain consists of multiple cell types including neurons and other glial cells. To purify microglia from mouse brain, we used a flow-cytometry based sorting strategy. Single cells obtained from mouse brain were stained with antibodies recognizing CD45 and CD11b, two commonly used markers to identify macrophages. Live cells with intermediate levels of CD45 and CD11b were sorted out as microglia (**Figure 9**), which were used in the following procedures.

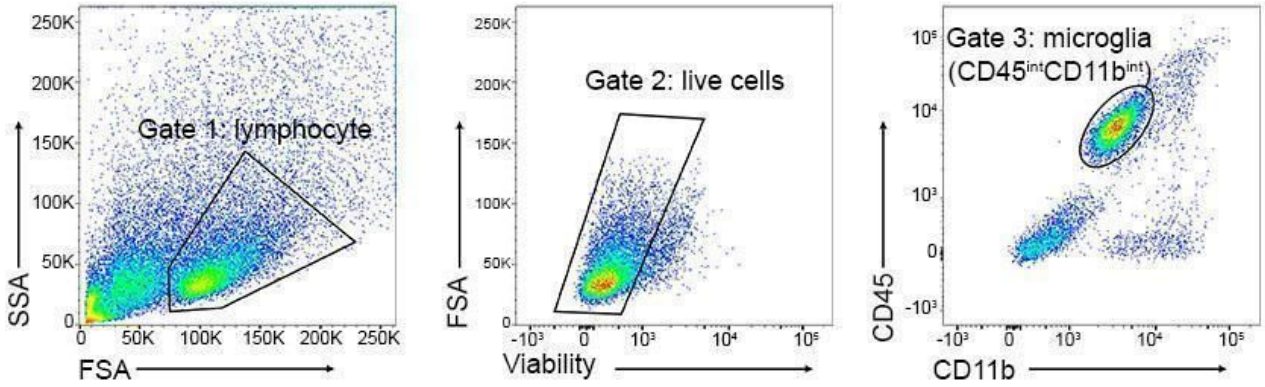


Figure 9: *sorting strategy to purify microglia from mouse brain. Antibody labeled brain cells were analyzed by BD Aria B. Debris were gated out in Gate 1 by relative cell size (FSC-A) and cellular granularity (SSC-A) and immune cells were enriched in Gate 1. Dead cells were gated out in Gate 2 by a fluorescence dye that could stain all dead cells. Microglia were selected in Gate 3 by expression levels of CD45 and CD11b. Cells with intermediate levels of CD45 and CD11b were sorted out .*

To assess the phagocytic ability, we incubated microglia with fluorescence dye-labeled synaptosome, and the intensity of fluorescence reflects the total amount of synaptosome engulfed by microglia. To distinguish synaptosome that has been "eaten" by microglia from those stuck on the cell surface, we chose pHrodo Red, a pH-sensitive dye that only emits strong fluorescence signal when pH is low[10] (**Figure 10**). Thereby only the synaptosome within the endosome or lysosome where pH is low, will be measured.

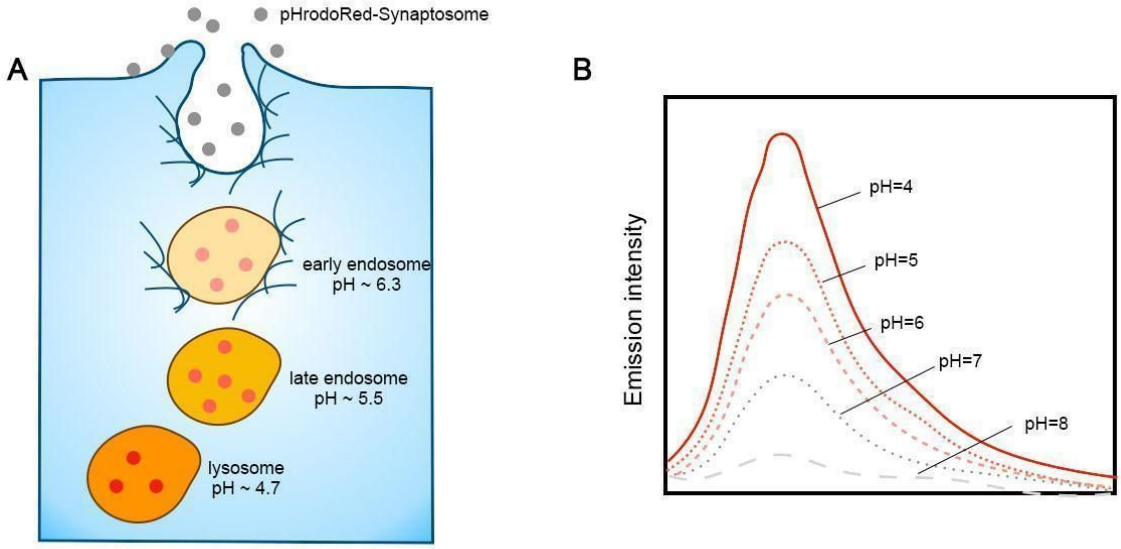


Figure 10: *pH effects on fluorescence intensity of pHrodo Red conjugated synaptosome (adapted from Dolman et al 2013[3]). (A) Relative increase of pHrodo Red intensity from nonfluorescent state at neutral pH in cellular medium to gradually higher fluorescence intensity through progressive state of phagocytosis by microglia. (B) Solution-based emission profile of pHrodo Red at different pH levels, with gradually higher peak emission at lower pH.*

To evaluate the reliability of our assay, we incubated freshly sorted microglia in the absence or presence of pHrodo Red labeled synaptosome. As expected, pHrodo Red signal was only detected in microglia incubated with pHrodo Red labeled synaptosome (**Figure 11**).

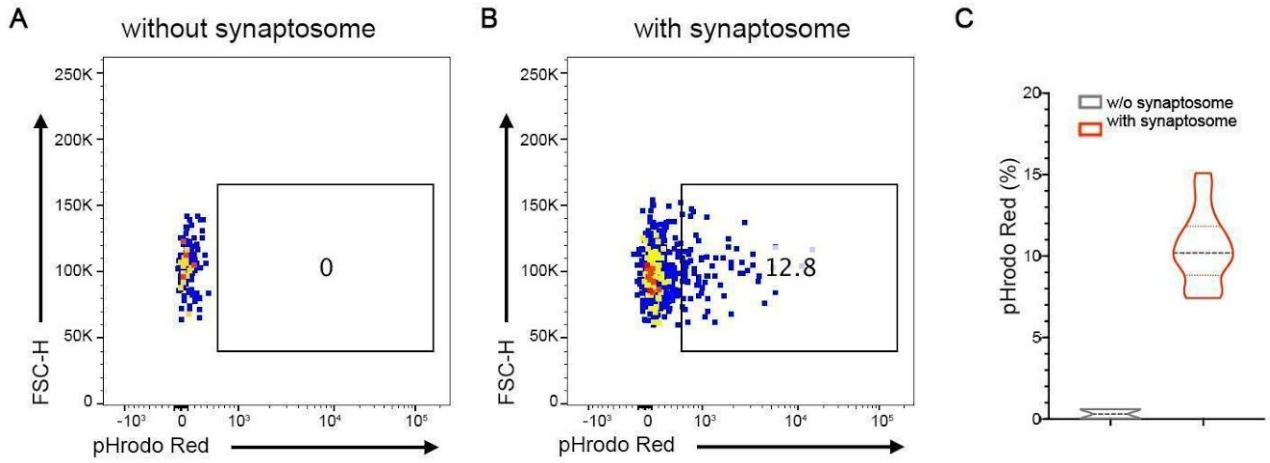


Figure 11: *microglia engulf synaptosome ex vivo. Freshly sorted microglia were incubated with pHrodo Red labeled synaptosome for 2hrs and then analyzed by flow cytometry. Microglia incubated in the absence of synaptosome served as negative control (shown in A), and pHrodo Red signals could only be detected in microglia incubated with pHrodo Red labeled synaptosome (shown in B). Quantification of the proportion of microglia with pHrodo Red signals is shown in C.*

Next, we asked if IL-33 could regulate synaptosome engulfment by microglia. Postnatal day 5 mice received 4 injections of either PBS or IL-33 intraperitoneally, and microglia were purified from postnatal day 9 brains. Freshly sorted microglia from both groups were incubated with pHrodo Red conjugated synaptosome for 2 hours, and the fluorescence intensity was analyzed by flow cytometry. We found IL-33 administration increased the total amount of synaptosome engulfed by microglia (**Figure 12**), suggesting that IL-33 promotes the phagocytic activity of microglia.

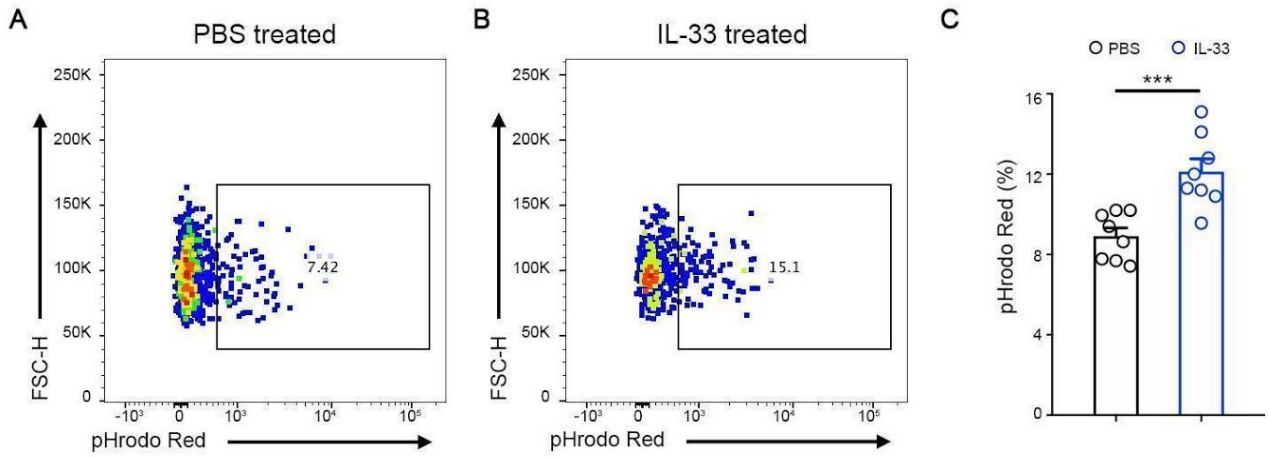


Figure 12: *IL-33 stimulates synaptosome engulfment by microglia. Microglia were acutely isolated from postnatal day 9 mice that have received either PBS or IL-33 intraperitoneally, and then incubated with pHrodo Red conjugated synaptosome for 2hrs. The intensity of pHrodo Red was analyzed by flow-cytometry. PBS-treated microglia are shown in A and IL-33 treated microglia are shown in B. Quantification of the proportion of microglia with pHrodo Red signals is shown in C.*

Summary

In summary, we have concluded that the cytokine IL-33 indeed regulates synaptic pruning by microglia. Through previous knowledge, we garnered that microglial cells express ST2 and that ST2 is the receptor for IL-33. This connection revealed a relationship between microglia and neurodevelopment. Through our experimentation, we furthered quantified the relationship, where IL-33 promotes the phagocytic activity of microglia. In observation, we discovered that ST2 KO mice exhibit seizures. This phenomenon results from altered synaptic functioning of the brain. As ST2 is knocked out, fewer IL-33 can bind and promote synaptosome engulfment by microglia. Furthermore, we observed additional synaptic impairment. Through electrophysiology, we discovered that ST2 KO mice display LTP impairment, signifying reduced synaptic plasticity in seizure mice. Previous research indicates that seizures may cause synaptic impairment. Such observations suggest a repetitive cycle with synaptic impairment. Reduced synaptosome engulfment causes seizures, and in turn, seizures lower synaptic plasticity.

As a result, synaptic impairment, specifically underregulated synapses, correlates to the presence of epileptic behaviors and neurological disorders. Conveying this research into real-life circumstances, we can indicate a relationship between microglial abnormalities and epileptic and autisitic behaviors.

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