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Role of astroglial cell hemichannels in prenatal LPS-induced neurotoxicity in the offspring

Background: Several epidemiological studies indicate that children from mothers exposed to infections during gestation (e.g., sinusitis, pneumonia and pyelonephritis), have an increased risk to develop neurological disorders including schizophrenia, autism and cerebral palsy (**Boksa, 2010**). Up to now, all these epidemiological data have been supported by a large body of experimental evidence in rodents mostly based in the administration of lipopolysaccharide (LPS) during pregnancy (**Boksa, 2010**). Indeed, offspring from LPS-exposed pregnant rats exhibit brain cell apoptosis, morphological and structural brain damage, neuronal proliferation deficit, memory and learning impairment and increased anxiety-like behaviors (**Golan et al., 2005, Meyer et al., 2006, Rousset et al., 2006**). While is clear that chronic brain inflammation in the offspring contribute to the neurological abnormalities induced by prenatal inflammation, the mechanisms are still unclear (**Gilmore and Jarskog, 1997**).

For a long time, astrocytes were considered to be part of connective tissue or simple support cells in the central nervous system (CNS). Nonetheless, with the emerging concept of the tripartite synapse, astrocytes are now recognized as essential protagonists in brain processing, learning and memory (**Araque et al., 2014**). To accomplish their regulatory functions on synaptic transmission, most astrocytes express a large repertoire of neurotransmitter receptors, allowing them to sense the neuronal activity and respond locally by Ca^{2+}-dependent release of "gliotransmitters", such as glutamate, D-serine and ATP (**Araque et al., 2014**). In addition to their trophic and synaptic role in the CNS, astrocytes are key players in the maintenance of homeostatic balance of pH, neurotransmitters and ions, as well as the control of cell-to-cell Ca^{2+} signaling and communication (**Volterra et al., 2014**). The fact that gliotransmitter release from astrocytes could be governed by pro-inflammatory molecules, including cytokines and prostaglandins, indicates that astrocyte-to-neuron signaling may be sensitive to changes in the production of these mediators occurring in pathological conditions (**Agulhon et al., 2012**). In fact, in the CNS, a local inflammatory reaction characterized by astroglial and microglial activation has been described in different brain pathologies, such as stroke, Alzheimer's disease and meningitis (**Verkhratsky et al., 2014**). In this process, astrocytes undergo molecular, functional and morphological changes that result in the consequent impairment of intercellular communication normally occurring between these cells and neurons (**Rossi and Volterra, 2009**). Among these alterations are found changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) dynamics, cytokine release and production of nitric oxide (NO) (**Agulhon et al., 2012**).

In the CNS, intercellular communication and gliotransmitter release is in part mediated through hemichannels formed by connexins or pannexins (**Montero and Orellana 2014; Orellana and Stehberg 2014**). Hemichannels are plasma membrane channels that under physiological conditions enable diffusional exchange between the intra- and extracellular compartments, allowing cellular release of relevant quantities of autocrine/paracrine signaling molecules (**MacVicar and Thompson 2010; Wang et al., 2013**). Nevertheless, it has been proposed that dysregulation of hemichannel properties could be critical in the beginning and during the maintenance of homeostatic imbalances observed in several diseases (**Orellana et al., 2012c, Peñuela et al., 2014, Bosch and Kielian 2014**). Until now, there is no evidence whether glial activation and glia-to-neuron communication is altered by prenatal inflammation.

Hypothesis and aims: Accordingly, the hypothesis of the current proposal is "***Maternal inflammation increase astroglial hemichannel activity affecting neuronal survival in the offspring***". The general goals of this proposal will be: 1) To investigate whether astrocytes obtained from the offspring of LPS-exposed dams exhibit an increased hemichannel activity as a result of changes in the pro-inflammatory profile and signaling of astrocytes; 2) To investigate whether astrocytes obtained from the offspring of LPS-exposed dams exhibit an increased release of glutamate and/or ATP via hemichannels, which further could promote neurotoxicity.

Plan of work: To generate a maternal inflammatory condition we will perform a single intraperitoneal injection of LPS (10 µg/Kg) in pregnant mice at gestation day 17. Control pregnant mice will be injected with PBS at the same gestation stage. We will use glial cultures and hippocampal brain slices to study how maternal inflammation during pregnancy affects hemichannel activity in glial cells of the offspring. Hemichannel activity will be evaluated through dye uptake experiments, whereas the pro-inflammatory profile of glial cells will be examined by measuring the intracellular Ca^{2+} levels, iNOS expression and release of cytokines and nitric oxide. To examine whether maternal LPS administration make glial cell cultures of the offspring neurotoxic, glutamate and ATP will be measured on glial conditioned media (CM) from neonatal mice of untreated or LPS treated mothers. To determine which hemichannel forming proteins could be involved in the above mentioned phenomena, we will use primary cell cultures and specific hemichannel blockers and siRNAs or cell cultures of null mice for hemichannel forming proteins.

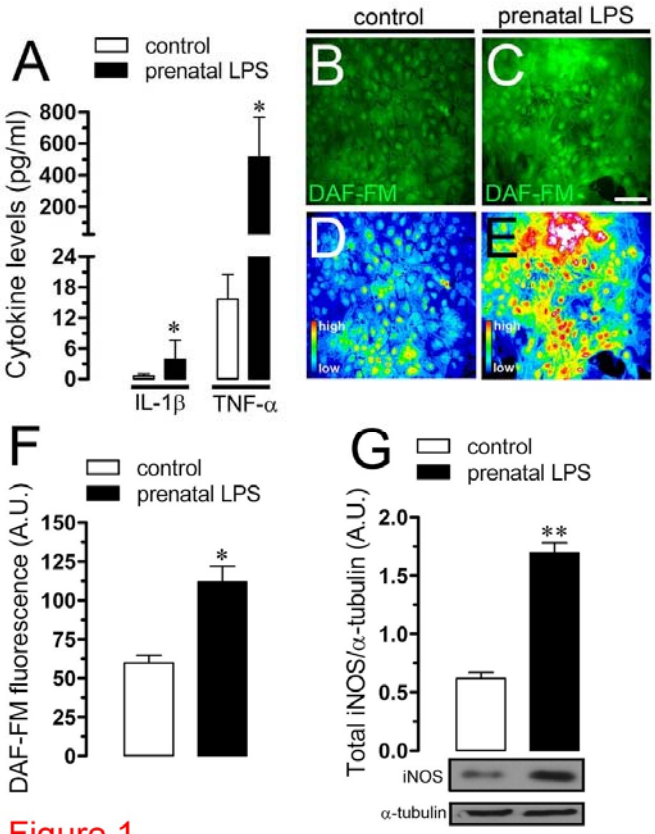

Figure 1

Expected outcomes and significance: The relevance of this proposal is that both addresses mechanisms involved in the brain disorders induced by maternal inflammation and offers new horizons for possible therapies.

Results:

Specific Goal 1.1: To study whether glial cells obtained from offspring of mothers exposed to LPS exhibit an increase in TNF-α/IL-1β release, levels of iNOS, NO production and basal levels of $[Ca^{2+}]_i$.

Systemic administration of LPS in rodents has been used to demonstrate that prenatal inflammation during pregnancy induces apoptosis, structural damage and behavior impairment in the offspring. Although it has been proposed that prenatal inflammation may lead to persistent fetal brain inflammation, is still unclear whether glial cells could participate in this phenomenon. In the CNS, microglia and astrocytes are the instrumental cells of inflammatory response and serve as the main source of cytokines (e.g., TNF-α/IL-1β) for the brain parenchyma. Most of these cytokines control and regulate glial and neuronal functions in response to pathological conditions, including iNOS expression, NO production and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). In this context, we investigated whether astrocytes obtained from the offspring of dams exposed to LPS exhibit an increased release of TNF-α and IL-1β. To address that, mice were injected (i.p) on gestation day 17 with PBS (prenatal control group) or E. Coli LPS (0.01 µg/gr, prenatal inflammatory group). Following full term delivery, offspring were housed with their dams until postnatal day 2. At this stage, astroglial cell cultures were made from the offspring of control or LPS-exposed dams and then were held in culture for two weeks. Afterwards, conditioned media (CM) were collected, filtered and were used to measure TNF-α and IL-1β by ELISA. Astrocytes from offspring of LPS-treated dams (black bars) showed a ~40-fold and ~5-fold increase in the release of TNF-α and IL-1β compared to control conditions (white bars), respectively (Fig. 1A). To investigate whether astrocytes obtained from offspring of dams exposed to LPS exhibit an activation in iNOS pathway, we measured iNOS levels and NO production, by western blot and DAF-FM imaging, respectively. Basal levels of NO determined by DAF-FM imaging were incremented in ~2-fold in astrocytes from offspring of LPS-treated mothers (Fig. 1C, E and F; black bars) compared to control conditions (Fig. 1B, D and F: white bars). Astrocytes coming from prenatal inflammation protocol exhibited a ~3-fold increase in iNOS expression at the protein level compared to control conditions (Fig. 1G).

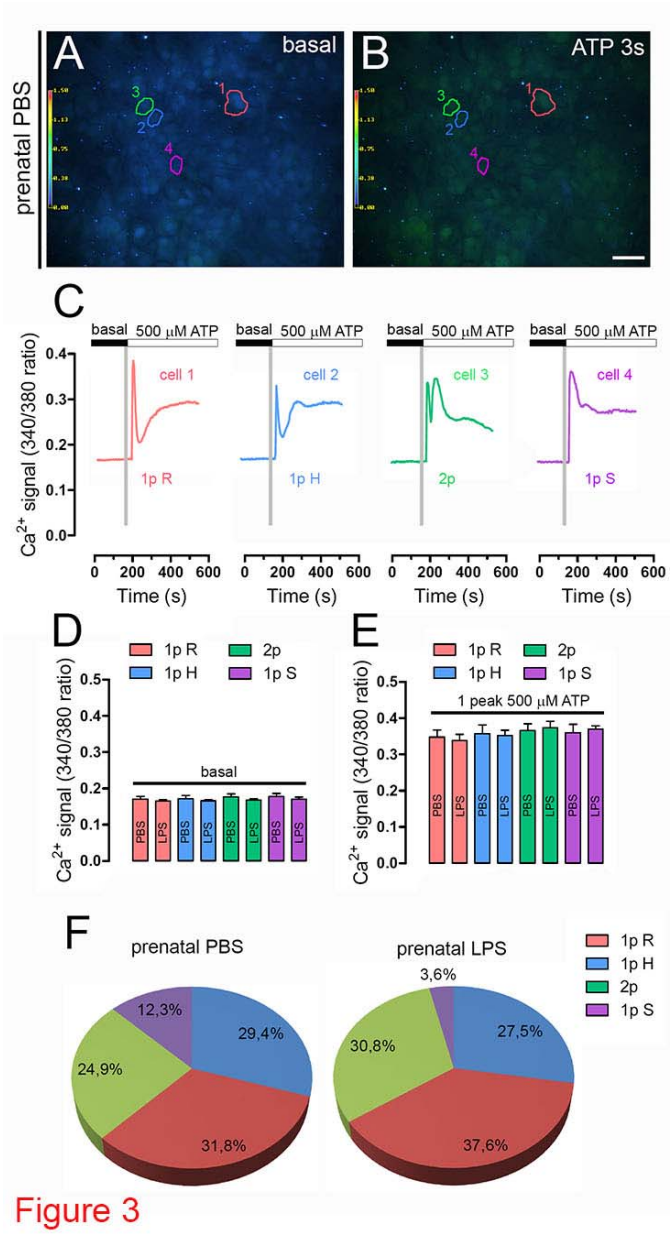

Figure 3

Several studies show that intracellular Ca^{2+} dynamics plays a critical role on the pro-inflammatory profile of glial cells, and serves as a tight sensitive system to mediate release of gliotransmitters, cytokines and growth factors, representing the primary pathway by which glial cells respond to neural activity. Therefore, we examined the effect of prenatal inflammation on basal levels of $[Ca^{2+}]_i$ of astrocytes. As indicated by measurements of Fura-2AM ratio (340/380), astrocytes from offspring of LPS-treated dams (Fig. 2C and E; black bars) showed similar basal levels of Ca^{2+} signal that control astrocytes (Fig. 2A and E; white bars). To study the impact of prenatal inflammation on receptor mediated Ca^{2+} signaling on astrocytes, we elicited transient increases in $[Ca^{2+}]_i$ with ATP, known to activate both ionotropic and metabotropic purinergic receptors, P2X and P2Y, respectively. Interestingly, astrocytes from offspring of LPS-treated or control dams showed similar peaks of Ca^{2+} signal upon stimulation with 500 µM ATP (Fig. 2B, D and E). Up to now, all these data indicate that prenatal inflammation could increase the release and production of TNF-α/IL-1β and NO, but not change the basal levels of $[Ca^{2+}]_i$. Previous studies performed in astrocytes have revealed that ATP (500 µM) produce a biphasic $[Ca^{2+}]_i$ response: the release of stored Ca^{2+} (first spike) and Ca^{2+} influx from the extracellular medium (second shoulder). These studies showed that the first spike in the ATP-induced $[Ca^{2+}]_i$ response depend on P2Y receptors, whereas the second shoulder response occurred via activation of $P2X_7$ receptors. Therefore, we analyzed the pattern of response in the ATP-evoked changes in Ca^{2+} signal in our system. Either control astrocytes (Fig. 3A, B and C) as astrocytes from LPS-treated dams (not shown) showed four different patterns of Ca^{2+} responses upon ATP application (Fig. 3C): 1) one peak followed by an slight rise in $[Ca^{2+}]_i$ (1p R, cell 1 in red); 2) one peak followed by a horn rise in $[Ca^{2+}]_I$ (1p H, cell 2 in blue); 3) two peaks followed by different changes in $[Ca^{2+}]_I$ (2p, cell 3 in green) and 4) one peak followed by an slight decrease and plateau in $[Ca^{2+}]_i$ (1p S, cell 4 in magenta). When basal levels and ATP-evoked changes in $[Ca^{2+}]_i$ when analyzed considering the specific pattern response, no differences were detected between control astrocytes and astrocytes from LPS-treated mothers (Fig. 3D and E). However, when compared with control astrocytes, only a few of astrocytes from LPS-treated mothers showed a 1p S Ca^{2+} pattern (3.6% ± 2), whereas most of them responded more with a 2p or 1p R Ca^{2+} pattern (30.8% ± 4.1 and 37.6% ± 3.9, respectively) (Fig. 3F, Annex 1). These results support the idea that prenatal inflammation could affect the distribution of astrocytes that respond with a specific ATP-evoked Ca^{2+} pattern in the offspring. This idea results interesting given that 1p R and 1p S Ca^{2+} pattern, fit with a P2Y/P2X and P2Y ATP-evoked response, respectively. In other words, prenatal inflammation could affect the distribution and activation of P2 receptors in astrocytes of the offspring. Astrocytes display spontaneous $[Ca^{2+}]_i$ oscillations that were previously shown to correlate with the release of gliotransmitters regulating local neuronal networks. Accordingly, we evaluated whether prenatal inflammation could affect $[Ca^{2+}]_i$ levels in offspring's

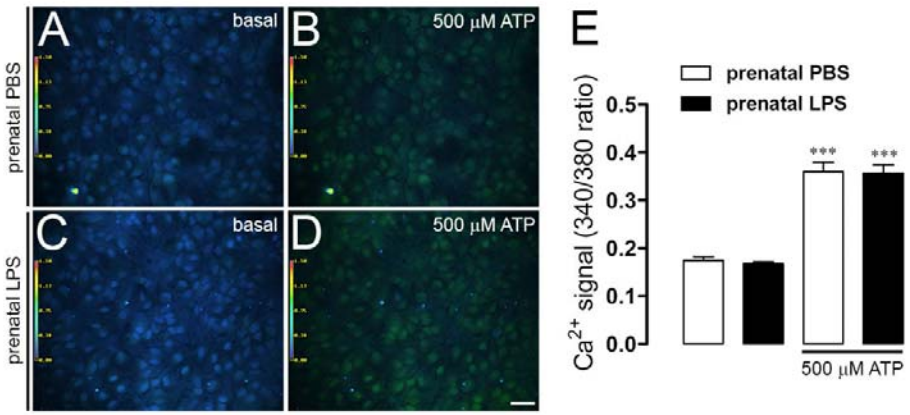

Figure 2

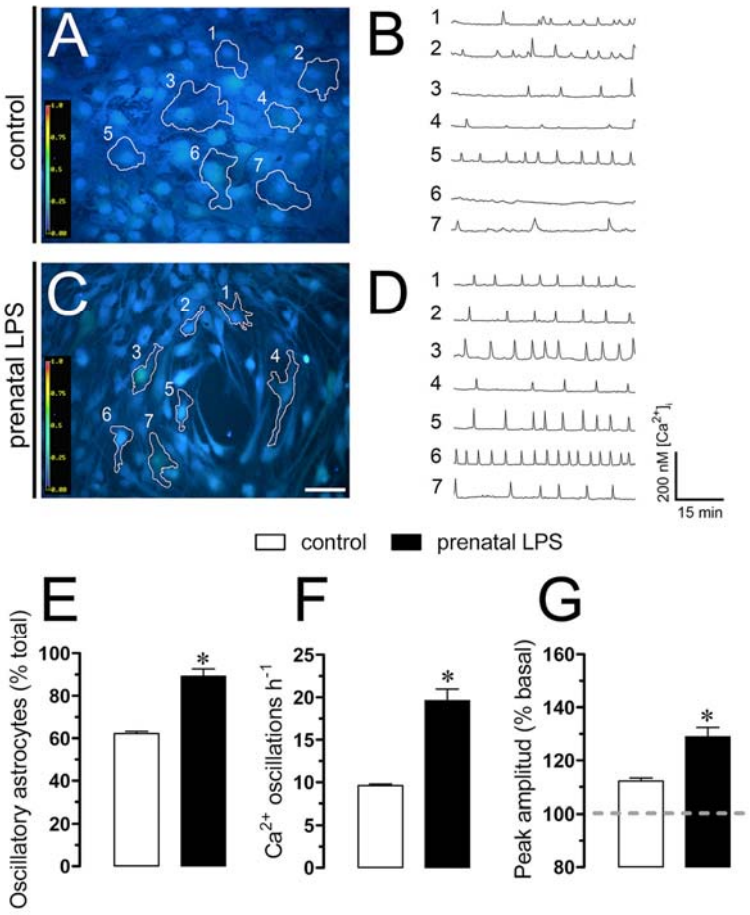

Figure 4

astrocytes. As indicated by the assessment of Fura-2AM ratio (340/380), astrocytes from offspring of LPS-exposed dams showed basal levels of Ca^{2+} signal that were similar to control astrocytes (203.1 ± 9 nM and 221.2 ± 10 nM, respectively, n=4) (Fig. 4A–D). Notably, the percentage of astrocytes that showed oscillatory activity was significantly increased by prenatal LPS exposure (from ~62% to ~89%; P> 0.05), when compared with control conditions (Fig. 4E). Moreover, astrocytes from the offspring of LPS-exposed dams exhibited a ~2-fold increase in spontaneous $[Ca^{2+}]_i$ oscillations (Fig. 4F) and displayed higher Ca^{2+} peaks than control astrocytes (Fig. 4F). Most of these data have been recently published by our laboratory (**Avendaño et al., 2015. Glia. 65(11): 2058–2072**)

Specific Goal 1.2. To study whether glial cells obtained from offsprings of mothers exposed to LPS exhibit an increased hemichannel activity, and to identify the connexins and/or pannexins responsible of this phenomenon using specific hemichannel blockers, connexin or pannexin siRNAs or primary glial cell cultures from connexin or pannexin null mice.

To investigate whether astrocytes obtained from the offspring of LPS-exposed dams exhibit an increase in hemichannel activity, the functional activity of hemichannels was measure by recording the rate of uptake of ethidium (Etd, 5 µM). Etd only crosses the plasma membrane in healthy cells by passing through specific large channels, such as connexin or pannexin hemichannels, and its intracellular fluorescence is reflective of channel opening. When astrocytes from offspring of control dams were exposed to 10 min of Etd, they showed a low Etd uptake (Fig. 5A) as occur with astrocytes under control conditions. However, astrocytes from offspring of LPS-treated dams exhibited an increased Etd uptake upon 10 min of exposure (Fig. 5B). Because astrocytes express an enhanced Cx43 hemichannel activity in pathological conditions, we employed Gap19, a mimetic peptide that specifically blocks Cx43 hemichannels, but not gap junction channels. Interestingly, when astrocytes from the offspring of LPS-treated dams were pre-treated with Gap19 (100 µM) for 15 min and exposed to Etd in co-incubation with Gap19 for 10 min, they showed a low Etd uptake, similar to control conditions (Fig. 5C). Similarly, when Etd uptake is observed in time-lapse recordings (Fig. 5D), astrocytes from offspring of LPS-treated dams exhibit a curve with a high slope (black circles) of Etd uptake compared to control astrocytes (white circles). Importantly, Gap19 fully reduced the effect on Etd uptake induced by prenatal inflammation in astrocytes (gray circles) (Fig. 5D). The Etd uptake rate obtained from the slopes of curves similar to that showed in Fig. 5D, reflect an instantaneous measure of hemichannel activity. We observed that prenatal inflammation induced a ~4-fold increases in the Etd uptake rate compared to control astrocytes, whereas several pharmacological inhibitors of Cx43 hemichannels, including Gap26 (100 µM); Gap19 (100 µM) and TAT-L2 (100 µM) strongly inhibited this response (Fig. 5E). Supporting these results, downregulation of Cx43 with $siRNA^{Cx43}$, but not scrambled siRNA completely inhibited the Etd uptake induced by prenatal inflammation (Fig. 5E). Interestingly, ^{10}panx1 or Probenecid, both blockers of Panx1 hemichannels and downregulation of Panx1 with $siRNA^{Panx1}$ partially inhibit the Etd uptake induced by prenatal inflammation (Fig. 5E). This evidence suggests strongly that Cx43 and Panx1 hemichannels are the main contributors to the above response.

To confirm these findings, we employed another experimental approach to measure hemichannel activity: whole-cell voltage-clamp experiments. Thus, macroscopic membrane current (total current measured in the absence of other active membrane channels) was recorded and the presence of hemichannel unitary events by applying positive voltage (+40 mv) were assessed (Fig. 6). In control astrocytes, a few unitary current events were detected in 14 cells (e.g., Fig. 6A). In contrast, astrocytes from offspring of LPS-treated dams exhibited numerous unitary current events at positive potentials (Fig. 6B, Annex 1). In those astrocytes, unitary current events were recorded, and point-by-point conversion of current to conductance values revealed single channels of ~220 and ~500 pS (see inset in Fig. 6B, Annex 1). Those conductance values are close to the expected unitary conductance of Cx43 (220 ps) and Panx1 (500 ps) hemichannels, respectively. Altogether these data, support strongly the hypothesis that prenatal inflammation increase hemichannel activity in astrocyte of the offspring. Most of these data have been recently published by our laboratory (**Avendaño et al., 2015. Glia. 65(11): 2058–2072**)

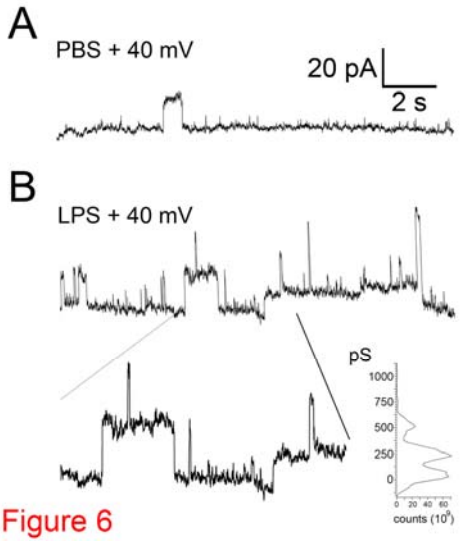

Figure 6

Specific Goal 1.3. <u>To study whether the glial cell hemichannel activity observed in offspring of mothers exposed to LPS is inhibited by neutralization of TNF-α/IL-1β, inhibition of iNOS and/or p38 MAP kinase.</u>

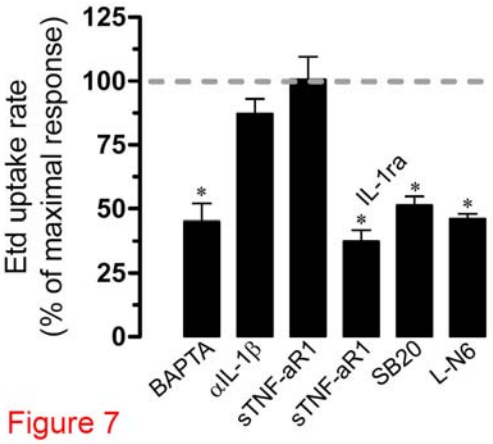

Figure 7

Given that previous studies have demonstrated that increased $[Ca^{2+}]_i$, TNF-α/IL-1β release, iNOS/NO and p38MAPk pathways participate in the opening of glial cell hemi-channels, we examined whether these factors were involved in the prenatal LPS exposure-induced unopposed channel activity in astrocytes. BAPTA strongly reduced the prenatal LPS-induced Etd uptake in astrocytes, whereas pretreatment with a soluble form of TNF-α receptor that binds TNF-α(sTNF-aR1) and a recombinant antagonist for IL-1β receptor (IL-1ra) induced similar effects (Fig. 7). When sTNF-aR1 or IL-1ra were applied by hem selves, no prevention was observed (Fig. 7). Moreover, the prenatal LPS-induced Etd uptake in astrocytes was robustly blunted by inhibition of p38 MAPk with 10 μM SB202190 or iNOS by 5 μM L-N6 (Fig. 7). These findings suggest that increased $[Ca^{2+}]_i$, release of TNF-α/IL-1β, and activation of iNOS/p38 MAPk pathways appear to be critical for the opening of astrocyte unopposed channels evoked on the offspring by prenatal LPS exposure. Most of these data obtained in our laboratory have been recently published (**Avendaño et al., 2015. Glia. 65(11): 2058–2072**).

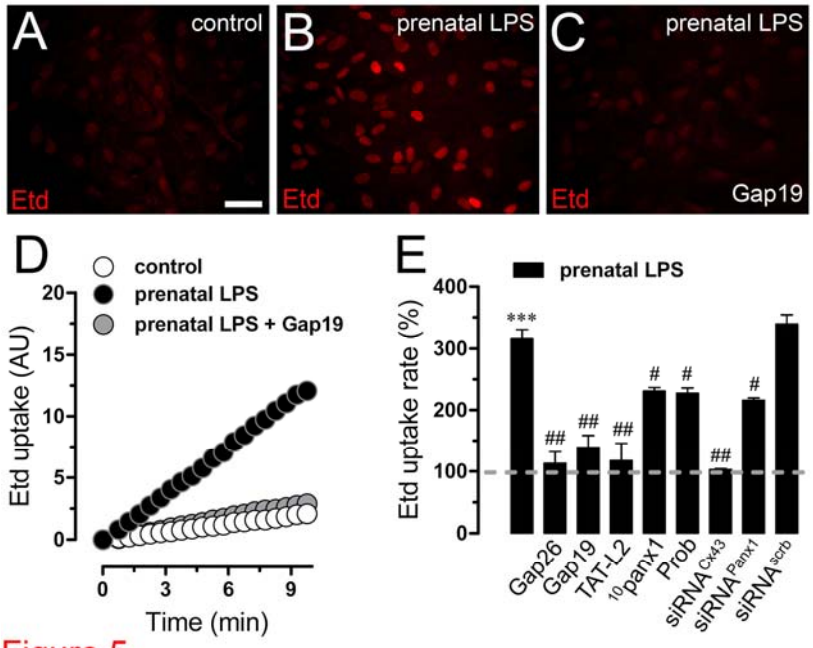

Figure 5

Given that an acute LPS injection during pregnancy affect the astroglial hemichannel activity in the offspring, we evaluated in addition the acute effect of LPS on hemichannel activity in control astrocytes. LPS (1 µg/ml) increased the Etd uptake in a time-dependent manner (Fig. 8A). To examine whether LPS could change the production of NO in glial cells, we measured nitrite levels in astrocytes and microglia upon several times of stimulation with LPS. Control astrocytes did not exhibit a significant variation in nitrite levels (Fig. 8B), whereas after LPS stimulation for 24 h exhibited a ~4-fold increase in nitrites reaching a plateau at 96 h of LPS stimulation that persisted for at least 192 h (Fig. 8B). Interestingly, after LPS stimulation for 24 h, microglia showed a ~2-fold increase in nitrites that increased over time up to ~11-fold after 168 h of stimulation (Fig. 8C). Interestingly, microglia stimulated with LPS for 96 h showed increased level of intracellular Ca^{2+} signal (Fig. 8D) and ATP release (Fig. 8E) compared to control conditions, as measured by FURA-2-AM ratio and luciferin/luciferase assay, respectively. Both responses were inhibited when iNOS was blocked with LN-6, indicating the involvement of NO production in these processes. Similarly, LPS-treated microglia exhibit a high Etd uptake compared to control conditions, which was completely by pharmacological and molecular inhibition of Panx1 (probenecid, ^{10}Panx1 and siRNA^{Panx1}) but not Cx43 (Gap26, Cx43^{E2} and siRNA^{Cx43}) hemichannels (Fig. 8F). This indicate that in vitro treatment with LPS promote activation of Panx1 hemichannels in microglia. Interestingly, LPS-induced Etd uptake in microglia was inhibited when the CM of LPS-treated astrocytes was co-incubated with the LPS in microglia (not shown). Interestingly, these results indicate that astrocytes are able to inhibit Panx1 hemichannels activity in activated microglia. Most of these data were published two years ago by our laboratory (**Orellana et al., 2013. Glia. 61(12):2023-2037**).

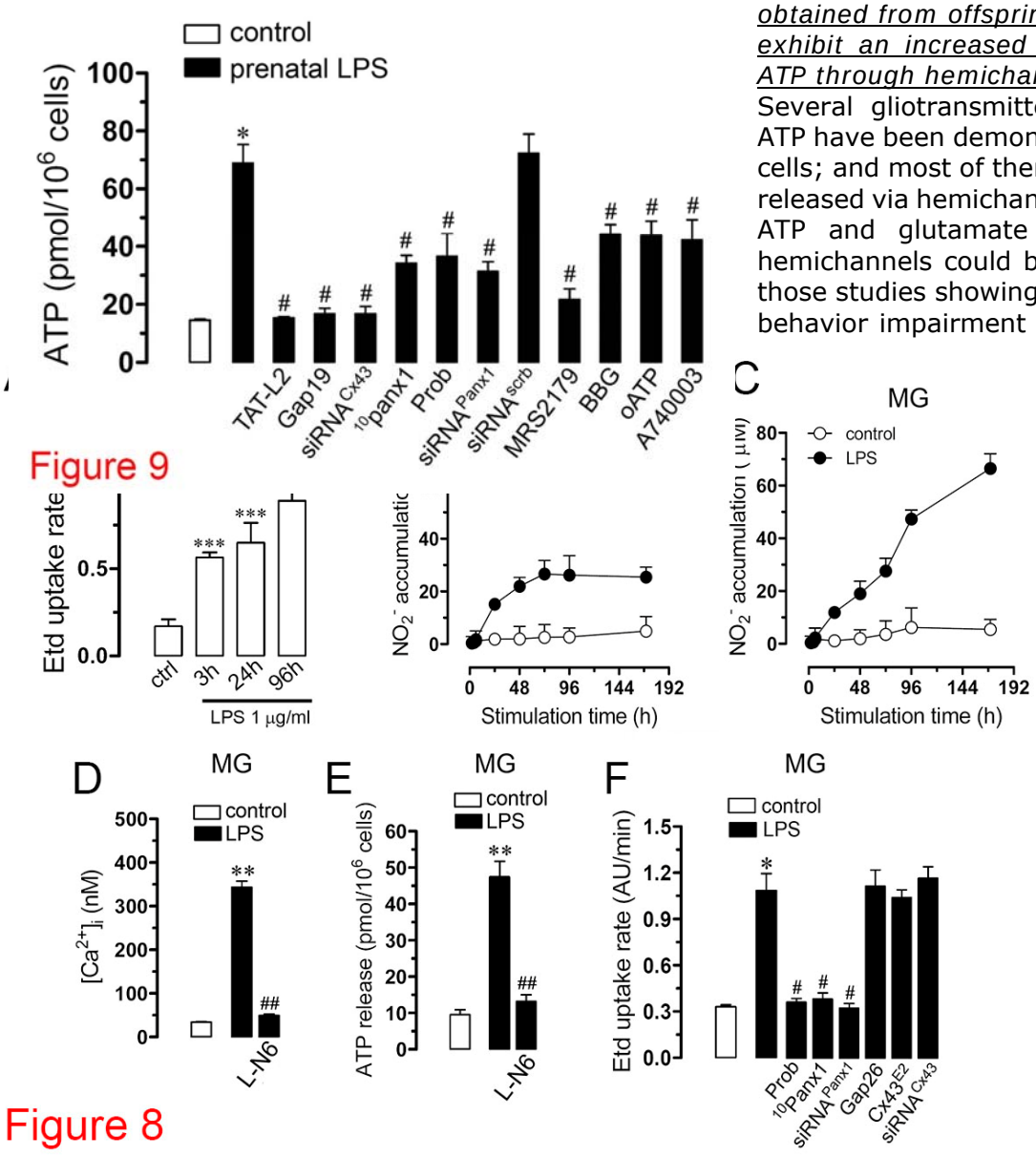

Figure 9

Figure 8

Specific Goal 2.1. *To study whether glial cells obtained from offspring of mothers exposed to LPS exhibit an increased release of glutamate and/or ATP through hemichannels.*

Several gliotransmitters including glutamate and ATP have been demonstrated to be released by glial cells; and most of them have been recognized to be released via hemichannels by astrocytes. Therefore, ATP and glutamate released by astroglial cell hemichannels could be good candidates to explain those studies showing a morphological damage and behavior impairment in offspring of dams exposed to inflammatory conditions during pregnancy. Thus, we investigate whether astrocytes obtained from offspring of LPS-exposed dams exhibit an increased release of glutamate and/or ATP through hemichannels. Astrocytes from offspring of LPS-exposed dams showed a prominent increase in the release of ATP when compared to astrocytes from offspring of control dams (from 14.6 ± 0.6 pmol/mg to 69.0 ± 11 pmol/mg, respectively, n=3) (Fig. 9). Interestingly, blockers of Cx43 (TAT-L2 and Gap19, 100 µM) and Panx1 (^{10}panx1 and probenecid, 100 µM) hemichannels, strongly reduced the release of ATP in astrocytes evoked by prenatal LPS (from 69.0 ± 11 pmol/mg to 15.4 ± 0.6 pmol/mg, 16.7 ± 3.3 pmol/mg, 34.2 ± 4.7 pmol/mg and 36.5± 13.7 pmol/mg, respectively, n=3) (Fig. 9). These findings indicate that prenatal LPS increases the

release of ATP via opening of Cx43 and Panx1 hemichannels. In support to this notion, knockdown of Cx43 or Panx1 using siRNAs nearly abolished the increase in prenatal LPS-induced ATP release in astrocytes (from 69.0 ± 11 pmol/mg to 16.8 ± 4.4 pmol/mg and 31.3 ± 5.9 pmol/mg, respectively, n=3) (Fig. 9). In addition, astrocytes from offspring of LPS-treated dams showed similar levels of glutamate release than control astrocytes (not shown). These findings indicate that contrary to that observed under other pro-inflammatory conditions (e.g., amyloid-β peptide treatments), prenatal LPS increase the release of ATP but not glutamate via hemichannels in astrocytes.

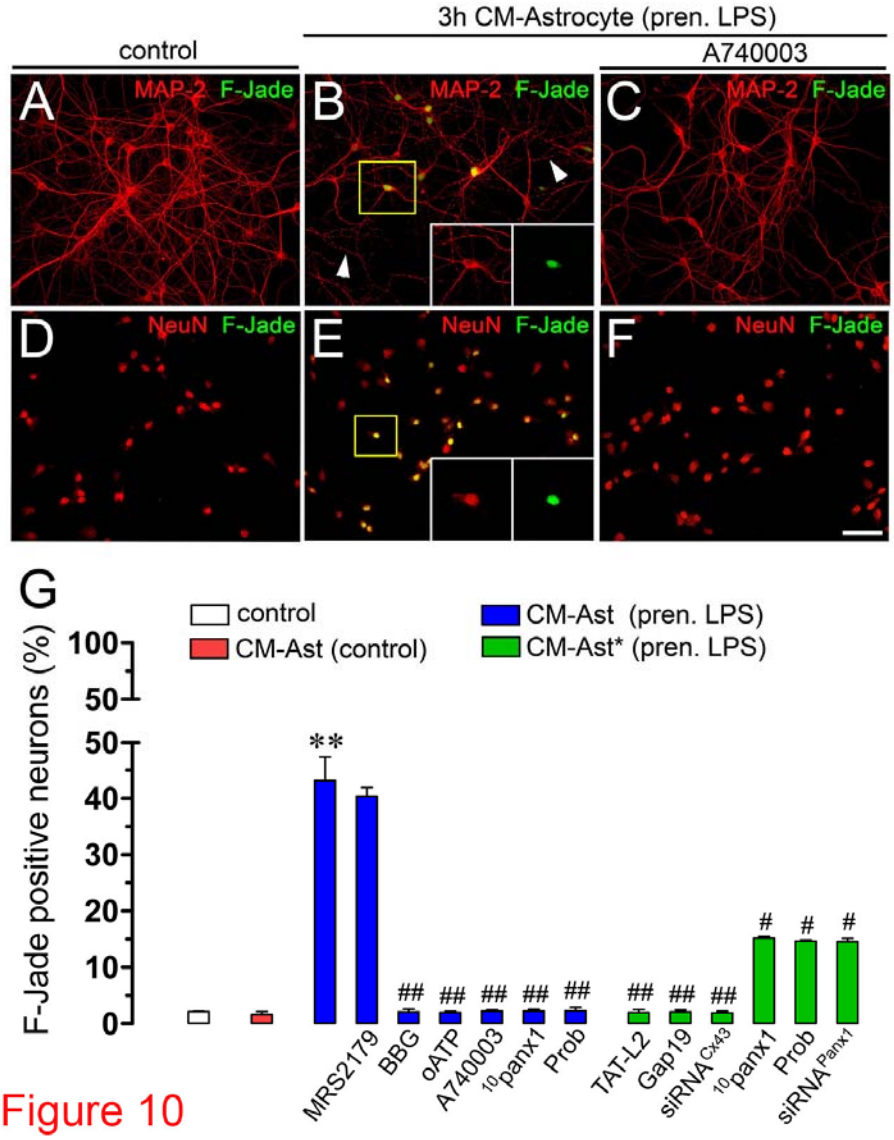

Figure 10

It has been demonstrated that gliotransmitters elicit their own release in an autocrine manner via Cx43 and Panx1 hemichannels. Thus, we next evaluated whether ATP receptors could be involved in the ATP release from astrocytes triggered by prenatal LPS. Blockade of $P2Y_1$ receptors with MRS2179 induced a prominent reduction on prenatal LPS-induced ATP release in astrocytes (from 69.0 ± 11 pmol/mg to 21.7 ± 6.3%, respectively, n=3) (Fig. 9). To elucidate if in addition to $P2Y_1$ receptors, ionotropic $P2X_7$ receptors might also be involved in prenatal LPS-induced ATP release, we used BBG, oATP and A740003, blockers of $P2X_7$ receptors which have been previously linked to hemichannel-dependent ATP release in the CNS. BBG (10 μM), oATP (200 μM) and A740003 (10 μM) partially abolished the release of ATP evoked by prenatal LPS (from 69.0 ± 11 pmol/mg to 44.2 ± 5.9 pmol/mg, 44.8 ± 8.8 pmol/mg and 42.2 ± 12.2 pmol/mg, respectively, n=3). This evidence suggest that ATP, could evokes its own release by an autocrine pathway possibly mediated by Cx43 and Panx1 hemichannels (Fig. 9). Most of these data obtained in our laboratory have been recently published (**Avendaño et al., 2015. Glia. 65(11): 2058–2072**)

Specific Goal 2.2. *To study whether glial cells obtained from offspring of mothers exposed to LPS are neurotoxic and if this can be abolished by blockade of glutamate/ATP released via hemichannels using specific hemichannel blockers, connexin or pannexin siRNAs or primary glial cell cultures from connexin or pannexin null mice.*

Although it is expected that gliotransmitters released via hemichannels possibly modulate presynaptic efficacy and postsynaptic responses at the synapse, several studies indicate that under pro-inflammatory conditions this paracrine pathway could affect neuronal survival. Indeed, activated astrocytes release ATP and glutamate via hemichannels, evoking activation of neuronal $P2X_7$ and NMDA receptors, triggering further neuronal death. Accordingly, we examined whether the increased hemichannel activity observed in astrocytes obtained from offspring of LPS-exposed dams could affect neuronal survival. Therefore, enriched neuronal cultures were incubated for 3h with conditioned medium (CM) of astrocytes obtained from offspring of LPS-exposed or control dams. Under control conditions most 12 day-old MAP-2 and NeuN positive neurons were not labeled with F-Jade, a marker of neurodegeneration and neuronal death (2.1 ± 0.1% of total cells, n=3) (Fig. 10A, D and G). However, after treatment with CM of astrocytes from prenatal LPS protocol, a prominent increase in neuronal death was observed (43.1 ± 7.2% of total cells, n=3) (Fig. 10B, E and G). In contrast, CM from control astrocytes did not alter neuronal survival, supporting the idea that astrocytes obtained from offspring of dams exposed to LPS are neurotoxic. Importantly, neuronal death was associated with focal beadlike swellings in dendrites and axons (neuritic beading), which has been proposed as an early pathological feature of neuronal

cell dysfunction that precedes neuronal death (Fig. 10B, white arrowheads, Annex 1). To elucidate the contribution of astroglial ATP and neuronal purinergic receptors in the CM-induced neuronal death, we employed blockers of $P2Y_1$ (MRS2179) and $P2X_7$ receptors (BBG, oATP, A740003) to inhibit the response. MRS2179 failed on inhibit the CM-induced neuronal death (Fig. 10G, Annex 1), whereas BBG, oATP and A740003 greatly reduced it (from 43.1 ± 7.2% to 2.1 ± 0.9%, 2.0 ± 0.3%, 2.3 ± 0.3% of total cells, respectively, n=3) (Fig. 10C, F and G). All these findings suggest that ATP contained in the CM from astrocytes and its action on neuronal $P2X_7$ receptors decrease neuronal survival. Because activation of neuronal purinergic receptors and further activation of Panx1 hemichannels lead to neuronal death, we inhibited neuronal Panx1 hemichannels with ^{10}panx1 and probenecid to examine their contribution to the CM-induced neuronal death. ^{10}panx1 and probenecid fully reduced the CM-induced neuronal death (from 43.1 ± 7.2% to 2.2 ± 0.6% and 2.3 ± 1.0% of total cells, respectively, n=3) (Fig. 10G), indicating that astrocytes from prenatal LPS protocol release ATP that lead to activation of $P2X_7$ receptors and further opening of neuronal Panx1 hemichannels. Since activation of $P2X_7$ receptors raises $[Ca^{2+}]_i$, and increased levels of $[Ca^{2+}]_i$ trigger opening of Panx1 hemichannels, it is plausible to suggest that CM-induced neuronal death could be related to ionic, osmotic and Ca^{2+} imbalance evoked by $P2X_7$ receptor and Panx1 hemichannel activation.

Notably, when CM from astrocytes was made in the presence of TAT-L2 or Gap19 neuronal death was fully abolished compared to the effect induced by CM from astrocytes made in the absence of these blockers (from 43.1 ± 7.2% to 2.0 ± 0.9% and 2.1 ± 0.6% of total cells, respectively, n=3) (Fig. 10G Annex 1). Similar results were also observed in neurons incubated with CM from siRNA^{Cx43}-treated astrocytes (from 43.1 ± 7.2% to 1.9 ± 0.6% of total cells, respectively, n=3) (Fig. 10G), whereas a partial reduction on cell death was observed in neurons incubated with CM from astrocytes treated with ^{10}panx1, probenecid or siRNA^{Panx1} (from 43.1 ± 7.2% to 15.2 ± 0.5%, 14.7 ± 0.3% and 14.5 ± 1.0% of total cells, respectively, n=3) (Fig. 10G). Altogether this evidence suggest that neuronal death was due to release of ATP by astrocytes obtained from offspring of LPS-exposed dams and that astroglial cell Cx43 and/or Panx1 hemichannels might be key pathways involved in this process. Most of these data obtained in our laboratory have been recently published (**Avendaño et al., 2015. Glia. 65(11): 2058–2072**)

Conclusions:

Despite that previous studies have demonstrated that prenatal inflammation increases astrogliosis and GFAP expression in the offspring (**Hao et al. 2010; Samuelsson et al. 2006**), whether astrocytes contribute to brain dysfunction induced by the latter condition has remained unknown (**Gilmore and Jarskog 1997**). Our model, consisting in a single LPS injection during pregnancy, increased the production of inflammatory mediators (cytokines and NO) and altered intracellular Ca^{2+} dynamics in offspring astrocytes. Due to these functional changes, astrocytes exhibited an enhanced p38MAPK/iNOS-dependent release of ATP via astroglial cell Cx43 and Panx1 unopposed channels, which in consequence caused the impairment of neuronal survival.

Acknowledgements:

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Scientific Production derived from the Grant:

This grant did not only support the current proposal but also other research focused in astroglial and hemichannel function. Most of these data were published and properly acknowledged in the following publications:

- **Orellana JA**. Physiological functions of glial cell hemichannels. Adv Exp Med Biol. 2016. In press
- Del Rio R, Quintanilla RA, **Orellana JA**, Retamal MA. Neuron-Glia Crosstalk in the Autonomic Nervous System and Its Possible Role in the Progression of Metabolic Syndrome: A New Hypothesis. Front Physiol. 2015 Dec 1;6:350. doi: 10.3389/fphys.2015.00350
- Avendaño BC, Montero TD, Chávez CE, von Bernhardi R, **Orellana JA**. Prenatal exposure to inflammatory conditions increases Cx43 and Panx1 unopposed channel opening and activation of astrocytes in the offspring effect on neuronal survival. Glia. 2015 Jun 19. doi: 10.1002/glia.22877.
- **Orellana JA**, Moraga-Amaro R, Díaz-Galarce R, Rojas S, Maturana CJ, Stehberg J, Sáez JC. Restraint stress increases hemichannel activity in hippocampal glial cells and neurons. Front Cell Neurosci. 2015 Apr 2;9:102. doi: 10.3389/fncel.2015.00102

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