Oxytocin: Mediating the Beneficial Effects of Social Interaction on Health

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SUMMARY:
Social interaction is an important component of both mental and physical health, and despite a plethora of studies demonstrating the influence of social support on neurological disease processes, whether there are quantifiable baseline differences between the neurons of socially isolated and integrated individuals remains unknown. Here we report that social isolation in mice leads to a decrease in the expression of CD200 and Crry on neurons; both of these surface proteins suppress microglial activation. Indeed, microglia obtained from socially isolated mice demonstrate greater neuron-directed phagocytic activity *in vitro* than those collected from socially paired mice. Furthermore, phagocytosis is directed against CD200- neurons and Crry- neurons. Collectively, these data suggest that in otherwise healthy animals, the neurons of socially isolated mice may be more vulnerable to phagocytosis than neurons from socially paired mice.

Running title: Social isolation compromises neurons

Highlights:

Social isolation compromises neuronal integrity.

Social isolation results in decreased expression of CD200 and Crry on surface neurons.

CD200- neurons from socially isolated mice are distinct from CD200- neurons from paired mice based on Crry expression.

Microglia-mediated phagocytosis in socially isolated mice targets CD200- neurons and Crry- neurons.

INTRODUCTION

Social isolation is an important risk factor and strongly influences morbidity and mortality for a myriad of human health conditions, including cancer, cerebrovascular disease, and cardiovascular disease (Reblin and Uchino, 2008; House et al., 1988). The effects of social environment on disease course have been reproduced in animal models and provide insight into potential underlying mechanisms (Craft et al., 2005; Karelina et al., 2009; Weil et al., 2008; Pyter et al., 2009). Indeed, the extent of microglial activation, pro-inflammatory cytokine expression, and neuronal damage that occurs after experimental stroke or cardiac arrest among socially isolated mice is substantially higher than among socially housed mice (Craft et al., 2005; Karelina et al., 2009; Weil et al., 2008; Pyter et al., 2009). All mechanistic studies to date have focused on the evolution of microglial activation and neuronal damage following a neurological insult; whether neuron-microglia interactions are altered among socially isolated, but otherwise healthy, mice is not known but is critically important to understanding the influence of social environment on brain function in the contexts of both health and disease states.

Although neurons comprise approximately 10% of the total number of cells in the brain, they are vital to maintaining a homeostatic environment through communication with surrounding glia cells, such as microglia (Fuhrmann et al., 2009; Galea et al., 2007). Disruption of this communication could result in both direct and indirect infliction of damage on neurons. Two proteins expressed on the surface of neurons, CD200 and Crry, are particularly important in modulating microglial activation. CD200 (Ox2) is a neuronal glycoprotein expressed on the surface of neurons which functions to hold microglia in a quiescent state through engagement of the CD200 receptor expressed by microglial cells (Hoek et al., 2000; Webb and Barclay., 1984; Wright et al., 2000). In the absence of neuronal CD200 expression in diseases such as

Alzheimers, Parkinson's and EAE, microglia become activated, suggesting that microglial activation is mediated, at least in part, by the integrity of surrounding neurons (Fuhrmann et al., 2009; Le et al., 2001; Hoek et al., 2000). The autoimmune nature of these diseases suggests loss of neuronal CD200 expression and subsequent activation of microglia may initiate a cascade of events that can damage neurons.

Additionally, loss of neuronal integrity may cause neurons to become susceptible to complement-mediated phagocytosis by activated microglia through loss of complement regulatory proteins such as Crry. Crry is a membrane bound molecule which protects against the classical and alternative complement pathways (Foley et al., 1993; Li et al., 1993; Kim et al., 1995). Its expression has been shown throughout the mouse brain, including neurons (Davoust et al., 1999).

In the current study, we sought to identify phenotypic and functional differences in the neurons and microglia of healthy animals with disparate social environments. The data demonstrate an increase in neuronal vulnerability of socially isolated mice relative to paired controls. Although total frequencies of neurons and microglia do not change due to housing conditions, there is a decrease in surface expression of CD200 and Crry on neurons of socially isolated mice compared to paired controls. Further, we provide functional data demonstrating an increase in the phagocytic activity of microglia in response to social isolation and loss of CD200 and Crry. Collectively, these data suggest that neuron-microglial communication is altered in socially isolated mice and may lead to increased phagocytic activity of microglia and ultimately neuronal death.

RESULTS

Neuronal and microglial frequencies in various housing conditions: Cells isolated from the brains of male mice that had been socially isolated (n=5) or pair housed with an ovariectomized female (n=5) for one week were stained with anti-NeuN (Figures 1A-1C) or anti-CD11b (Figures 1D-1F), markers specific for neurons and microglia, respectively. Flow cytometry revealed no significant difference in the frequency of neurons ($P > 0.05$; Figure 1B) or microglia ($P > 0.05$; Figures 1E) when comparing socially isolated and pair housed animals. These findings are corroborated with immunohistochemical analysis for NeuN (Figure 1C) and CD11b (Figure 1F). Surface expression of NeuN was confirmed by immunohistochemistry and used for flow cytometry analysis in subsequent studies (supplemental data).

Neuronal surface expression of CD200: When following the staining pattern of the NeuN+ population, distinct populations of CD200+ and CD200- cells become apparent (Figures 2A and 2B). The ratio of NeuN+CD200+ cells to NeuN+CD200- cells is decreased in socially isolated mice relative to pair housed controls $P < 0.05$; Figure 2C). When examining specific regions of the brain tissue, we find a population of CD200- cells in hippocampus sections of socially isolated mice that is not present in cortex sections (Figure 2D). Immunohistochemical analysis of hippocampus from socially isolated mice demonstrates the disparity of NeuN+ cells expressing CD200 (Figures 2E-2G). A time course study investigating socially isolated mice and mice paired Day 1, Day 3 and Day 7 demonstrate a gradual increase in the surface expression on CD200 by NeuN+ cells overtime (Figure 2H). Further, removal of a partner after 7 days of pairing resulted in a striking decrease in CD200 expression by NeuN+ cells as early as Day 1. This value remained below baseline at Day 3 and returned to a level similar to isolated mice

(Day 0) at Day 7. In contrast, expression of the CD200 receptor (CD200R) on microglial cells was not affected by social environment (data not shown).

Neuronal expression of regulatory receptors: An increase in CD200- cells during social isolation may make these cells more susceptible targets of phagocytosis (Hoek et al., 2000; Graeber et al., 1988). Therefore, we sought to identify the expression of the phagocytosis inhibitor, Crry, by neurons obtained from paired and socially isolated mice (n= 5/group). Social isolation was associated with an increase in Crry- neurons (Figure 3A) and reduction in Crry+ neurons (Figures 3B), suggesting that neurons from socially isolated mice could be more vulnerable to phagocytosis. Similarly, purified neurons from socially isolated mice demonstrate a significant reduction in relative expression of Crry compared to paired control mice (Figure 3C). Furthermore, when looking specifically within the CD200- population, significantly more cells from socially isolated mice are also Crry- (Figure 3D). Thus, not only is the frequency of CD200+ cells decreased in socially isolated mice (Figure 2), it appears that the CD200- population in socially isolated mice is phenotypically distinct from the CD200- population in paired mice with regard to Crry expression. Similar to the pattern observed with CD200 in Figure 2H, the increased expression of Crry by NeuN+ cells is gradual from isolated mice to Day 7 pairing (Figure 3E). One day after separation, we observe a reduction in Crry expression followed by a gradual increase returning to baseline isolated levels at Day 7.

Microglial activation based on housing conditions: As CD200 and Crry expressing neurons communicate with microglia, we sought to characterize the microglia phenotype in response to social isolation. Microglia from socially isolated mice exhibit an activated phenotype as evidenced by increased CD80 surface expression (Figure 4A). Further, social isolation induces an increase in the pro-inflammatory cytokine IL-1β (Figure 4B) and decrease in the anti-

inflammatory cytokine IL-10 (Figure 4C) demonstrating a general pro-inflammatory profile of microglia from socially isolated mice relative to paired control mice.

Microglia-mediated phagocytosis of neurons: Decreased surface expression of CD200 and Crry suggest that social isolation could make neurons more susceptible to phagocytosis. To directly address this, a functional assay was performed to determine the phagocytic activity of microglia from socially isolated and paired mice. Microglia were enriched as described in Materials and Methods section and incubated for 30 minutes with CFSE-labeled non-microglia CNS cells from the same mice (Figure 5A). The percent of CD11b+CFSEint microglia cells in isolated mice is significantly increased (25%) relative to CD11b+CFSEint microglia cells in paired mice (15%; Figures 5B and 5C). We then sought to directly determine the impact of neurons and microglia in this assay. Neurons were purified from isolated mice followed by incubation with microglia from paired mice and similarly neurons were purified from paired mice followed by incubation with microglia from isolated mice. Figure 5D demonstrates that increased phagocytosis is dictated by the housing condition of the neuron. To further support this, microglia from paired or socially isolated mice were incubated with opsonized microspheres to determine phagocytic potential of microglia in the absence of neurons. The data show that, in contrast to uptake of neurons in Figure 5D, microglia from paired and socially isolated mice present no significant difference in uptake of opsonized microspheres (Figure 5E).

CD200-dependent and Crry-dependent phagocytosis of neurons: Socially isolated mice demonstrate reduced CD200 (Figure 2) and Crry (Figure 3) expression and increased phagocytosis (Figure 5). Therefore, we sought to determine the influence of CD200 and Crry directly in the phagocytic potential of microglia cells. NeuN+ cells sorted into CD200+ and CD200- neurons were labeled with CFSE and incubated with matching microglia cells (Figure

6A). Purity of CD200 sort is shown in representative dot plots in Figure 7B. We demonstrate that microglia incubation with CD200- cells results in a significant increase in the frequency of CD11b+CFSEint cells (17%) relative to CD200+ cells (57%) indicating increased engulfment of CD200- cells by microglia (Figures 6C and 6D). To determine the mechanism of phagocytosis, microglia were pre-treated with cytochalasin D as described in Materials and Methods. We show in Figure 6E that co-incubation of micoglia and CD200-sorted neurons in the presence of cytochalasin D does not eliminate the different levels of uptake between CD200+ and CD200- cells by microglia. Additionally, NeuN+ cells were sorted based on expression of Crry as shown in Figure 6F. Crry+ and Crry- cell populations were labeled with CFSE and incubated with matching microglia cells. Similar to CD200 expression, the absence of Crry expression induces an increase in neuronal uptake as determined by an increase in CD11b+CFSEint cells relative to cells expressing Crry (Figures 6G and 6H). However, in contrast to CD200, pre-treatment of enriched microglia with cytochalasin D prior to incubation with Crry+ or Crry- cells resulted in a dramatic reduction of Crry- cell uptake (Figure 6I) relative to no pre-treatment with cytochalasin D (Figure 6H).

FIGURE LEGENDS

Figure 1: Frequency of neurons and microglia remain constant in paired and socially isolated mice. (A, B, C) Representative dot plots (A) or summary bar diagram (B) of whole brain preparations from paired or isolated mice labeled with anti-NeuN marking neurons by flow cytometry or immunohistochemistry (C). (D, E, F) Representative dot plots (D) or summary bar diagram (E) of whole brain preparations from paired or isolated mice labeled with anti-CD11b marking microglia by flow cytometry or immunohistochemistry (F). Gates outlined in dot plots are used for all subsequent studies. Bar diagrams represent 5 mice/group and error bars represent

SEM. There were no statistically significant differences ($P > 0.05$) between groups based on housing condition .

Figure 2: Social isolation reduces neuronal surface expression of CD200. (A) Representative dot plots of whole brain preparations from paired or isolated mice labeled with anti-NeuN and anti-CD200. (B) Representative histogram of NeuN+ neuronal cells labeled for CD200 surface expression. Data from paired mice and socially isolated mice are highlighted in red and blue, respectively. (C) Summary bar diagram of NeuN+ neuronal cells plotting ratio of NeuN+CD200+ and NeuN+CD200- frequency in paired and socially isolated mice. (D) Representative histograms of CD200 expression by NeuN+ cells in hippocampus and cortex sections from socially isolated mice. (E, F, G) Immunohistochemical analysis of a hippocampal slice from a representative socially isolated mouse staining for NeuN (E), CD200 (F) and merge (G). (H) Time course diagram of the percent of NeuN+CD200+ cells from Isolated, Day 1, Day 3, and Day 7 paired mice and mice separated Day 1, Day 3, Day 7 after pairing. Bar diagrams represent 5 mice/group. Error bars represent SEM. An asterisks (*) indicates a statistically significant difference between at $P < 0.05$. Similar letters above error bars indicate no significant difference between groups ($P > 0.05$), whereas different letters represent a significant difference between groups ($P < 0.05$).

Figure 3: Neurons from socially isolated mice reduce surface expression of Crry. (A) Representative histogram of NeuN+ neuronal cells from socially isolated or paired mice labeled for Crry surface expression. (B) Summary bar diagram of NeuN+ neuronal cells from socially isolated or paired mice co-expressing Crry. (C) Summary bar diagram of relative Crry expression in samples of purified neurons from paired and socially isolated mice. (D) Summary bar diagram of percent NeuN+ CD200- Crry- neuronal cells. (E) Time course diagram of the percent of

NeuN+Crry+ cells from Isolated, Day 1, Day 3, and Day 7 paired mice and mice separated Day 1, Day 3, Day 7 after pairing. Bar diagrams represent 5 mice/group. Error bars represent SEM. Asterisks indicate a statistically significant difference between groups at $P < 0.05$. Similar letters above error bars indicate no significant difference between groups ($P > 0.05$), whereas different letters represent a significant difference between groups ($P < 0.05$).

Figure 4: Microglia in socially isolated mice exhibit an activated, pro-inflammatory profile. Bar diagrams summarizing microglial expression of (A) surface CD80, (B) intracellular IL-1β, and (C) intracellular IL-10. Bar diagrams represent 5-8 mice/group. Error bars represent SEM. Asterisks indicate a statistically significant difference between groups at $P < 0.05$.

Figure 5: Social isolation induces increased microglia-mediated phagocytosis. (A) Experimental design of microglia-mediated phagocytosis as described in Materials and Methods. (B) Representative dot plots of cells from enriched microglia population and CNS cell population mixed and stained for co-expression of CD11b and CFSE. Gate represents CD11b+CFSEint cells. (C) Summary bar diagram of CD11b+CFSEint microglial cells after incubation with non-microglia CNS cells. (D) Summary bar diagram of CD11b+CFSEint cells after incubation of paired neurons with isolated microglia and isolated neurons with paired microglia. (E) Summary bar diagram of enriched microglial cells following 30 minute incubation with opsonized microspheres. Bar diagrams represent 4 mice/group. Error bars represent SEM. An asterisks indicates a statistically significant difference between groups at $P < 0.05$.

Figure 6: (A) Experimental design of cell-sorted phagocytosis as described in Materials and Methods. (B) Representative dot plots of CD200 expression in sorted CD200+ and sorted CD200- populations. (C) Representative histogram of CD11b+ cells incubated with sorted CD200+ cells or sorted CD200- cells. (D) Summary bar diagram of CD11b+CFSEint microglial

cells after incubation with CD200+ or CD200- cells. (E) Summary bar diagram of CD11b+CFSEint microglia cells treated with cytochalasin D prior to incubation with CD200+ or CD200- cells. (F) Representative dot plots of Crry expression in sorted Crry+ and sorted Crry- populations. (G) Representative histogram of CD11b+ cells incubated with sorted Crry+ cells or sorted Crry- cells. (H) Summary bar diagram of CD11b+CFSEint microglial cells after incubation with Crry+ or Crry- cells. (I) Summary bar diagram of CD11b+CFSEint microglia cells treated with cytochalasin D prior to incubation with Crry+ or Crry- cells. Bar diagrams represent 3 mice/group. Error bars represent SEM. An asterisks indicates a statistically significant difference between groups at $P < 0.05$.

Supplemental Figure 1: (A) Immunohistochemical staining of hippocampal slices from isolated mice stained for surface or intracellular NeuN. (B) NeuN surface staining of Cath.a neuronal cell line. (C) Surface expression of 2F7 following purification of brain cells based on NeuN surface expression.

DISCUSSION

Social isolation has long been recognized as a risk factor for mental disorders and more recently as an exacerbating factor for somatic disorders as well (Nesse and Finlayson., 1996; Patten et al., 2005; Chapman et al., 2005). However, a physiological link between social isolation and disease has been less obvious, and whether quantifiable physiological differences exist prior to the onset of disease is not known. The current study suggests that subtle changes in neurons and microglia do occur in otherwise healthy mice following social isolation; the neurons of socially isolated mice are phenotypically distinct and more susceptible to phagocytosis than neurons from socially integrated mice. Furthermore, microglia from socially isolated mice exhibit a more pro-

inflammatory profile than those from mice housed in affiliative pairs. These data have implications for neuron-immune interactions in both healthy and diseased brains.

CD200 and CD200R surface expression

CD200 surface expression is often used as a marker of neuronal health and integrity as its expression is reduced during neurological disease and aging (Chitnis et al., 2007; Walker et al., 2009; Koning et al., 2009; Frank et al., 2006). CD200 holds microglia quiescent through engagement of the microglial CD200 surface receptor (CD200R) (Hoek et al., 2000). Thus, reduced neuronal CD200 expression is associated with increased microglial activation. A reduction in CD200 expression has been reported in rodent models of bacterial infections (Masocha, 2009), and aging (Frank et al., 2006) as well as in human brain tissue collected from Alzheimer's (Walker et al., 2009) and multiple sclerosis (Koning et al., 2009) patients. In contrast, upregulation of CD200 expression attenuates the development of symptoms associated with experimental autoimmune encephalomyelitis, an experimental model of multiple sclerosis (Chitnis et al., 2007). Thus, a potential role for CD200 has been suggested in several neurodegenerative disorders. The current study suggests that social environment can modulate CD200 expression in otherwise healthy animals (Figure 2); neuronal CD200 expression was reduced in socially isolated mice compared to pair housed controls. In contrast, there were no significant group differences in the expression of CD200R on the surface of microglia (data not shown), thus there was not a compensatory increase in CD200R expression in response to the reduction in CD200 signal among socially isolated mice, suggesting that the reduction in CD200 could be functionally significant. In addition to the observation of a CD200- population in whole brain preparations described above, we identified a striking increase in CD200- frequency of

hippocampal relative to cortex preparations suggesting a link to long-term memory and learning (Figure 2D).

Crry Expression on Neurons

In a healthy environment, phagocytosis is occurring at basal levels to maintain homeostasis through removal of cellular debris (Nimmerjahn et al., 2005). However, reduced surface expression of Crry enhances the progression of complement-mediated phagocytosis. Crry is a protective marker expressed on the surface of cells resulting in disruption of the complement cascade involving cleavage of complement component C3 into the proteins C3a and C3b (Foley et al., 1993; Davoust et al., 1999). We show that the loss of Crry expression on neurons from socially isolated mice relative to paired control mice (Figure 3) is associated with an increase in neuron-directed phagocytosis (Figure 6). In addition to overall reduction in Crry expression by isolated mice, Crry expression is also significantly reduced within the CD200- population (Figure 3D). The combined loss of CD200 and Crry expression by isolated mice suggests that neurons have a reduced capacity to inhibit phagocytosis in an environment that encourages microglial activation. Further, separation of mice following 7 days of pairing resulted in a reduction in CD200 (Figure 2H) and Crry (Figure 3E) expression as early as Day 1 returning to baseline isolated levels at Day 7. These data support our hypothesis by demonstrating an immediate physiologic change in neurons due solely to removal of a partner mouse.

Microglia

In the current study, CD11b surface expression marked microglial cells in the whole brain preparations, while the concurrent intermediate expression of CD45 suggests that the samples were not contaminated by CD11b+ infiltrating macrophages (data not shown). Microglia from socially isolated mice had elevated expression of the activation marker CD80 and the pro-

inflammatory cytokine interleukin 1-β (IL-1β) as well as decreased expression of the anti-inflammatory cytokine IL-10 relative to pair housed mice (Figure 4). Together, these data suggest that the microglia in socially isolated mice are exhibiting a pro-inflammatory bias (Leonard, 2007).

Phagocytosis

A microglial phagocytosis assay was performed *in vitro* to determine the potential functional significance of changes in neuronal expression of CD200 and Crry. Microglia were enriched through the use of a density gradient while neurons were purified via cell sorting or magnetic beads. The increase in phagocytosis among microglia from socially isolated mice was evident using non-microglia CNS cells (Figure 5C) or magnetic bead-purified neurons (Figure 5D). Indeed, after incubation of microglia with magnetic bead-purified neurons, we observed a more pronounced shift in the CFSEint population (60-70%) relative to non-microglia CNS cells (15-25%) suggesting neurons are a major target of microglia engulfment. Further, our data suggest neurons dictate the phagocytosis response as incubation of neurons from paired mice with microglia from isolated mice follow the reduced phagocytosis pattern of paired mice whereas incubation of neurons from isolated mice with microglia from paired mice follow the increased phagocytosis pattern of isolated mice (Figure 5D). A similar pattern was noted when we observed no significant difference in uptake of opsonized beads between microglia from paired or isolated mice (Figure 5E) further suggesting the role of neuronal signals. The observed increase in microglial phagocytosis of neurons from socially isolated mice is consistent with our phenotypic observations of decreased neuronal CD200 and Crry expression in this experimental group. The importance of CD200 and Crry as markers of phagocytosis is confirmed as phagocytosis is significantly increased in the absence of either of these markers (Figure 6). The

data are consistent with the hypothesis that the loss of CD200 expression is inducing a sensitized microglial response due to the lack of CD200R engagement.

It is interesting to note that while Crry- cells are susceptible to cytochalisin D-mediated inhibition of phagocytosis (Figure 6I), CD200- cells maintain high levels of neuronal uptake in the presence of this inhibitor (Figure 6E). We conclude from these data that a population of CD200- cells likely induce a mechanism of neuronal uptake that is not susceptible to cytochalasin D. This may be explained by 1) the observation that 20-30% of Crry- cells are capable of neuronal uptake in the presence of the inhibitor (Figure 6I) and 2) although a proportion of CD200- cells are also Crry- (Figure 3D), there remains a population of CD200- cells that are Crry+ and therefore not susceptible to cytochalasin D. This alternate mechanism may include clatharin-mediated endocytosis and/or caveolae, among others. The exact mechanism of CD200- neuronal uptake is yet to be determined.

Summary

Social environment has a profound effect on mental and physical health. The current study provides the first evidence that social isolation alters neuro-immune interaction in a manner that compromises neurons in otherwise healthy individuals. Furthermore, social modulation of microglial behavior via neuronal expression of CD200 and Crry could increase susceptibility to neurodegenerative disorders and precipitate affective or cognitive disorders.

EXPERIMENTAL PROCEDURES

Animals: Adult male C57/BL6 mice (Charles River, Wilmington, Mass.) were maintained in micro-isolator cages on a 14:10 light/dark cycle, in a temperature and humidity-controlled vivarium. All animals were allowed *ad libitum* access to food and water. The mice were housed

either individually (socially isolated) or with an ovariectomized female (paired) for one week prior to tissue collection unless otherwise noted.

Reagents: Stain wash buffer consisted of PBS, Na azide, and newborn calf serum. Anti-CD11b (clone M1/70), anti-CD200 (clone OX90), anti-NeuN (clone A60), anti-Crry (clone 1F2), anti-IL-1b, anti-IL-10 (clone JES5-16E3), anti-CD80 (clone 16-1OA1) and CD200R (clone OX110) antibodies were obtained from ebioscience (San Diego, CA) and used per manufacturer's instructions. Cytochalasin D was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell isolation and flow cytometry: Brain tissue was collected immediately following euthanasia and passed through 70uM cell strainers to obtain single-cell suspensions. Red blood cells were lysed and a minimum of 5x106 cells/sample were washed and resuspended in stain wash buffer. Cell surface Fc receptors were blocked via incubation with anti-CD16/32 antibody (ebioscience, San Diego, CA) and then washed with stain wash buffer. All antibody incubations were performed on ice, in the absence of light. In general, the cells are incubated for 30 min with mixtures of specific concentrations of fluorochrome-labeled antibodies. Flow cytometry data were acquired using a BD LSRII instrument (San Jose, CA) and analyzed using TreeStar FlowJo software (Ashland, Or). For any given marker, all of the analysis gates were identical in size and position.

For CD200 cell sorting, microglia were removed using Percoll density gradient as described below and cells were incubated with Fc block (CD16/32), washed and stained with anti-NeuN and anti-CD200. NeuN+ cells were selected and purified into CD200+ and CD200- populations (CD200+ purity 72%, CD200- purity 97%). For Crry cell sorting, neurons were first enriched using neuron enrichment protocol as described below followed by incubation with Fc block (CD16/32) and washed and stained with anti-Crry and purified into Crry+ and Crry- populations

(Crry+ purity 83%, Crry- purity 95%). All samples were purified using BD FACSAria II with Diva option.

Microglial enrichment: Brain tissue isolated from paired or socially isolated mice was minced and homogenized. Single cell suspensions were resuspended in 70% Percoll to which 50% Percoll and 0% Percoll were layered atop respectively to generate a density gradient during a 45 minute centrifugation (2400 rpm). Non-microglia CNS cells generated a thick, white interface between 0% and 50% Percoll layers. The interface generated between the CNS interface and the pellet consisting of enriched microglia was removed, washed and used for subsequent studies. CD11b+ enrichment was confirmed using flow cytometry staining of surface CD11b before and after enrichment. Microglial purity was confirmed as CD11b+ cells at >85%.

Neuron enrichment: Anti-NeuN clone A60 (Millipore, Billerica, MA) was labeled using DSB-X Biotin Protein Labeling Kit according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Neurons were enriched using Dynabeads FlowComp Flexi Kit according to manufacturer's instructions (Invitrogen, Carlsbad, CA).

Phagocytic assay: The CNS interface components separated from microglia using a density gradient as described above were labeled with 0.05uM Carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR). The CFSE-labeled CNS cell suspension was incubated with gentle rotation at 37°C with enriched microglial cells from the matching paired or socially isolated mice in serum-free DMEM. After 30 minutes, cold HBSS was added to stop phagocytosis and cells were immediately analyzed by flow cytometry. Analysis determined the % of CFSEint cells (<105) within the CD11b+ gate to identify CFSE-labeled cell uptake by microglial cells. The analysis accounted for the potential contamination of CD11b+ cells directly labeled with CFSE (>105). For specified experiments, 1uM cytochalasin D was added to

enriched microglia for 30 minutes prior to addition of sorted NeuN+ cells. For studies co-incubating isolated microglia with paired neurons and vice versa, microglia were enriched as described above and dynabead-purified neurons were labeled as described above. The mixed cell populations were incubated for 30 minutes prior to analysis to determine the % of CFSEint cells.

Real-Time PCR: Total RNA was extracted from purified neurons by using an ultrasonic cell disruptor (Microson XL-2000, Misonix) and an RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. Extracted RNA was suspended in 30 μL of RNase-free water, and RNA concentration was determined by a spectrophotometer (NanoDrop ND-1000). M-MLV Reverse Transcriptase was used to create cDNA according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Crry inventoried primer probe kit was purchased from Applied Biosystems (Carlsbad, CA). A Taqman 18S ribosomal RNA primer and probe set, labeled with VIC dye (Applied Biosystems), was used as a control gene for relative quantification. Amplification was performed on an ABI 7000 Fast Sequencing Detection System using Taqman Universal PCR master mix.

Immunohistochemistry: Mice were anesthetized (pentobarbital) and perfused transcardially with 0.1 mol/L phosphate-buffered saline followed by 4% paraformaldehyde. Brains were removed, post-fixed for 4 h, and then cryoprotected in 30% sucrose in 0.2 mol/L phosphate-buffered saline. The brains were sectioned coronally at 50 μm on a freezing microtome and rinsed 3 times for 5 minutes each in 0.1 mol/L phosphate-buffered saline. The sections were then incubated for 40 hours at 4°C with mouse anti-NeuN (Millipore Bioscience Research Reagents, 1:100). After 3 five minute rinses, the tissue was incubated with secondary antibodies for 2 hours at room temperature Donkey anti-mouse Dylight 649 (Jackson ImmunoResearch Laboratories, 1:1000). The tissue was again rinsed 3 times and then mounted onto Super Frost Plus slides

(Fisher, Hampton, NH, USA). All images were captured using a Zeiss LSM 510 confocal microscope.

Data Analysis: Differences between the paired and socially isolated experimental groups in each experiment was evaluated by Student's t-test. Differences were considered statistically significant if P values were less than 0.05. All error bars represent standard error.

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FIGURE 1

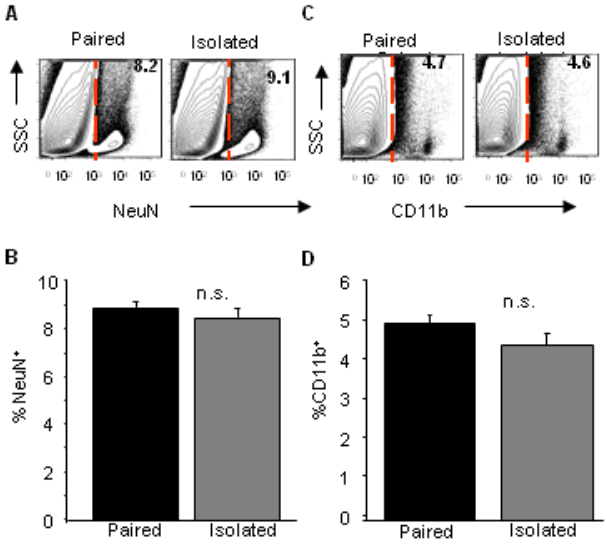

A
Paired
Isolated
8.2
9.1
SSC
NeuN
C
Paired
Isolated
4.7
4.6
SSC
CD11b
B
% NeuN+
n.s.
Paired
Isolated
D
%CD11b+
n.s.
Paired
Isolated

FIGURE 2

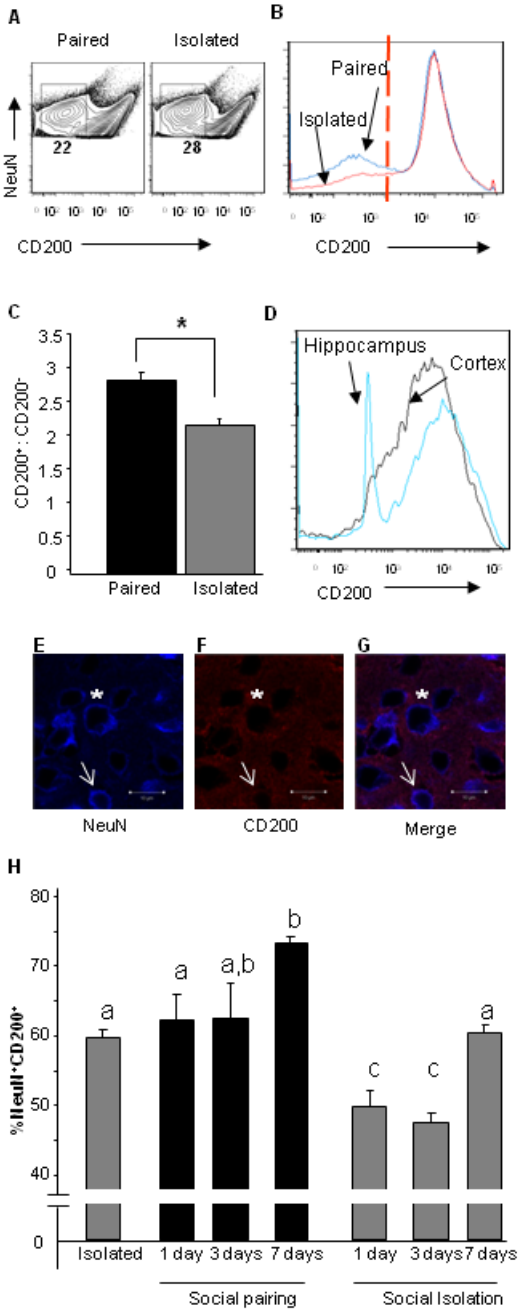

A
Paired
Isolated
NeuN
22
28
CD200
B
Paired
Isolated
CD200
C
CD200+ : CD200-
Paired
Isolated
D
Hippocampus
Cortex
CD200
E
F
G
NeuN
CD200
Merge
H
%NeuN+CD200+
Isolated
1 day
3 days
7 days
Social pairing
Social Isolation

FIGURE 3

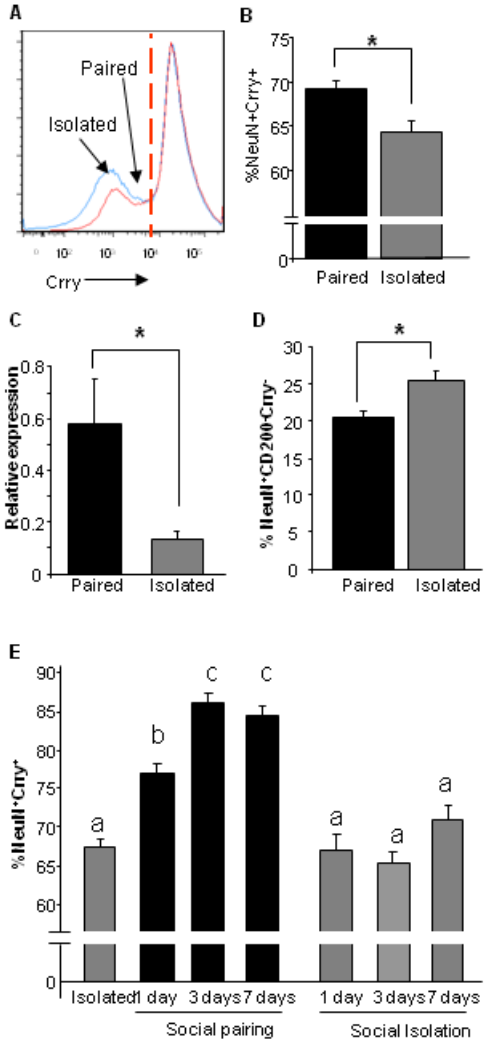

A
Paired
Isolated
Crry
B
%NeuN+Crry+
75
70
65
60
0
*
Paired
Isolated
C
Relative expression
0.8
0.6
0.4
0.2
0
*
Paired
Isolated
D
% Ileuil+CD200-Cnry-
30
25
20
15
10
5
0
*
Paired
Isolated
E
%Ileuil+Cnry+
90
85
80
75
70
65
60
0
a
b
c
c
a
a
a
Isolated
1 day
3 days
7 days
1 day
3 days
7 days
Social pairing
Social Isolation

FIGURE 4

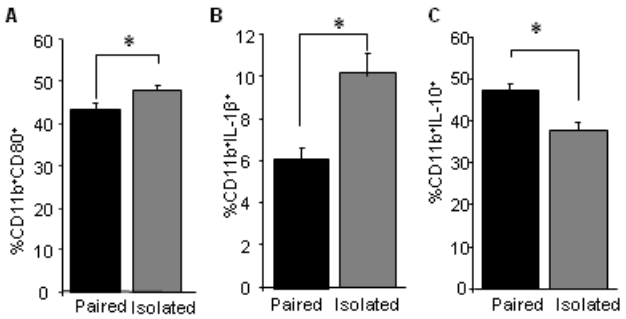

A
B
C
%CD11b+CD80+
%CD11b+IL-1β+
%CD11b+IL-10+
*
Paired
Isolated
0
10
20
30
40
50
60
2
4
6
8
10
12

FIGURE 5

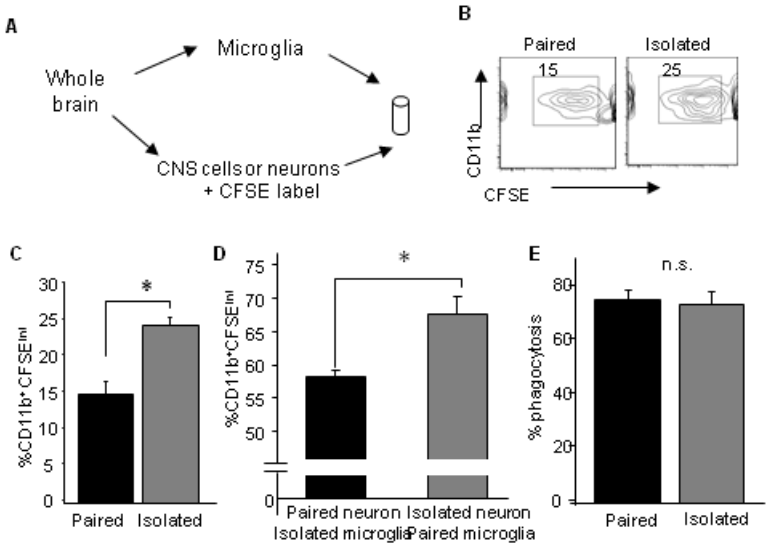
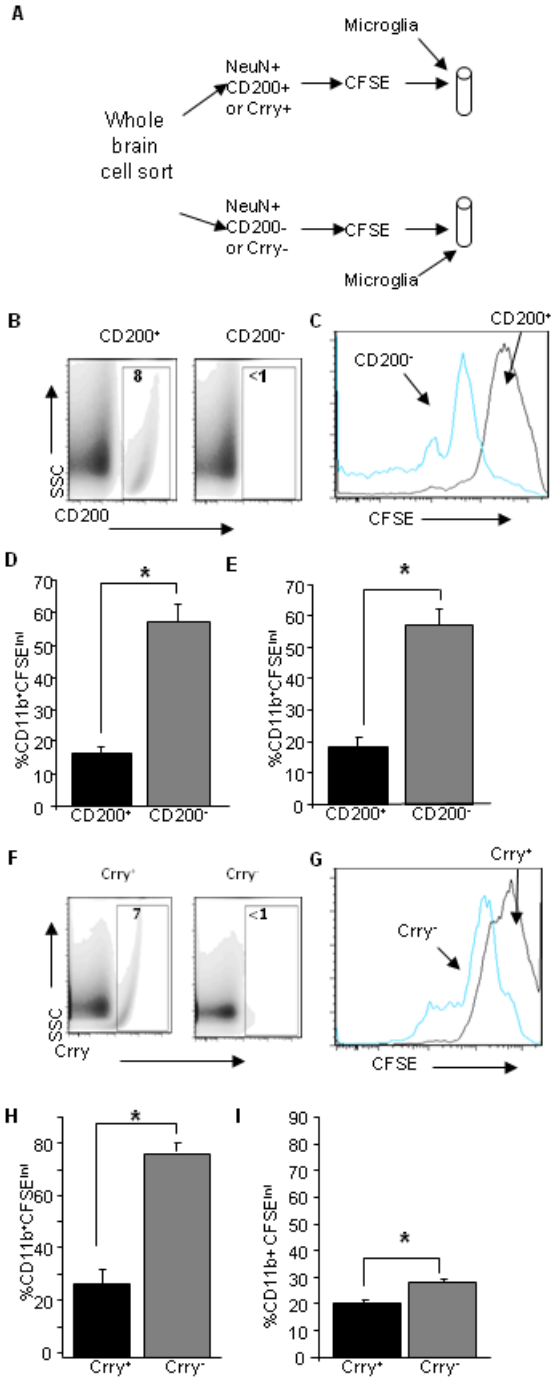
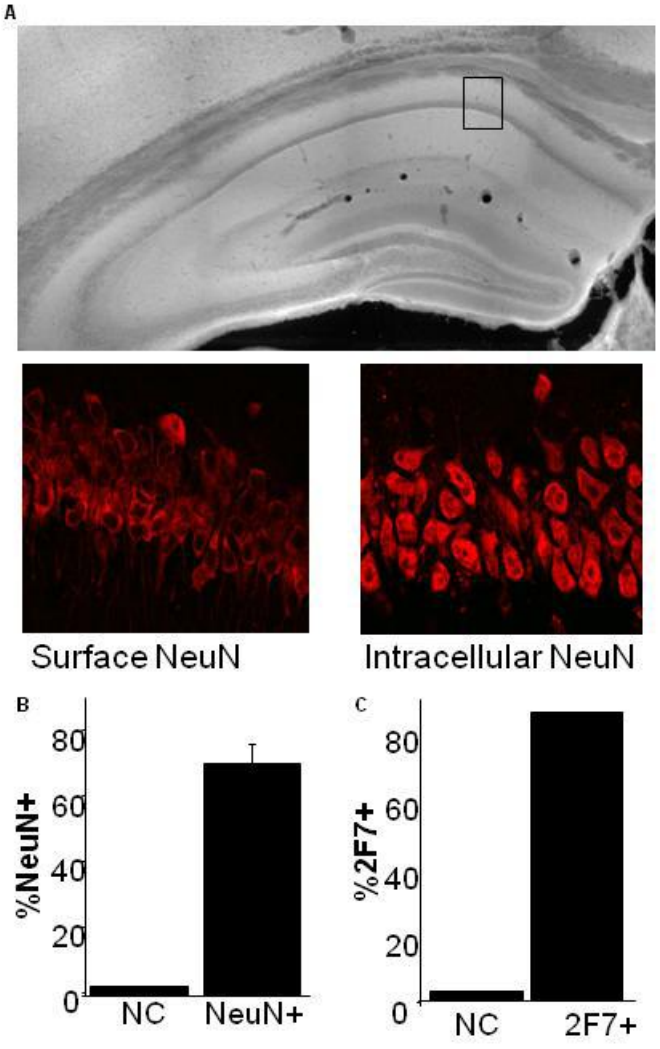

A
Whole brain
Microglia
CNS cells or neurons + CFSE label
B
Paired
Isolated
15
25
CD11b
CFSE
C
%CD11b+CFSEint
30
25
20
15
10
5
0
*
Paired
Isolated
D
%CD11b+CFSEint
75
70
65
60
55
50
0
*
Paired neuron Isolated microglia
Isolated neuron Paired microglia
E
% phagocytosis
80
60
40
20
0
n.s.
Paired
Isolated

FIGURE 6


A
Microglia
NeuN+
CD200+
or Crry+
CFSE
Whole
brain
cell sort
NeuN+
CD200-
or Crry-
CFSE
Microglia
B
CD200+
CD200-
8
<1
SSC
CD200
C
CD200+
CD200-
CFSE
D
%CD11b+CFSEint
CD200+
CD200-
E
%CD11b+CFSEint
CD200+
CD200-
F
Crry+
Crry-
7
<1
SSC
Crry
G
Crry+
Crry-
CFSE
H
%CD11b+CFSEint
Crry+
Crry-
I
%CD11b+ CFSEint
Crry+
Crry-

Supplement


A
Surface NeuN
Intracellular NeuN
B
%NeuN+
80
60
40
20
0
NC
NeuN+
C
%2F7+
80
60
40
20
0
NC
2F7+