PURINERGIC REGULATION OF NEUROGENESIS FOLLOWING SPINAL CORD INJURY IN *DANIO RERIO*

PURINERGIC REGULATION OF NEUROGENESIS FOLLOWING SPINAL CORD INJURY IN *DANIO RERIO*

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Lay Abstract

Spinal cord injury in mammals causes widespread neuronal cell death and paralysis. In comparison, zebrafish regenerate damaged neurons and restore motor function. Radial glial cells within the zebrafish spinal cord maintain stem-cell properties. Following injury, these cells divide and replace motor neurons. Since mammals have similar cell-types within the spinal cord, understanding the molecular cues driving this adaptive response is of great interest. Here, we examined the evolutionarily conserved purinergic signaling system and found that the expression of the $P2X_7$ receptor varies significantly from mammals and promotes radial glia division following injury.

Abstract

In contrast to mammals, adult zebrafish undergo successful neural regeneration following spinal cord injury (SCI). Radial glia (RG) lining the zebrafish central canal undergo injury-induced proliferation and subsequent neuronal differentiation to replace damaged cells and restore motor function. However, the molecular mechanisms that underlie these processes remain elusive. Here, we demonstrate that signaling through the evolutionarily conserved purinergic $P2X_7$ receptor is involved. Within the zebrafish spinal cord, $P2X_7$ receptors have widespread distribution with specific localization to neurons and radial glia. At the protein level, the predominant $P2X_7$ receptor isoforms in zebrafish did not include the full-length variant expressed throughout the murine central nervous system, but two truncated splice variants. In response to SCI, protein expression of the 50 kDa isoform became downregulated at 7 dpi and returned to basal levels of expression at 14 and 21 dpi when compared to naïve controls. Meanwhile, expression of the 37 kDa isoform did not change following injury. Pharmacological activation of $P2X_7$ following SCI resulted in a greater number of proliferating cells around the central canal by 7 dpi, while $P2X_7$ inhibition appeared to have no effect. At 14 dpi, these treatments did not have a significant effect on the number of neurons within the injured spinal cord. This data indicates that $P2X_7$ receptor activation is sufficient to induce cellular proliferation, but not a necessary mediator of either proliferation or neurogenesis following SCI in adult zebrafish. Our findings suggest that unlike in humans, $P2X_7$ signaling may not play a maladaptive role following SCI in adult zebrafish.

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List of Abbreviations and Symbols

SCI	Spinal Cord Injury
CNS	Central Nervous System
RG	Radial Glia
DPI	Day(s) Post Injury
ATP	Adenosine 5′-Triphosphate
ADP	Adenosine Diphosphate
UTP	Uridine Triphosphate
UDP	Uridine Diphosphate
CD39	Cluster of Differentiation 39
CREB	cAMP response element-binding protein
ERK1/2	Extracellular signal-regulated kinases 1/2
IL	Interleukin
NGF2	Nerve Growth Factor-2
NLRP1/3	NLR family pyrin domain containing 1/3
NT-3	Neurotrophin-3
STAT3	Signal Transducer and Activator of Transcription 3
TNF-α	Tumor Necrosis Factor Alpha
Akt	Protein Kinase B
Wnt	Wingless-Related Intergration Site
BzATP	2'(3')-O-(4-Benzoylbenzoyl) adenosine-5'-triphosphate tri(triethylammonium)
GFAP	Glial Fibrillary Acidic Protein
PCNA	Proliferating Cell Nuclear Antigen
NeuN	Neuronal Nuclei
Ca^{2+}	Calcium
AAD	Acute Axonal Degeneration
KD	Knockdown
KO	Knockout

Declaration of Academic Achievement

I hereby declare that this thesis is based on my original work except for citations which have been duly acknowledged. Research contributors include Dr. Angela Scott, Jasleen Jagayat, Julian Dychiao, and Matin Borhani.

Chapter 1

Preface to Chapter 1

Chapter 1 is a comprehensive peer-reviewed article that was recently published in Neural Regeneration Research.

Article Title: Purinergic signaling systems across comparative models of spinal cord injury

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1.1 Abstract

Within the last several decades, the scientific community has made substantial progress in elucidating the complex pathophysiology underlying spinal cord injury. However, despite the many advances using conventional mammalian models, both cellular and axonal regeneration following spinal cord injury have remained out of reach. In this sense, turning to non-mammalian, regenerative species presents a unique opportunity to identify pro-regenerative cues and characterize a spinal cord microenvironment permissive to re-growth. Among the signaling pathways hypothesized to be dysregulated during spinal cord injury is the purinergic signaling system. In addition to its well-known role as energy currency in cells, ATP and its metabolites are small molecule neurotransmitters that mediate many diverse cellular processes within the central nervous system. While our understanding of the roles of the purinergic system following spinal cord injury is limited, this signaling pathway has been implicated in all injury-induced secondary processes, including cellular death, inflammation, reactive gliosis, and neural regeneration. Given that the purinergic system is also evolutionarily conserved between mammalian and non-mammalian species, comparisons of these roles may provide important insights into conditions responsible for recovery success. Here, we compare the secondary processes between key model species and the influence of purinergic signaling in each context. As our understanding of this signaling system and pro-regenerative conditions continues to evolve, so does the potential for the development of novel therapeutic interventions for spinal cord injury.

1.2 Introduction

Following spinal cord injury (SCI), functional recovery is extremely limited in adult mammals. This lack of recovery is reflective of the collective failure of lesioned axons to re-grow and damaged neurons to regenerate in the spinal cord following the primary trauma. The result is a debilitating reduction in sensorimotor function and quality of life in approximately 770,000 people of all ages, genders, ethnicities, and socioeconomic backgrounds each year (Kumar et al., 2018). In contrast to the devastating consequence of spinal cord injury in mammals, several non-mammalian species within the vertebrate subphylum have a much higher regenerative capacity within the central nervous system (CNS) and can undergo functional recovery in adulthood. Although the underlying source of the regenerative differences between regenerating and non-regenerating species is not fully understood, landmark work studying these comparisons has begun to shed light on the defining features and characteristics of successful regeneration and functional recovery.

While all vertebrates have a certain degree of regenerative capacity in the CNS during development, by adulthood, this plasticity is highly restricted in birds and mammals. Limitations to regeneration are largely attributed to a secondary cellular insult involving acute and chronic processes of neuroinflammation, cellular death, reactive gliosis, and axonal degeneration within the spinal cord following injury. The species who maintain some regenerative ability beyond development appear to be members of the more primitive classes of vertebrates including Agnatha (jawless fish), Reptilia, Amphibia, and Osteichthyes (teleost fish), suggesting that regeneration is an ancestral trait that is diminished over evolution. Indeed, zebrafish (*Danio rerio*) (Goldshmit et al., 2012; Hui et al., 2010; Reimer et al., 2008), sea lamprey (*Petromyzon marinus*) (Herman et al., 2018), axolotl (*Ambystoma mexicanum*) (Sabin et al., 2019), knifefish

(*Apteronotus leptorhynchus*) (Vitalo et al., 2016), goldfish (*Carassius auratus*) (Takeda et al., 2015; Takeda et al., 2017), and newts (*Notophthalmus viridescens*) (Zukor et al., 2011) are all non-mammalian vertebrate species with a preserved high degree of CNS regeneration capacity. One particularly distinct difference between these select species and mammals is the ability to undergo injury-induced neurogenesis following SCI (Goldshmit et al., 2012; Hui et al., 2010; Joven & Simon, 2018; Reimer et al., 2008; Sirbulescu et al., 2009; Takeda et al., 2008). In adult zebrafish for instance, newly generated motoneurons are integrated into the existing circuitry to restore locomotion below the level of the lesion within 6 weeks post SCI (Reimer et al., 2008). A further understanding of the neuroprotective and regenerative mechanisms in these species will help to uncover the molecular cues associated with limiting secondary injury and promoting regeneration and recovery.

One evolutionarily conserved family of signaling factors involved in various aspects of secondary responses to injury in both regenerative and non-regenerative species is the purinergic signaling system. Purinergic signaling has been associated with regulating cellular proliferation, migration, differentiation, and survival during development and in adulthood (Burnstock & Ulrich, 2011; Gomez-Villafuertes, 2016; Oliveira et al., 2016; Ribeiro et al., 2016). Purines and pyrimidines, such as adenosine triphosphate (ATP) and uridine triphosphate (UTP) respectively, along with their metabolites, function as extracellular signaling molecules that mediate cellular communication via purinergic receptors (Burnstock, 2018).

These receptors have extensive protein families, including metabotropic adenosine A_1, A_{2A}, A_{2B}, A_3, ionotropic nucleotide $P2X_{1–7}$, and metabotropic nucleotide $P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$ receptors (Burnstock, 2018). Following mammalian SCI, Wang et al. (2004) found that extracellular ATP is elevated within the lesion site for several hours, thus suggesting a role for

injury-induced purinergic signaling. Since then, it has been proposed that ATP may function as an early distress signal within the tissue that is involved in the chemoattraction of various cell types and induction of cell reactivity and proliferation (Dou et al., 2012; Haynes et al., 2006; Kobayakawa et al., 2019; Quintas et al., 2018; Shinozaki et al., 2017; Suadicani et al., 2006). While accumulating evidence supports a role for purinergic signaling in the CNS injury response of mammals, how this complex system may be adapted in regeneratively competent, non-mammalian species is less clear. Here, we review on the role of purinergic signaling during each phase of the secondary injury [(1) cellular death, (2) neuroinflammation, (3) reactive gliosis, and (4) axonal degeneration] and examine the variations of this signaling system in regenerative and non-regenerative species.

1.3 Cell Death

Following an initial mechanical trauma to the spinal cord, secondary injury is associated with a wave of cell death that exacerbates damage and expands the lesion size (Figure 1). In rodent models of SCI, the predominant mechanism of neuronal cell death is necrosis. This begins almost immediately, peaks at 1–2 days, and continues for months (Hassannejad et al., 2018; Kwiecien et al., 2020). Glial cells similarly reach peak levels of necrotic cell death between 1–2 days following SCI (Kwiecien et al., 2020). Meanwhile, apoptosis of neurons peaks first around 4–8 hours and then again at 3 days (Hassannejad et al., 2018). In oligodendrocytes, it peaks within hours and continues for weeks (Crowe et al., 1997), and in microglia, it peaks at 1 day (Bellver-Landete et al., 2019). Additional mechanisms of secondary cell death include intracellular calcium dysregulation, excitotoxicity, and mitochondrial dysfunction/oxidative stress (Ohishi et al., 2016; Reigada et al., 2017).

In contrast, regeneratively competent species predominantly display apoptotic, as opposed to necrotic, cell death following a CNS injury (Figure 2) (Sirbulescu et al., 2009). Notably, necrosis stimulates a pro-inflammatory immune response that leads to further damage, whereas apoptosis is typically anti-inflammatory (Kono & Rock, 2008). The presence of secondary cell death in regenerative species suggests that some degree of cell death following injury may be tolerated, and perhaps essential, for successful CNS regeneration. Indeed, inhibition of apoptosis following caudal fin amputation in adult zebrafish reduced blastema formation and sensory neuron regrowth (Rampon et al., 2014). Similarly, inhibition of caspase-mediated cell death within the first 24 hours post injury was found to prevent tail regeneration following amputation in the African clawed frog tadpole (*Xenopus laevis*) (Tseng et al., 2007). However, the duration of cell death differs significantly between regenerative and non-regenerative species. In teleosts, apoptosis peaks between 15–24 hours post SCI and then declines sharply after 3–5 days (Hui et al., 2010; Hui et al., 2014; Sirbulescu et al., 2009). The sustained expression of apoptotic markers observed 3 weeks post SCI in rodents (Crowe et al., 1997) and 8 weeks in humans (Emery et al., 1998) suggests that regenerative failure in mammals may not be caused by the presence of secondary cell death, but by its necrotic and prolonged nature.

Activation of purinergic signaling is kicked off very early and known to promote cell death in the damaged mammalian spinal cord (Figure 3). Apoptosis stimulates damaged cells to release nucleotides into the extracellular space, further stimulating additional ATP release from nearby cells (Chekeni et al., 2010; Elliott et al., 2009). In addition, excess glutamate within the lesion site induces increased Ca^{2+} influx in adjacent neurons and glia that also stimulates ATP release (Sieger et al., 2012). As a result, an elevated concentration of extracellular ATP in and around the lesion is sustained for several hours following injury (Wang et al., 2004). Local activation of

both adenosine and P2X receptors have been implicated in dysregulation of intracellular calcium homeostasis and cell death induction. In particular, activation of both A_{2A} and $P2X_7$ enables rapid cytosolic Ca^{2+} overload and subsequent cell death soon after mouse SCI (Leeson et al., 2018; Paterniti et al., 2011; Wang et al., 2004). Moreover, $P2X_4$ receptor activation potentiates $P2X_7$-mediated cell death (Kawano, Tsukimoto, Noguchi, et al., 2012). Purinergic activation of microglia and macrophages within the lesion site also facilitates $P2X_7$-mediated cytotoxic cell death of oligodendrocytes (Lopez-Serrano et al., 2019) and apoptosis of many other potential cell types expressing $P2X_7$, including spinal neurons, ependymal cells, astrocytes, and inflammatory cells (Bidula et al., 2019; Gandelman et al., 2013; He et al., 2017; Marichal et al., 2016; Wang et al., 2012). Interestingly, $P2X_7$ is conserved and expressed ubiquitously within most tissues of zebrafish and other bony fish species (Ogryzko et al., 2014; Rump et al., 2020). However, the exact role of $P2X_7$ in mediating injury-induced cell death within the spinal cord of these species has not been explored.

While purines may exacerbate the initial injury by inducing cell death, there is evidence that they may also induce cell proliferation and neuroprotection. Following caudal fin amputation in zebrafish, activation of A_{2B} receptors via adenosine enhanced progenitor cell recruitment, which consequently increased cell proliferation within the lesion beyond spontaneous levels (Rampon et al., 2014). In mammals, microglia $P2Y_1$ receptor stimulation enhanced neuronal survival following transient forebrain ischemic injury (Fukumoto et al., 2019), whereas astrocyte and neuronal $P2Y_2$ receptor stimulation was associated with the inhibition of apoptosis through activation of ERK1/2 and Akt signaling pathways and upregulation of neurotrophins, growth factors, and anti-apoptotic genes (Arthur et al., 2006; Chorna et al., 2004). Furthermore, pre-treatment of neuroblastoma cells with diadenosine tetraphosphate prior to strong ATP

stimulation decreased excitotoxicity (Reigada et al., 2017). This reduced not only $P2X_7$, but also $P2X_2$, $P2Y_1$, and $P2Y_2$ expression, probably through activation of $P2Y_2$ receptors (Reigada et al., 2017). In the rodent spinal cord, $P2Y_2$ is widely expressed by neurons, glia, and immune cells and is upregulated from 4–28 days following injury (Rodriguez-Zayas et al., 2010). Interestingly, stimulation of astrocyte $P2X_7$ receptors induces phosphorylation of ERK1/2 that subsequently increases $P2Y_2$ mRNA expression (D'Alimonte et al., 2007). Whether $P2Y_1$ and $P2Y_2$ play similar roles in regeneratively competent vertebrates following SCI remains unknown.

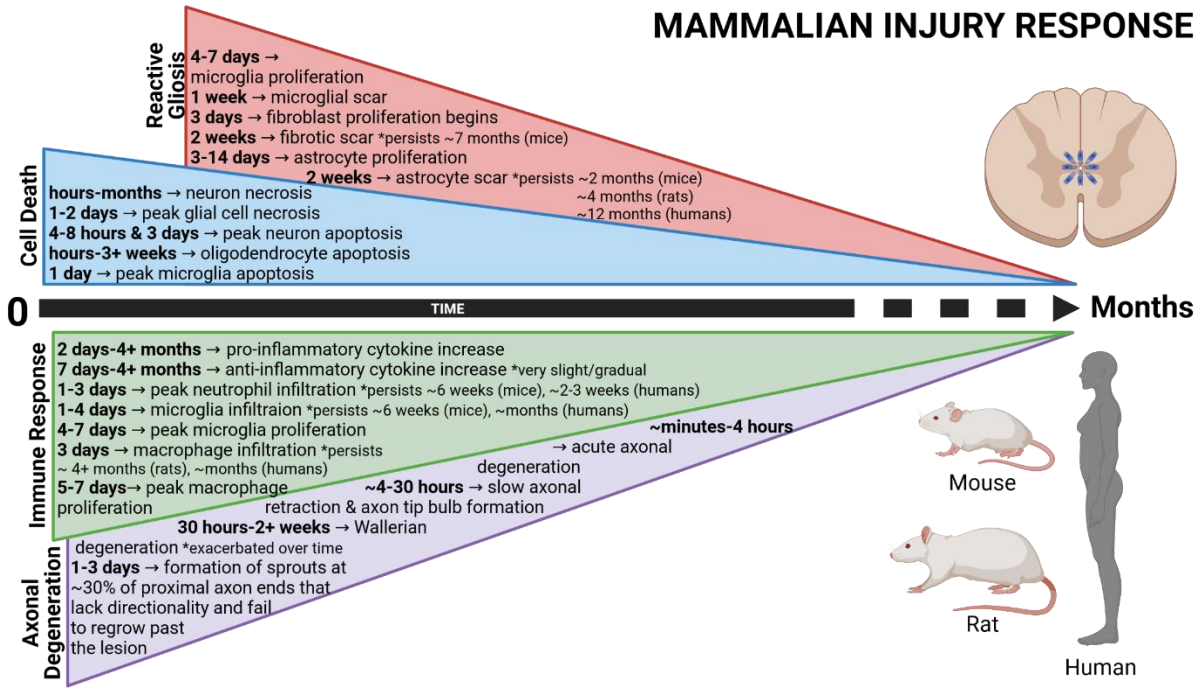

Figure 1. Timeline depicting the secondary cellular injury response following mammalian spinal cord injury. The primary mechanical trauma is exacerbated by prolonged cell death, widespread inflammation, reactive gliosis, and axonal degeneration. These events prevent successful regeneration and limit sensorimotor recovery. Created with BioRender.com with permissions and publication license.

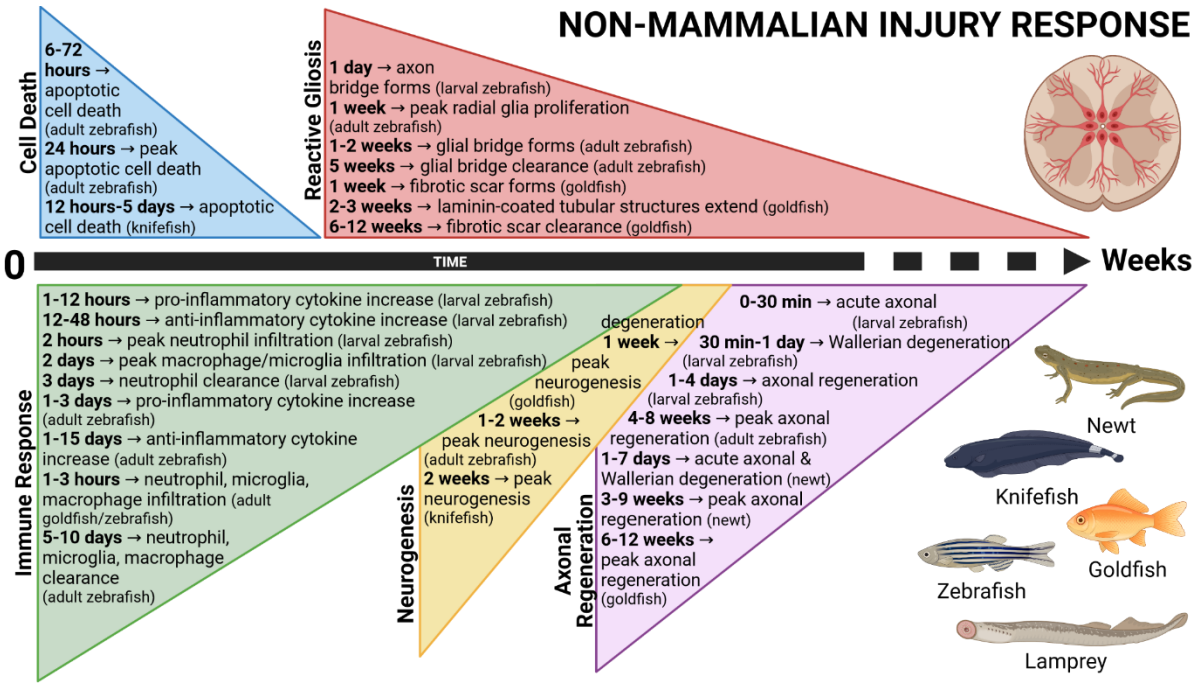

Figure 2. Timeline depicting the secondary cellular injury response following non-mammalian spinal cord injury. The primary mechanical trauma induces transient cell death, controlled inflammation, reactive gliosis, neurogenesis, and axonal regeneration. These events conclude within weeks and facilitate recovery and restoration of locomotor function in non-mammalian vertebrates. Created with BioRender.com with permissions and publication license.

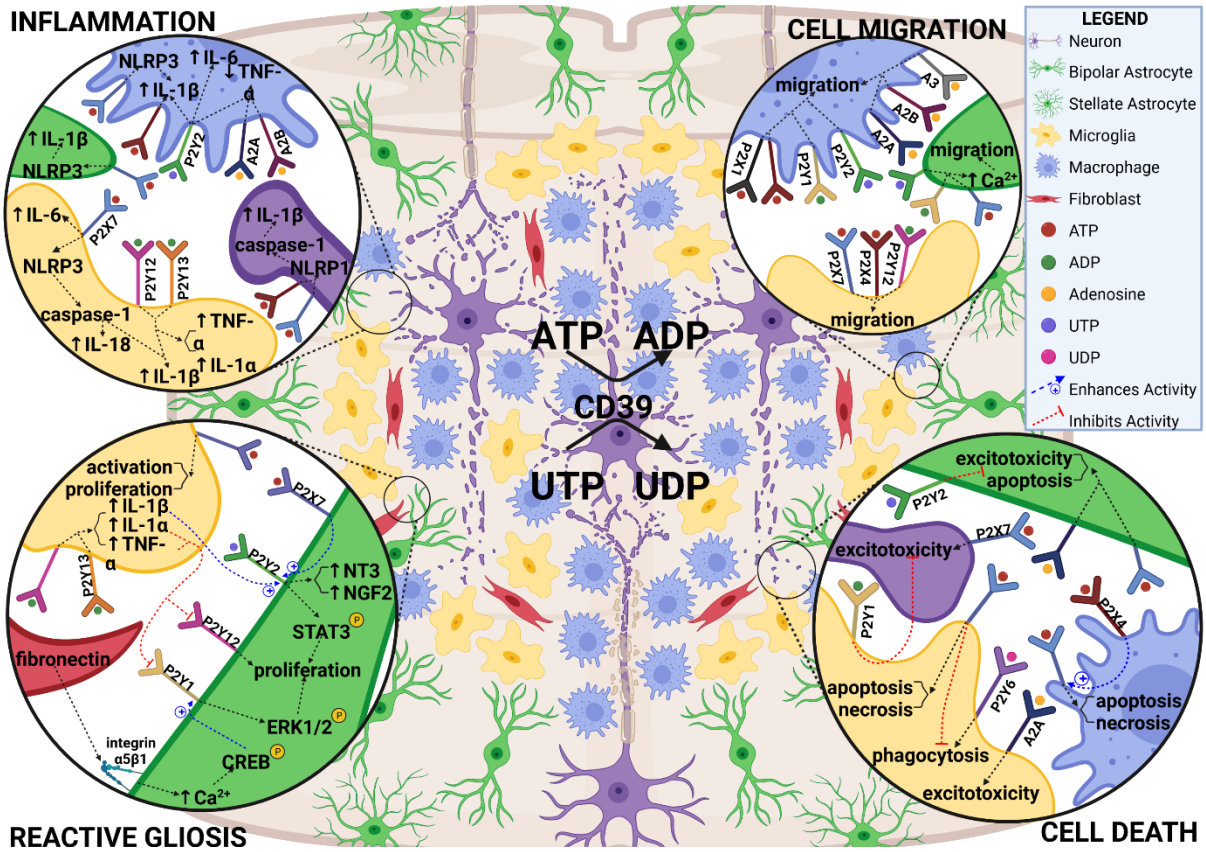

Figure 3. Purinergic signaling within the spinal cord microenvironment during the early injury response. The first several days following mammalian SCI are characterized by widespread cell death, migration of various cell types to the lesion, inflammation, and reactive gliosis. Identified roles for purinergic receptors in these processes is summarized. Created with BioRender.com with permissions and publication license. ADP: adenosine diphosphate; ATP: adenosine triphosphate; CD39: cluster of differentiation 39; Ca2+: calcium; CREB: cAMP response element-binding protein; ERK1/2: extracellular signal-regulated kinases 1/2; IL: interleukin; NGF2: nerve growth factor-2; NLRP1/3: NLR family pyrin domain containing 1/3; NT3: neurotrophin-3; STAT3: signal transducer and activator of transcription 3; TNF-α: tumor necrosis factor alpha; UDP: uridine diphosphate; UTP: uridine triphosphate. Created with BioRender.com with permissions and publication license.

1.4 Immune Response

Shortly after the primary trauma, the microenvironment of the injured mammalian spinal cord is largely occupied by resident and infiltrating inflammatory phagocytic cells (Figure 1). Microglia, CNS-derived macrophages, undergo cell death within the first 24 hours post injury like other cell types; however, residual microglia also undergo activation and proliferation to subsequently repopulate the spinal cord region (Bellver-Landete et al., 2019). Indeed, rapidly proliferating microglia are the predominant phagocytes of cellular and myelin debris within the first few days after injury (Bellver-Landete et al., 2019). By 7 days, microglia proliferation peaks, and by 14 days, the number of microglia within and around the lesion core reaches maximal levels corresponding to the transformation of the area into a 'lesion cavity' (Bellver-Landete et al., 2019; Kwiecien et al., 2020). While few peripheral macrophages and fibroblasts occupy the lesion site at 72 hours post injury, these cells rapidly infiltrate over the course of the following week (Zhu et al., 2015) and soon outnumber microglia (Greenhalgh & David, 2014). The occurrence of phagocytic activity within the spinal cord appears important for recovery, as depletion of spinal cord microglia results in delayed myeloid cell recruitment, increased lesion size, enhanced neuronal and oligodendrocyte cell death, and exacerbated locomotor dysfunction (Bellver-Landete et al., 2019). However, harmful inflammatory cells have been found to persist and phagocytose myelin beyond 4 months post SCI in rats (Kwiecien et al., 2020) and up to one year in humans (Fleming et al., 2006). Thus, the beneficial *versus* detrimental effects of inflammatory cells within the spinal cord is largely dependent on their spatial and temporal presence following injury.

In comparison to macrophage recruitment in mammals, regeneratively competent teleosts appear to have accelerated recruitment of resident and peripheral immune cells to the injury site

(Figure 2). In goldfish, SCI induces reactive microglia, macrophage, and neutrophil migration to the lesion site within 1 hour post injury, and migration to damaged neurons by 3 hours (Koganti et al., 2020). Similarly, neutrophils, followed by microglia and macrophages, infiltrate the larval zebrafish spinal cord within 2 hours post injury (Tsarouchas et al., 2018). In addition to the advanced infiltration of inflammatory cells, clearance of these cells also occurs at a higher rate in teleost fish. Accumulating macrophages, neutrophils, and monocytes within the spinal lesion of zebrafish are cleared by 5–10 days post injury (Hui et al., 2010), a much faster process than the prolonged rate in mammals. Indeed, the enhanced immune cell recruitment to, and clearance from, the injury site following a CNS trauma may be one of the key adaptations by this species that distinguishes their regenerative capacity from others.

One of the primary signals that draws the migration of immune cells to the lesion site is ATP (Elliott et al., 2009; Kronlage et al., 2010). An ATP gradient, enhanced by ATP-induced-ATP release from local astrocytes (Suadicani et al., 2006), microglia (Dou et al., 2012), and macrophages (Kobayakawa et al., 2019) provides necessary directional cues for their movement towards the injury site within minutes of a CNS trauma (Davalos et al., 2005). In particular, microglial migration in mammals is largely mediated via activation of $P2Y_{12}$ and $P2X_4$ receptors (Haynes et al., 2006; Ohsawa et al., 2007); while $P2X_1$, $P2Y_1$, $P2Y_2$, A_{2A}, A_{2B}, and A_3 receptors have been implicated to a lesser degree (Kobayakawa et al., 2019; Kronlage et al., 2010). ATP provides a chemotactic gradient that promotes the extension of pre-existing membrane lamellipodia and activates macrophage membrane propulsion machinery (Kronlage et al., 2010). Inhibition of metabotropic purinergic receptors caused macrophages to scatter throughout the injured cord and enhanced secondary axonal dieback outside the lesion core (Evans et al., 2014). In larval zebrafish, microglial migration is also driven by $P2Y_{12}$ (Sieger et al., 2012), whereas

macrophage and neutrophil migration is mediated by $P2X_7$ receptors (Ogryzko et al., 2014). The recruitment of peripheral macrophages in larval zebrafish appeared necessary for regeneration as mutants lacking macrophages displayed an extended period of neuroinflammation and impaired axonal regrowth (Tsarouchas et al., 2018).

Once migration to the lesion site occurs, mammalian microglial cells downregulate $P2Y_{12}$ and subsequently upregulate $P2X_7$ and $P2Y_6$ receptors to stimulate proliferation and phagocytotic behavior (Bellver-Landete et al., 2019; Koizumi et al., 2007; Monif et al., 2009). In addition to proliferation, $P2X_7$ upregulation also induced the activation of microglia and an increase in pro-inflammatory gene expression (He et al., 2017; Monif et al., 2009). Among the purinergic receptors, $P2X_7$ has been most strongly implicated in mediating pro-inflammatory cytokine expression and secretion by various cell types within the mammalian CNS (Giuliani et al., 2017; He et al., 2017). Resident activated microglia and, to an even greater extent, infiltrating macrophages lead to an early (~2 days post SCI) and late wave (~14 days post SCI) upregulation and release of pro-inflammatory cytokines, including interleukins (IL-1α, IL-1β, IL-6) and tumor necrosis factor-α (TNF-α) (Bellver-Landete et al., 2019; Kwiecien et al., 2020; Pineau & Lacroix, 2007).

In comparison to mammals, SCI in zebrafish induces a brief pro-inflammatory response characterized by elevated expression of IL-1β and TNF-α that also appears to be driven by $P2X_7$ activation (Ogryzko et al., 2014; Tsarouchas et al., 2018). Interestingly, inhibiting the initial pro-inflammatory TNF-α and IL-1β upregulation in larval zebrafish reduced axonogenesis, whereas IL-1β downregulation at later time points was required for successful regeneration (Tsarouchas et al., 2018). The decrease in expression of IL-1β also corresponds with a longer anti-inflammatory response characterized by elevated expression of transforming growth factor- β1a

(TGF-β1a) and transforming growth factor-β3 (TGF-β3) by 3 days following injury (Hui et al., 2014; Tsarouchas et al., 2018). This supports the view that an early pro-inflammatory response is adaptive and required for regeneration but sustained immune responses can be detrimental to recovery.

$P2X_7$-mediated effects within the spinal cord appear to be regulated by the purinergic $P2X_4$ receptor. Macrophage $P2X_4$ receptors enhance $P2X_7$-mediated pro-inflammatory cytokine release in mice and in this way, help to sustain the neuroinflammatory response following SCI (Kawano, Tsukimoto, Mori, et al., 2012). In contrast, $P2X_4$ mRNA expression is downregulated with SCI in zebrafish (Hui et al., 2014). While it is unclear what this means in terms of regenerative success, deletion of $P2X_4$ decreases inflammasome activation, IL-1β secretion, and neuronal degeneration, while reducing microglial/macrophage activation, immune cell infiltration, and lesion size following SCI in mice (de Rivero Vaccari et al., 2012). In addition to $P2X_4$ and $P2X_7$, several other purinergic receptors have also been implicated in driving cytokine release in mammals. These include macrophage $P2Y_2$ receptors, which induce an increase in IL-1β, IL-6, and a decrease in TNF-α (de la Rosa et al., 2020), and $A_{2A/2B}$ receptors, which similarly decrease TNF-α release (Cohen et al., 2013). Lastly, stimulation of microglial $P2Y_{12}$ and $P2Y_{13}$ receptors also appears to induce IL- 1β, IL-6, and TNF-α release (Liu et al., 2017). Given some of the key roles of purinergic receptors across various cell types in neuroinflammation (Figure 3), evidence for a therapeutic advantage by targeting this signaling system during late response phases is growing.

1.5 Reactive Gliosis

The migration of macrophages and microglia to the lesion core is known to recruit other cell types, including perivascular fibroblasts and astrocytes, that form a multi-layer ‘glial scar’ in

mammalian SCI (Figure 1). Peripheral macrophages appear to be largely responsible for the formation of an inner fibrotic scar around the core of the lesion site that is fully encircled by reactive microglia within the first week following SCI (Bellver-Landete et al., 2019; Zhu et al., 2015). The reactive microglia then work to orchestrate the formation of the outermost portion of the glial scar comprised of newly proliferating $GFAP^+$ astrocytes (Wanner et al., 2013) (Figure 4). Depletion of reactive microglia reduces astrocyte proliferation by approximately 40–55%, which disrupts the organization of the glial scar and further impairs locomotor function (Bellver-Landete et al., 2019). On the flip side, augmenting microglial proliferation during the first week post injury enhances astroglial scar formation, increases cell survival, and improves recovery (Bellver-Landete et al., 2019). This suggests that the astroglial scar component is particularly important for preventing secondary cellular death and promoting functional recovery (Bellver-Landete et al., 2019; Okada et al., 2006; Wanner et al., 2013).

The formation of the astroglial scar begins as early as 3 days post SCI, peaks around 7 days, and is fully formed by 14 days (Bellver-Landete et al., 2019; Wanner et al., 2013). During early glial scar formation (5–7 days), newly proliferated astrocytes become oriented perpendicular to the lesion core and display a bipolar radial-glia-like morphology, unlike their ‘inactivated’ stellate counterparts distal from the lesion (Wanner et al., 2013). Indeed, a gradient spanning the lesion site consists of increasing astroglial proliferation, density, radial-like morphology, and $GFAP^+$ expression. During chronic glial scar formation (9–21 days), reactive bipolar astrocytes transform into scar-forming astrocytes as they form a ‘dense meshwork’ of intersecting and overlapping cell processes that express the inhibitory factors: chondroitin sulfate proteoglycans (CSPGs) (Hara et al., 2017; Wanner et al., 2013). Disrupting early glial scar formation through the inhibition of STAT3 signaling, a critical regulator of astrogliosis, led to a scattering of

inflammatory immune cells throughout the injured spinal cord that augmented secondary neuronal and oligodendrocyte cell death (Okada et al., 2006; Wanner et al., 2013). Deletion of the chronic glial scar using diphtheria toxin similarly exacerbated tissue damage (Anderson et al., 2016). However, attenuating the transformation of reactive astrocytes to those that are scar forming during later time frames post injury had a positive effect on axonal regeneration (Hara et al., 2017). Thus, while injury-induced astrogliosis may corral inflammatory cells at the lesion core and promote regeneration early on, it is equally important to limit this process in chronic conditions.

Both purinergic and cytokine-mediated signaling are involved in astrocyte reactivity and proliferation (Figure 3). How factors of these signaling families contribute to astroglial scar formation appears to be part of a delicate balance of local interactions between astrocytes and microglia. Purinergic signaling via $P2Y_1$ and $P2Y_{12}$ receptors significantly promotes astrocyte proliferation (Quintas et al., 2018). However, both the presence of microglia and IL-1α/ TNF-α prevented $P2Y_{1, 12}$ activation and astrocyte proliferation in response to agonist ADPβS. In the study, authors proposed this was due to the expression of $P2Y_{13}$ receptors by microglia. $P2Y_{13}$ is abundantly and exclusively expressed by microglia *in vivo* and $P2Y_{13}$ antagonism rescues ADPβS mediated astrocyte proliferation in astrocyte/microglia co-cultures (Quintas et al., 2018; Stefani et al., 2018). Thus, stimulation of microglial $P2Y_{13}$ receptors may result in the release of IL-1α and TNF-α, inhibition of astrocyte $P2Y_{1, 12}$ receptors, and prevention of astrocyte proliferation. It is likely that while microglia draw astrocytes to the lesion site, they also act to modulate the degree of astrocyte proliferation in response to elevated local purine levels. Indeed, astrocyte proliferation following SCI peaks within 3–5 days (Wanner et al., 2013): a point in

time when proinflammatory IL-1α and TNF-α expression is also strongly upregulated (Kwiecien et al., 2020).

In conjunction with proliferation, cytokine signaling is also involved in regulating astrocyte reactivity and cell survival. Microglia secretion of cytokines IL-1β, IL-6, and TNF-α promotes astrocyte transformation from a basal to a reactive phenotype by the concurrent downregulation of astrocyte $P2Y_1$ expression and upregulation of STAT3 phosphorylation (Shinozaki et al., 2017). In this study, $P2Y_1$ knockdown (KD) in astrocytes promoted astrocyte process extension, $GFAP^+$ expression, STAT3 phosphorylation, as well as a smaller injury core, decreased neuronal cell death, and decreased immune cell infiltration (Shinozaki et al., 2017). In contrast, others have demonstrated that astrocyte $P2Y_1$ upregulation protects against oxidative stress-mediated neuronal damage by inducing increased release of IL-6: a factor that induces astrogliosis, enhances spinal cord progenitor cell proliferation via activation of JAK/STAT3, and promotes neuronal differentiation of neural progenitor cells (Fujita et al., 2009; Kang & Kang, 2008; Oh et al., 2010; Okada et al., 2004). It is likely that the apparent conflicting roles of $P2Y_1$ stem from the action of additional P2Y receptors also expressed within the glial scar.

In addition to other proinflammatory cytokines, IL-1β expression is also elevated within the first week following SCI in mice (Kwiecien et al., 2020) and treatment of mouse primary striatal astrocytes or mouse primary cortical neurons with exogenous IL-1β increases $P2Y_2$ mRNA expression (Peterson et al., 2013; Stella et al., 1997). Similar to $P2Y_1$, $P2Y_2$ receptor stimulation has also been shown to induce astrocyte proliferation and STAT3 activation (Chorna et al., 2004; Wu et al., 2018). Thus, $P2Y_2$ may work to compensate for $P2Y_1$ inhibition, particularly in knockout and KD manipulations, and continue to promote astrogliosis independently. Interestingly, treatment with pan P2Y antagonists, which target both $P2Y_1$ and $P2Y_2$ receptors,

significantly reduce $GFAP^+$ expression within the lesion site and exacerbate secondary cell death (Rodriguez-Zayas et al., 2012), demonstrating the overall neuroprotective role of these factors and the astroglial scar.

While reactive gliosis in regeneratively competent teleosts is reflective of that in mammals, key differences do exist (Figure 2). Similar to mammals, goldfish and zebrafish generate a fibrotic scar within 1–3 weeks following a spinal cord hemisection; however, this scar does not impede regeneration as it does in mammals (Takeda et al., 2015; Wehner et al., 2017). Glial cells with elevated $GFAP^+$ expression surround the fibrotic scar but do not display a hypertrophied morphology (Goldshmit et al., 2012; Herman et al., 2018; Takeda et al., 2015). In the weeks after injury, laminin-coated tubular structures develop across the fibrous scar, and by 6–12 weeks, glial processes along with regenerating axons utilize these 'tunnels' to cross the injury site (Goldshmit et al., 2012; Takeda et al., 2015). Interestingly, deposition of neural/glial antigen 2, an inhibitory CSPG also upregulated in mammals, is present within the goldfish fibrous scar at 3 weeks post injury but its reduction by 6 weeks aligns with the increase in axon infiltration (Takeda et al., 2015).

Fibroblast growth factor (FGF) signaling appears to be particularly important for the formation of the glial bridge. Inhibition of FGF in zebrafish following SCI results in significant reductions to $GFAP^+$ expression, as well as a decline in axonal regeneration and functional recovery (Goldshmit et al., 2012). Treatment of primary astrocyte cultures derived from postnatal day 14 marmoset (*Callithrix jacchus*) cerebral cortex with human recombinant FGF2 accelerated wound closure in a scratch-wound assay by increasing proliferation, migration, and astrocyte transformation from a stellate/ multipolar to an elongated/bipolar morphology (Goldshmit et al., 2012). These findings were replicated in a mouse model of SCI where

application of exogenous FGF2 dampened the neuroinflammatory response, decreased levels of CSPGs, and promoted the phenotypic transformation of astrocytes into a bipolar morphology resembling the glial bridge in teleost species (Goldshmit et al., 2014). Interestingly, purinergic signaling governs FGF-induced astrogliosis. On one hand, P2Y activation enhances proliferation of astrocytes induced by FGF2; while $P2X_7$ stimulation inhibits proliferation by inducing a state of growth arrest (Neary et al., 2008). In addition to FGF, expression of connective tissue growth factor a (CTGFA) by glial cells accelerates glial bridge formation, enhances axonogenesis, and promotes functional recovery in zebrafish (Mokalled et al., 2016). Purinergic-mediated CTGFA secretion via A_{2A} receptors also supports a role for purines in promoting glial bridge formation (Chan et al., 2013). Future research is required to improve the current understanding of purinergic influences in mammalian and non-mammalian injury-induced gliosis.

1.6 Neurogenesis and Axonal Regeneration

In the mid 1990's, a latent neural stem cell niche lining the rodent central canal was identified (Weiss et al., 1996). It was found that under certain conditions, mammalian spinal ependymal cells give rise to new neurons and glia *in vitro*: a feat not normally present *in vivo*. The exception to this is the result of a direct injury, but even then, migration is almost absent, proliferation is minimal, and exclusive glial differentiation accounts for a very small percentage of newly derived astrocytes (Ren et al., 2017). Yet transplantation of spinal ependymal cells into the dentate gyrus of the hippocampus, a neurogenic niche within the adult mammalian brain, induced differentiation of these cells into neurons (Shihabuddin et al., 2000). This emphasized the influence of the spinal cord microenvironment and prompted much work into identifying potential cues responsible for determining the cell fate of endogenous spinal ependymal cells (Becker et al., 2018; Liu & Chen, 2019).

Apart from other mammalian species, the existence of injury-induced spinal progenitors in humans has been recently challenged. One study examining the central canal in humans suggested that in contrast to the ependymal layer in rodents, the central canal closes caudal to the brainstem beyond 18 years of age (Garcia-Ovejero et al., 2015). The authors show that the ependymal layer is replaced by a dense accumulation of $GFAP^+$ astroglial processes, ependymocytes, and perivascular pseudorosettes. Furthermore, expression of stem cell markers is highly limited within this region (Garcia-Ovejero et al., 2015). In conjunction, a post-mortem study of human SCI patients (aged 33–88) found no evidence for injury-induced proliferation of cells within the ependymal region (Paniagua-Torija et al., 2018). Together, these studies suggest that morphological and molecular profiles in adult humans may be quite unique compared to other vertebrate species, and approaches to induce endogenous neurogenesis could be more applicable to both younger individuals and those with higher level injuries.

Another regenerative strategy to encourage functional recovery following SCI is the re-growth of damaged axons or compensatory axonal sprouting. Unfortunately, axonal regeneration and sprouting are also highly restricted in mammalian species (Figure 1). Within 30 minutes following injury, neurons undergo rapid and symmetrical proximal and distal axon end fragmentation, which is followed by a period of slow axonal retraction until ~30 hours post transection (Kerschensteiner et al., 2005). After this point the injury extends beyond the proximal axon ends. The distal axon ends begin to undergo Wallerian degeneration mediated by infiltrating peripheral macrophages for several days to weeks (Evans et al., 2014). Although limiting macrophage migration to the injury site does not promote axonal survival, restricting them to the injury site does promote distal axonal sparing (Kobayakawa et al., 2019). Interestingly, approximately 30% of transected axons do undergo compensatory sprouting at

their proximal ends (Kerschensteiner et al., 2005). While these sprouts grow relatively rapidly and straight within the first two days, they quickly lose directionality and are unable to grow across the injury site. This is largely due to the presence of numerous growth inhibitory environmental cues at the injury site that lead to dystrophic growth cone formation and prevent axonal regeneration (Filous & Schwab, 2018).

In contrast to mammals, adult teleost fish and urodele amphibians achieve functional recovery by not only successfully replacing lost neurons, but also regenerating damaged axons (Figure 1). Radial glia (RG), referred to as ependymoradial glia, are neural stem cells within the CNS of non-mammalian vertebrates that serve many homeostatic roles and are essential for regeneration in zebrafish (Briona & Dorsky, 2014; Hui et al., 2010; Reimer et al., 2008), goldfish (Takeda et al., 2008), brown ghost knifefish (Zupanc, 2019), and salamanders (Joven & Simon, 2018). Morphologically, they line the central canal and extend radial processes out to the pial surface. Functionally, they resemble mammalian astrocytes and ependymal cells (Becker et al., 2018). Following SCI, RG undergo a massive proliferative response reaching peak values at 7 days before differentiating into both neurons and glial cells that later populate the injured cord (Hui et al., 2015). In addition, a subpopulation of RG also appears to contribute to the formation of the astroglial bridge that promotes and guides axonal regeneration in these species (Goldshmit et al., 2012). RG cells are present in the mammalian spinal cord during development and give rise to new neurons, macroglia populations, and ependymal cells (Barry & McDermott, 2005; Malatesta et al., 2000; Xing et al., 2018). However, they undergo terminal differentiation into astrocytes during early postnatal development and are relatively absent from the adult spinal cord (Barry & McDermott, 2005).

The regeneration of axons across the lesion site in these non-mammalian species occurs simultaneously with injury-induced neurogenesis. In larval zebrafish, acute axonal degeneration occurs within the first 30 minutes following Mauthner cell axotomy, which is followed by additional retraction and Wallerian degeneration that concludes by 24 hours (Hu et al., 2018). Remarkably, this study showed that by 96 hours post axotomy, axonal regeneration across the lesion reached 78% of the uninjured axon length. More in line with axonal regeneration in mammals, spinal cord transection in newts results in axonal retraction and dystrophic growth cone formation within the first week post injury (Zukor et al., 2011). However, by 2 weeks, growth cones form at some axon tips and 'wisping axons' start to grow into the injury site in association with regenerating meninges and RG cell extensions that innervate their targets by 9 weeks (Zukor et al., 2011). Thus, the response to axonal transection in these species appears to be like mammals during early time points (AAD, retraction, Wallerian degeneration, sprouting), but differs significantly during more chronic responses (Figure 2). Identifying the factors and conditions present in regeneratively competent species, particularly at the later time points; therefore, may be of most use in promoting axonal regeneration in mammals.

Regardless of their ability to achieve functional recovery, non-mammalian vertebrate CNS regeneration does not result in a return to uninjured conditions. First, newly generated interneurons in larval zebrafish appear morphologically and functionally distinct from their uninjured counterparts following SCI (Vasudevan et al., 2021). In addition to having a smaller soma, they have a higher input resistance, a more depolarized resting membrane potential, lower firing frequencies, and lower spike frequencies; however, they are sufficient to restore function. Second, only a subset of neurons undergo axonal regeneration in non-mammalian species studied to date, including newts (Zukor et al., 2011), goldfish (Takeda et al., 2015), and zebrafish

(Goldshmit et al., 2012). Finally, while some regenerating axons travel along their original trajectories, as observed in newts (Zukor et al., 2011) and larval zebrafish (Hu et al., 2018), many take novel routes as seen in adult zebrafish (Becker & Becker, 2001). Notably, regenerative ability appears to decline with age in both mammals (Geoffroy et al., 2016) and non-mammalian vertebrates (Edelmann et al., 2013). Further examination of the post injury cytoarchitecture, the regulatory cues present in the microenvironment, and the changes to both over time are all important considerations in understanding regeneration in these species.

The role of purinergic signaling in injury-induced neurogenesis and axonal regeneration is quite limited (Figure 4). Expression of several purinergic receptors have been identified in mammalian spinal ependymal cells, including $P2Y_1$, $P2Y_4$, $P2X_4$, and $P2X_7$ (Gomez-Villafuertes et al., 2015). In response to SCI, the expression of $P2Y_1$ receptors is downregulated, whereas expression of $P2Y_4$, $P2X_4$, and $P2X_7$ receptors is upregulated (Gomez-Villafuertes et al., 2015). How the differential expression of these subtypes in ependymal cells influences their response to injury is largely unknown but has been shown to play roles in both proliferation and differentiation of other cell types. For instance, $P2Y_1$ receptor activation induces proliferation of astrocytes, RG, and tanycytes within the ependymal cell lining of the third ventricle (Quintas et al., 2018; Recabal et al., 2021; Weissman et al., 2004). Similarly, $P2X_4$ receptor expression has been associated with enhancing growth factor expression (e.g. brain-derived neurotrophic factor) and indirectly promoting ependymal cell proliferation (Ma et al., 2019; Su et al., 2019; Xu et al., 2018). In terms of differentiation, $P2Y_4$ expression accompanies glutamatergic differentiation of embryonic stem cells, and $P2X_7$ stimulation regulates neuronal differentiation of both embryonic and adult neural progenitor cells (Glaser et al., 2014; Leeson et al., 2018; Tsao et al., 2013; Uda

et al., 2016). It is likely that the balance and timing of purinergic receptor expression influences the response of ependymal cells to SCI, but further work is needed to determine their roles.

In addition to those mentioned above, purinergic receptors $P2Y_2$, $P2Y_{13}$, and A_{2A} have also been shown to regulate progenitor cell proliferation, prevent secondary injury, or promote functional recovery. In a recent study of $P2Y_2$ knockout mice, there was a significant reduction in the number of proliferating neural progenitor cells within the subgranular zone of the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles, demonstrating that $P2Y_2$ receptor expression is important for progenitor cell proliferation within the adult mammalian brain (Ali et al., 2021). Meanwhile, $P2Y_{13}$ knockout mice displayed enhanced progenitor cell proliferation within the dentate gyrus of the hippocampus in early and late adulthood, as well as an increase in neurogenesis (Stefani et al., 2018). Interestingly, inhibition of vesicular nucleotide transporter in postnatal cerebellar cells inhibited quiescence and self-renewal to drive cell-cycle exit toward a neurogenic fate (Paniagua-Herranz et al., 2020). Mechanistically, vesicular nucleotide transporter is responsible for promoting vesicular ATP storage; therefore, inhibition of this transporter decreases ATP signalling and suggests that variation in purinergic ligand alone is sufficient to influence neurogenesis (Paniagua-Herranz et al., 2020). Following SCI, activation of the A_{2A} receptor increases Wnt3a and β-catenin mRNA expression in mammals resulting in reductions of inflammation and neuronal cell death (Irrera et al., 2018). While a general reduction in Wnt/β-catenin signaling is typically observed with mammalian SCI (Gonzalez-Fernandez et al., 2014; Irrera et al., 2018), this pathway appears upregulated following SCI in zebrafish and in sea lamprey (Briona et al., 2015; Herman et al., 2018; Strand et al., 2016). Wnt/β-catenin specifically promotes and appears necessary for neuronal differentiation of RG following SCI in larval and adult zebrafish (Briona et al., 2015;

Strand et al., 2016). Various interventions targeting the upregulation of canonical Wnt/ β-catenin signaling, including A_{2A} agonism, following mammalian CNS injury have been widely successful in limiting neuroinflammation and secondary cell death, and enhancing functional recovery (Irrera et al., 2018; Shruster et al., 2012; Xu et al., 2019).

1.7 Concluding Remarks

Regenerative failure following injury to the mammalian CNS is the result of a microenvironment that enhances neuroinflammation, pathological reactive gliosis, and neurodegeneration. Studying regeneratively competent species provides an opportunity to elucidate critical pro-regenerative pathways. Given the evolutionary conservation and prevailing role of purinergic signaling in many of these processes throughout development and adulthood, identification of purinergic influences on regenerative processes may be integral to understanding differences among regeneratively competent and non-competent vertebrates. Ultimately, it is the hope that understanding these differences will lead to therapeutic approaches that target both the endogenous progenitor potential of spinal ependymal cells and functional axonal regeneration following spinal cord injury.

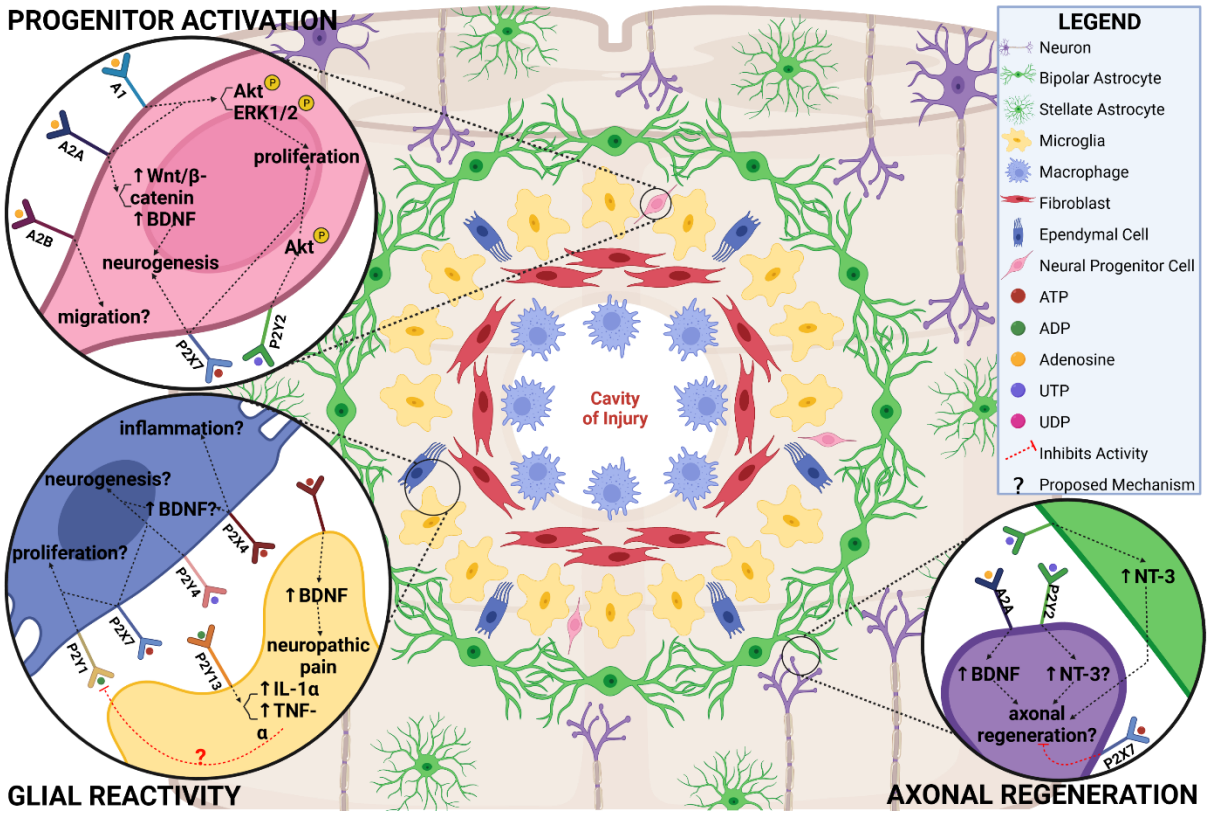

Figure 4. Purinergic signaling within the spinal cord microenvironment during the chronic injury response. After the first week following mammalian SCI, reactive astrocytes become scar forming, ependymal and neural progenitor cells fail to undergo injury-induced proliferation and neuronal differentiation, and axons continue to degenerate. Identified and hypothesized roles for purinergic receptors in these processes is summarized. Created with BioRender. com with publication permissions and publication license. ADP: Adenosine diphosphate; Akt: protein kinase B; ATP: adenosine triphosphate; BDNF: brain derived neurotrophic factor; CD39: cluster of differentiation 39; Ca2+: calcium; CREB: cAMP response element-binding protein; ERK1/2: extracellular signal-regulated kinases 1/2; IL: interleukin; NGF2: nerve growth factor-2; NLRP1/3: NLR family pyrin domain containing 1/3; NT3: neurotrophin-3; STAT3: signal transducer and activator of transcription 3; TNF-α: tumor necrosis factor alpha; UDP: uridine diphosphate; UTP: uridine triphosphate; Wnt: wingless-related intergration site. Created with BioRender.com with permissions and publication license.

1.8 References

Ali, A. A. H., Abdel-Hafiz, L., Tundo-Lavalle, F., Hassan, S. A., & von Gall, C. (2021). P2Y2 deficiency impacts adult neurogenesis and related forebrain functions. *FASEB J*, *35*(5), e21546. https://doi.org/10.1096/fj.202002419RR

Anderson, M. A., Burda, J. E., Ren, Y., Ao, Y., O'Shea, T. M., Kawaguchi, R., Coppola, G., Khakh, B. S., Deming, T. J., & Sofroniew, M. V. (2016). Astrocyte scar formation aids central nervous system axon regeneration. *Nature*, *532*(7598), 195-200. https://doi.org/10.1038/nature17623

Arthur, D. B., Georgi, S., Akassoglou, K., & Insel, P. A. (2006). Inhibition of apoptosis by P2Y2 receptor activation: novel pathways for neuronal survival. *J Neurosci*, *26*(14), 3798-3804. https://doi.org/10.1523/JNEUROSCI.5338-05.2006

Barry, D., & McDermott, K. (2005). Differentiation of radial glia from radial precursor cells and transformation into astrocytes in the developing rat spinal cord. *Glia*, *50*(3), 187-197. https://doi.org/10.1002/glia.20166

Becker, C. G., Becker, T., & Hugnot, J. P. (2018). The spinal ependymal zone as a source of endogenous repair cells across vertebrates. *Prog Neurobiol*, *170*, 67-80. https://doi.org/10.1016/j.pneurobio.2018.04.002

Becker, T., & Becker, C. G. (2001). Regenerating descending axons preferentially reroute to the gray matter in the presence of a general macrophage/microglial reaction caudal to a spinal transection in adult zebrafish. *J Comp Neurol*, *433*(1), 131-147. https://doi.org/10.1002/cne.1131

Bellver-Landete, V., Bretheau, F., Mailhot, B., Vallieres, N., Lessard, M., Janelle, M. E., Vernoux, N., Tremblay, M. E., Fuehrmann, T., Shoichet, M. S., & Lacroix, S. (2019). Microglia are an essential component of the neuroprotective scar that forms after spinal cord injury. *Nat Commun*, *10*(1), 518. https://doi.org/10.1038/s41467-019-08446-0

Bidula, S., Dhuna, K., Helliwell, R., & Stokes, L. (2019). Positive allosteric modulation of P2X7 promotes apoptotic cell death over lytic cell death responses in macrophages. *Cell Death Dis*, *10*(12), 882. https://doi.org/10.1038/s41419-019-2110-3

Briona, L. K., & Dorsky, R. I. (2014). Radial glial progenitors repair the zebrafish spinal cord following transection. *Exp Neurol*, *256*, 81-92. https://doi.org/10.1016/j.expneurol.2014.03.017

Briona, L. K., Poulain, F. E., Mosimann, C., & Dorsky, R. I. (2015). Wnt/ss-catenin signaling is required for radial glial neurogenesis following spinal cord injury. *Dev Biol*, *403*(1), 15-21. https://doi.org/10.1016/j.ydbio.2015.03.025

Burnstock, G. (2018). Purine and purinergic receptors. *Brain Neurosci Adv*, *2*, 2398212818817494. https://doi.org/10.1177/2398212818817494

Burnstock, G., & Ulrich, H. (2011). Purinergic signaling in embryonic and stem cell development. *Cell Mol Life Sci*, *68*(8), 1369-1394. https://doi.org/10.1007/s00018-010-0614-1

Chan, E. S., Liu, H., Fernandez, P., Luna, A., Perez-Aso, M., Bujor, A. M., Trojanowska, M., & Cronstein, B. N. (2013). Adenosine A(2A) receptors promote collagen production by a Fli1- and CTGF-mediated mechanism. *Arthritis Res Ther*, *15*(3), R58. https://doi.org/10.1186/ar4229

Chekeni, F. B., Elliott, M. R., Sandilos, J. K., Walk, S. F., Kinchen, J. M., Lazarowski, E. R., Armstrong, A. J., Penuela, S., Laird, D. W., Salvesen, G. S., Isakson, B. E., Bayliss, D. A., & Ravichandran, K. S. (2010). Pannexin 1 channels mediate 'find-me' signal release

and membrane permeability during apoptosis. *Nature*, *467*(7317), 863-867. https://doi.org/10.1038/nature09413

Chorna, N. E., Santiago-Perez, L. I., Erb, L., Seye, C. I., Neary, J. T., Sun, G. Y., Weisman, G. A., & Gonzalez, F. A. (2004). P2Y receptors activate neuroprotective mechanisms in astrocytic cells. *J Neurochem*, *91*(1), 119-132. https://doi.org/10.1111/j.1471-4159.2004.02699.x

Cohen, H. B., Briggs, K. T., Marino, J. P., Ravid, K., Robson, S. C., & Mosser, D. M. (2013). TLR stimulation initiates a CD39-based autoregulatory mechanism that limits macrophage inflammatory responses. *Blood*, *122*(11), 1935-1945. https://doi.org/10.1182/blood-2013-04-496216

Crowe, M. J., Bresnahan, J. C., Shuman, S. L., Masters, J. N., & Beattie, M. S. (1997). Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. *Nat Med*, *3*(1), 73-76. https://doi.org/10.1038/nm0197-73

D'Alimonte, I., Ciccarelli, R., Di Iorio, P., Nargi, E., Buccella, S., Giuliani, P., Rathbone, M. P., Jiang, S., Caciagli, F., & Ballerini, P. (2007). Activation of P2X(7) receptors stimulates the expression of P2Y(2) receptor mRNA in astrocytes cultured from rat brain. *Int J Immunopathol Pharmacol*, *20*(2), 301-316. https://doi.org/10.1177/039463200702000210

Davalos, D., Grutzendler, J., Yang, G., Kim, J. V., Zuo, Y., Jung, S., Littman, D. R., Dustin, M. L., & Gan, W. B. (2005). ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci*, *8*(6), 752-758. https://doi.org/10.1038/nn1472

de la Rosa, G., Gomez, A. I., Banos, M. C., & Pelegrin, P. (2020). Signaling Through Purinergic Receptor P2Y2 Enhances Macrophage IL-1beta Production. *Int J Mol Sci*, *21*(13). https://doi.org/10.3390/ijms21134686

de Rivero Vaccari, J. P., Bastien, D., Yurcisin, G., Pineau, I., Dietrich, W. D., De Koninck, Y., Keane, R. W., & Lacroix, S. (2012). P2X4 receptors influence inflammasome activation after spinal cord injury. *J Neurosci*, *32*(9), 3058-3066. https://doi.org/10.1523/JNEUROSCI.4930-11.2012

Dou, Y., Wu, H. J., Li, H. Q., Qin, S., Wang, Y. E., Li, J., Lou, H. F., Chen, Z., Li, X. M., Luo, Q. M., & Duan, S. (2012). Microglial migration mediated by ATP-induced ATP release from lysosomes. *Cell Res*, *22*(6), 1022-1033. https://doi.org/10.1038/cr.2012.10

Edelmann, K., Glashauser, L., Sprungala, S., Hesl, B., Fritschle, M., Ninkovic, J., Godinho, L., & Chapouton, P. (2013). Increased radial glia quiescence, decreased reactivation upon injury and unaltered neuroblast behavior underlie decreased neurogenesis in the aging zebrafish telencephalon. *J Comp Neurol*, *521*(13), 3099-3115. https://doi.org/10.1002/cne.23347

Elliott, M. R., Chekeni, F. B., Trampont, P. C., Lazarowski, E. R., Kadl, A., Walk, S. F., Park, D., Woodson, R. I., Ostankovich, M., Sharma, P., Lysiak, J. J., Harden, T. K., Leitinger, N., & Ravichandran, K. S. (2009). Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature*, *461*(7261), 282-286. https://doi.org/10.1038/nature08296

Emery, E., Aldana, P., Bunge, M. B., Puckett, W., Srinivasan, A., Keane, R. W., Bethea, J., & Levi, A. D. (1998). Apoptosis after traumatic human spinal cord injury. *J Neurosurg*, *89*(6), 911-920. https://doi.org/10.3171/jns.1998.89.6.0911

Evans, T. A., Barkauskas, D. S., Myers, J. T., Hare, E. G., You, J. Q., Ransohoff, R. M., Huang, A. Y., & Silver, J. (2014). High-resolution intravital imaging reveals that blood-derived macrophages but not resident microglia facilitate secondary axonal dieback in traumatic

spinal cord injury. *Exp Neurol*, *254*, 109-120. https://doi.org/10.1016/j.expneurol.2014.01.013

Filous, A. R., & Schwab, J. M. (2018). Determinants of Axon Growth, Plasticity, and Regeneration in the Context of Spinal Cord Injury. *Am J Pathol*, *188*(1), 53-62. https://doi.org/10.1016/j.ajpath.2017.09.005

Fleming, J. C., Norenberg, M. D., Ramsay, D. A., Dekaban, G. A., Marcillo, A. E., Saenz, A. D., Pasquale-Styles, M., Dietrich, W. D., & Weaver, L. C. (2006). The cellular inflammatory response in human spinal cords after injury. *Brain*, *129*(Pt 12), 3249-3269. https://doi.org/10.1093/brain/awl296

Fujita, T., Tozaki-Saitoh, H., & Inoue, K. (2009). P2Y1 receptor signaling enhances neuroprotection by astrocytes against oxidative stress via IL-6 release in hippocampal cultures. *Glia*, *57*(3), 244-257. https://doi.org/10.1002/glia.20749

Fukumoto, Y., Tanaka, K. F., Parajuli, B., Shibata, K., Yoshioka, H., Kanemaru, K., Gachet, C., Ikenaka, K., Koizumi, S., & Kinouchi, H. (2019). Neuroprotective effects of microglial P2Y1 receptors against ischemic neuronal injury. *J Cereb Blood Flow Metab*, *39*(11), 2144-2156. https://doi.org/10.1177/0271678X18805317

Gandelman, M., Levy, M., Cassina, P., Barbeito, L., & Beckman, J. S. (2013). P2X7 receptor-induced death of motor neurons by a peroxynitrite/FAS-dependent pathway. *J Neurochem*, *126*(3), 382-388. https://doi.org/10.1111/jnc.12286

Garcia-Ovejero, D., Arevalo-Martin, A., Paniagua-Torija, B., Florensa-Vila, J., Ferrer, I., Grassner, L., & Molina-Holgado, E. (2015). The ependymal region of the adult human spinal cord differs from other species and shows ependymoma-like features. *Brain*, *138*(Pt 6), 1583-1597. https://doi.org/10.1093/brain/awv089

Geoffroy, C. G., Hilton, B. J., Tetzlaff, W., & Zheng, B. (2016). Evidence for an Age-Dependent Decline in Axon Regeneration in the Adult Mammalian Central Nervous System. *Cell Rep*, *15*(2), 238-246. https://doi.org/10.1016/j.celrep.2016.03.028

Giuliani, A. L., Sarti, A. C., Falzoni, S., & Di Virgilio, F. (2017). The P2X7 Receptor-Interleukin-1 Liaison. *Front Pharmacol*, *8*, 123. https://doi.org/10.3389/fphar.2017.00123

Glaser, T., de Oliveira, S. L., Cheffer, A., Beco, R., Martins, P., Fornazari, M., Lameu, C., Junior, H. M., Coutinho-Silva, R., & Ulrich, H. (2014). Modulation of mouse embryonic stem cell proliferation and neural differentiation by the P2X7 receptor. *PLoS One*, *9*(5), e96281. https://doi.org/10.1371/journal.pone.0096281

Goldshmit, Y., Frisca, F., Pinto, A. R., Pebay, A., Tang, J. K., Siegel, A. L., Kaslin, J., & Currie, P. D. (2014). Fgf2 improves functional recovery-decreasing gliosis and increasing radial glia and neural progenitor cells after spinal cord injury. *Brain Behav*, *4*(2), 187-200. https://doi.org/10.1002/brb3.172

Goldshmit, Y., Sztal, T. E., Jusuf, P. R., Hall, T. E., Nguyen-Chi, M., & Currie, P. D. (2012). Fgf-dependent glial cell bridges facilitate spinal cord regeneration in zebrafish. *J Neurosci*, *32*(22), 7477-7492. https://doi.org/10.1523/JNEUROSCI.0758-12.2012

Gomez-Villafuertes, R. (2016). Contribution of purinergic receptors to spinal cord injury repair: stem cell-based neuroregeneration. *Neural Regen Res*, *11*(3), 418-419. https://doi.org/10.4103/1673-5374.179049

Gomez-Villafuertes, R., Rodriguez-Jimenez, F. J., Alastrue-Agudo, A., Stojkovic, M., Miras-Portugal, M. T., & Moreno-Manzano, V. (2015). Purinergic Receptors in Spinal Cord-Derived Ependymal Stem/Progenitor Cells and Their Potential Role in Cell-Based

Therapy for Spinal Cord Injury. *Cell Transplant*, *24*(8), 1493-1509. https://doi.org/10.3727/096368914X682828

Gonzalez-Fernandez, C., Fernandez-Martos, C. M., Shields, S. D., Arenas, E., & Javier Rodriguez, F. (2014). Wnts are expressed in the spinal cord of adult mice and are differentially induced after injury. *J Neurotrauma*, *31*(6), 565-581. https://doi.org/10.1089/neu.2013.3067

Greenhalgh, A. D., & David, S. (2014). Differences in the phagocytic response of microglia and peripheral macrophages after spinal cord injury and its effects on cell death. *J Neurosci*, *34*(18), 6316-6322. https://doi.org/10.1523/JNEUROSCI.4912-13.2014

Hara, M., Kobayakawa, K., Ohkawa, Y., Kumamaru, H., Yokota, K., Saito, T., Kijima, K., Yoshizaki, S., Harimaya, K., Nakashima, Y., & Okada, S. (2017). Interaction of reactive astrocytes with type I collagen induces astrocytic scar formation through the integrin-N-cadherin pathway after spinal cord injury. *Nat Med*, *23*(7), 818-828. https://doi.org/10.1038/nm.4354

Hassannejad, Z., Zadegan, S. A., Shakouri-Motlagh, A., Mokhatab, M., Rezvan, M., Sharif-Alhoseini, M., Shokraneh, F., Moshayedi, P., & Rahimi-Movaghar, V. (2018). The fate of neurons after traumatic spinal cord injury in rats: A systematic review. *Iran J Basic Med Sci*, *21*(6), 546-557. https://doi.org/10.22038/IJBMS.2018.24239.6052

Haynes, S. E., Hollopeter, G., Yang, G., Kurpius, D., Dailey, M. E., Gan, W. B., & Julius, D. (2006). The P2Y12 receptor regulates microglial activation by extracellular nucleotides. *Nat Neurosci*, *9*(12), 1512-1519. https://doi.org/10.1038/nn1805

He, Y., Taylor, N., Fourgeaud, L., & Bhattacharya, A. (2017). The role of microglial P2X7: modulation of cell death and cytokine release. *J Neuroinflammation*, *14*(1), 135. https://doi.org/10.1186/s12974-017-0904-8

Herman, P. E., Papatheodorou, A., Bryant, S. A., Waterbury, C. K. M., Herdy, J. R., Arcese, A. A., Buxbaum, J. D., Smith, J. J., Morgan, J. R., & Bloom, O. (2018). Highly conserved molecular pathways, including Wnt signaling, promote functional recovery from spinal cord injury in lampreys. *Sci Rep*, *8*(1), 742. https://doi.org/10.1038/s41598-017-18757-1

Hu, B. B., Chen, M., Huang, R. C., Huang, Y. B., Xu, Y., Yin, W., Li, L., & Hu, B. (2018). In vivo imaging of Mauthner axon regeneration, remyelination and synapses re-establishment after laser axotomy in zebrafish larvae. *Exp Neurol*, *300*, 67-73. https://doi.org/10.1016/j.expneurol.2017.10.028

Hui, S. P., Dutta, A., & Ghosh, S. (2010). Cellular response after crush injury in adult zebrafish spinal cord. *Dev Dyn*, *239*(11), 2962-2979. https://doi.org/10.1002/dvdy.22438

Hui, S. P., Nag, T. C., & Ghosh, S. (2015). Characterization of Proliferating Neural Progenitors after Spinal Cord Injury in Adult Zebrafish. *PLoS One*, *10*(12), e0143595. https://doi.org/10.1371/journal.pone.0143595

Hui, S. P., Sengupta, D., Lee, S. G., Sen, T., Kundu, S., Mathavan, S., & Ghosh, S. (2014). Genome wide expression profiling during spinal cord regeneration identifies comprehensive cellular responses in zebrafish. *PLoS One*, *9*(1), e84212. https://doi.org/10.1371/journal.pone.0084212

Irrera, N., Arcoraci, V., Mannino, F., Vermiglio, G., Pallio, G., Minutoli, L., Bagnato, G., Anastasi, G. P., Mazzon, E., Bramanti, P., Squadrito, F., Altavilla, D., & Bitto, A. (2018). Activation of A2A Receptor by PDRN Reduces Neuronal Damage and Stimulates WNT/beta-CATENIN Driven Neurogenesis in Spinal Cord Injury. *Front Pharmacol*, *9*, 506. https://doi.org/10.3389/fphar.2018.00506

Joven, A., & Simon, A. (2018). Homeostatic and regenerative neurogenesis in salamanders. *Prog Neurobiol*, *170*, 81-98. https://doi.org/10.1016/j.pneurobio.2018.04.006

Kang, M. K., & Kang, S. K. (2008). Interleukin-6 induces proliferation in adult spinal cord-derived neural progenitors via the JAK2/STAT3 pathway with EGF-induced MAPK phosphorylation. *Cell Prolif*, *41*(3), 377-392. https://doi.org/10.1111/j.1365-2184.2008.00537.x

Kawano, A., Tsukimoto, M., Mori, D., Noguchi, T., Harada, H., Takenouchi, T., Kitani, H., & Kojima, S. (2012). Regulation of P2X7-dependent inflammatory functions by P2X4 receptor in mouse macrophages. *Biochem Biophys Res Commun*, *420*(1), 102-107. https://doi.org/10.1016/j.bbrc.2012.02.122

Kawano, A., Tsukimoto, M., Noguchi, T., Hotta, N., Harada, H., Takenouchi, T., Kitani, H., & Kojima, S. (2012). Involvement of P2X4 receptor in P2X7 receptor-dependent cell death of mouse macrophages. *Biochem Biophys Res Commun*, *419*(2), 374-380. https://doi.org/10.1016/j.bbrc.2012.01.156

Kerschensteiner, M., Schwab, M. E., Lichtman, J. W., & Misgeld, T. (2005). In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. *Nat Med*, *11*(5), 572-577. https://doi.org/10.1038/nm1229

Kobayakawa, K., Ohkawa, Y., Yoshizaki, S., Tamaru, T., Saito, T., Kijima, K., Yokota, K., Hara, M., Kubota, K., Matsumoto, Y., Harimaya, K., Ozato, K., Masuda, T., Tsuda, M., Tamura, T., Inoue, K., Edgerton, V. R., Iwamoto, Y., Nakashima, Y., & Okada, S. (2019). Macrophage centripetal migration drives spontaneous healing process after spinal cord injury. *Sci Adv*, *5*(5), eaav5086. https://doi.org/10.1126/sciadv.aav5086

Koganti, L., Liu, J., DeMajewski, A., Agostini, M. A., Wong, T. W., Faber, D. S., & Zottoli, S. J. (2020). Invasion of microglia/macrophages and granulocytes into the Mauthner axon myelin sheath following spinal cord injury of the adult goldfish, Carassius auratus. *J Morphol*, *281*(1), 135-152. https://doi.org/10.1002/jmor.21086

Koizumi, S., Shigemoto-Mogami, Y., Nasu-Tada, K., Shinozaki, Y., Ohsawa, K., Tsuda, M., Joshi, B. V., Jacobson, K. A., Kohsaka, S., & Inoue, K. (2007). UDP acting at P2Y6 receptors is a mediator of microglial phagocytosis. *Nature*, *446*(7139), 1091-1095. https://doi.org/10.1038/nature05704

Kono, H., & Rock, K. L. (2008). How dying cells alert the immune system to danger. *Nat Rev Immunol*, *8*(4), 279-289. https://doi.org/10.1038/nri2215

Kronlage, M., Song, J., Sorokin, L., Isfort, K., Schwerdtle, T., Leipziger, J., Robaye, B., Conley, P. B., Kim, H. C., Sargin, S., Schon, P., Schwab, A., & Hanley, P. J. (2010). Autocrine purinergic receptor signaling is essential for macrophage chemotaxis. *Sci Signal*, *3*(132), ra55. https://doi.org/10.1126/scisignal.2000588

Kumar, R., Lim, J., Mekary, R. A., Rattani, A., Dewan, M. C., Sharif, S. Y., Osorio-Fonseca, E., & Park, K. B. (2018). Traumatic Spinal Injury: Global Epidemiology and Worldwide Volume. *World Neurosurg*, *113*, e345-e363. https://doi.org/10.1016/j.wneu.2018.02.033

Kwiecien, J. M., Dabrowski, W., Dabrowska-Bouta, B., Sulkowski, G., Oakden, W., Kwiecien-Delaney, C. J., Yaron, J. R., Zhang, L., Schutz, L., Marzec-Kotarska, B., Stanisz, G. J., Karis, J. P., Struzynska, L., & Lucas, A. R. (2020). Prolonged inflammation leads to ongoing damage after spinal cord injury. *PLoS One*, *15*(3), e0226584. https://doi.org/10.1371/journal.pone.0226584

Leeson, H. C., Kasherman, M. A., Chan-Ling, T., Lovelace, M. D., Brownlie, J. C., Toppinen, K. M., Gu, B. J., & Weible, M. W., 2nd. (2018). P2X7 Receptors Regulate Phagocytosis and

Proliferation in Adult Hippocampal and SVZ Neural Progenitor Cells: Implications for Inflammation in Neurogenesis. *Stem Cells*, *36*(11), 1764-1777. https://doi.org/10.1002/stem.2894

Liu, P. W., Yue, M. X., Zhou, R., Niu, J., Huang, D. J., Xu, T., Luo, P., Liu, X. H., & Zeng, J. W. (2017). P2Y12 and P2Y13 receptors involved in ADPbetas induced the release of IL-1beta, IL-6 and TNF-alpha from cultured dorsal horn microglia. *J Pain Res*, *10*, 1755-1767. https://doi.org/10.2147/JPR.S137131

Liu, S., & Chen, Z. (2019). Employing Endogenous NSCs to Promote Recovery of Spinal Cord Injury. *Stem Cells Int*, *2019*, 1958631. https://doi.org/10.1155/2019/1958631

Lopez-Serrano, C., Santos-Nogueira, E., Francos-Quijorna, I., Coll-Miro, M., Chun, J., & Lopez-Vales, R. (2019). Lysophosphatidic acid receptor type 2 activation contributes to secondary damage after spinal cord injury in mice. *Brain Behav Immun*, *76*, 258-267. https://doi.org/10.1016/j.bbi.2018.12.007

Ma, Y., Deng, M., & Liu, M. (2019). Effect of Differently Polarized Macrophages on Proliferation and Differentiation of Ependymal Cells from Adult Spinal Cord. *J Neurotrauma*, *36*(15), 2337-2347. https://doi.org/10.1089/neu.2018.6133

Malatesta, P., Hartfuss, E., & Gotz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development*, *127*(24), 5253-5263. https://www.ncbi.nlm.nih.gov/pubmed/11076748

Marichal, N., Fabbiani, G., Trujillo-Cenoz, O., & Russo, R. E. (2016). Purinergic signalling in a latent stem cell niche of the rat spinal cord. *Purinergic Signal*, *12*(2), 331-341. https://doi.org/10.1007/s11302-016-9507-6

Mokalled, M. H., Patra, C., Dickson, A. L., Endo, T., Stainier, D. Y., & Poss, K. D. (2016). Injury-induced ctgfa directs glial bridging and spinal cord regeneration in zebrafish. *Science*, *354*(6312), 630-634. https://doi.org/10.1126/science.aaf2679

Monif, M., Reid, C. A., Powell, K. L., Smart, M. L., & Williams, D. A. (2009). The P2X7 receptor drives microglial activation and proliferation: a trophic role for P2X7R pore. *J Neurosci*, *29*(12), 3781-3791. https://doi.org/10.1523/JNEUROSCI.5512-08.2009

Neary, J. T., Shi, Y. F., Kang, Y., & Tran, M. D. (2008). Opposing effects of P2X(7) and P2Y purine/pyrimidine-preferring receptors on proliferation of astrocytes induced by fibroblast growth factor-2: implications for CNS development, injury, and repair. *J Neurosci Res*, *86*(14), 3096-3105. https://doi.org/10.1002/jnr.21765

Ogryzko, N. V., Hoggett, E. E., Solaymani-Kohal, S., Tazzyman, S., Chico, T. J., Renshaw, S. A., & Wilson, H. L. (2014). Zebrafish tissue injury causes upregulation of interleukin-1 and caspase-dependent amplification of the inflammatory response. *Dis Model Mech*, *7*(2), 259-264. https://doi.org/10.1242/dmm.013029

Oh, J., McCloskey, M. A., Blong, C. C., Bendickson, L., Nilsen-Hamilton, M., & Sakaguchi, D. S. (2010). Astrocyte-derived interleukin-6 promotes specific neuronal differentiation of neural progenitor cells from adult hippocampus. *J Neurosci Res*, *88*(13), 2798-2809. https://doi.org/10.1002/jnr.22447

Ohishi, A., Keno, Y., Marumiya, A., Sudo, Y., Uda, Y., Matsuda, K., Morita, Y., Furuta, T., Nishida, K., & Nagasawa, K. (2016). Expression level of P2X7 receptor is a determinant of ATP-induced death of mouse cultured neurons. *Neuroscience*, *319*, 35-45. https://doi.org/10.1016/j.neuroscience.2016.01.048

Ohsawa, K., Irino, Y., Nakamura, Y., Akazawa, C., Inoue, K., & Kohsaka, S. (2007). Involvement of P2X4 and P2Y12 receptors in ATP-induced microglial chemotaxis. *Glia*, *55*(6), 604-616. https://doi.org/10.1002/glia.20489

Okada, S., Nakamura, M., Katoh, H., Miyao, T., Shimazaki, T., Ishii, K., Yamane, J., Yoshimura, A., Iwamoto, Y., Toyama, Y., & Okano, H. (2006). Conditional ablation of Stat3 or Socs3 discloses a dual role for reactive astrocytes after spinal cord injury. *Nat Med*, *12*(7), 829-834. https://doi.org/10.1038/nm1425

Okada, S., Nakamura, M., Mikami, Y., Shimazaki, T., Mihara, M., Ohsugi, Y., Iwamoto, Y., Yoshizaki, K., Kishimoto, T., Toyama, Y., & Okano, H. (2004). Blockade of interleukin-6 receptor suppresses reactive astrogliosis and ameliorates functional recovery in experimental spinal cord injury. *J Neurosci Res*, *76*(2), 265-276. https://doi.org/10.1002/jnr.20044

Oliveira, A., Illes, P., & Ulrich, H. (2016). Purinergic receptors in embryonic and adult neurogenesis. *Neuropharmacology*, *104*, 272-281. https://doi.org/10.1016/j.neuropharm.2015.10.008

Paniagua-Herranz, L., Menéndez-Méndez, A., Gómez-Villafuertes, R., Olivos-Oré, L. A., Biscaia, M., Gualix, J., Pérez-Sen, R., Delicado, E. G., Artalejo, A. R., Miras-Portugal, M. T., & Ortega, F. (2020). Live Imaging Reveals Cerebellar Neural Stem Cell Dynamics and the Role of VNUT in Lineage Progression. *Stem Cell Reports*, *15*(5), 1080-1094. https://doi.org/10.1016/j.stemcr.2020.09.007

Paniagua-Torija, B., Norenberg, M., Arevalo-Martin, A., Carballosa-Gautam, M. M., Campos-Martin, Y., Molina-Holgado, E., & Garcia-Ovejero, D. (2018). Cells in the adult human spinal cord ependymal region do not proliferate after injury. *J Pathol*, *246*(4), 415-421. https://doi.org/10.1002/path.5151

Paterniti, I., Melani, A., Cipriani, S., Corti, F., Mello, T., Mazzon, E., Esposito, E., Bramanti, P., Cuzzocrea, S., & Pedata, F. (2011). Selective adenosine A2A receptor agonists and antagonists protect against spinal cord injury through peripheral and central effects. *J Neuroinflammation*, *8*, 31. https://doi.org/10.1186/1742-2094-8-31

Peterson, T. S., Thebeau, C. N., Ajit, D., Camden, J. M., Woods, L. T., Wood, W. G., Petris, M. J., Sun, G. Y., Erb, L., & Weisman, G. A. (2013). Up-regulation and activation of the P2Y(2) nucleotide receptor mediate neurite extension in IL-1beta-treated mouse primary cortical neurons. *J Neurochem*, *125*(6), 885-896. https://doi.org/10.1111/jnc.12252

Pineau, I., & Lacroix, S. (2007). Proinflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression pattern and identification of the cell types involved. *J Comp Neurol*, *500*(2), 267-285. https://doi.org/10.1002/cne.21149

Quintas, C., Vale, N., Goncalves, J., & Queiroz, G. (2018). Microglia P2Y13 Receptors Prevent Astrocyte Proliferation Mediated by P2Y1 Receptors. *Front Pharmacol*, *9*, 418. https://doi.org/10.3389/fphar.2018.00418

Rampon, C., Gauron, C., Meda, F., Volovitch, M., & Vriz, S. (2014). Adenosine enhances progenitor cell recruitment and nerve growth via its A2B receptor during adult fin regeneration. *Purinergic Signal*, *10*(4), 595-602. https://doi.org/10.1007/s11302-014-9420-9

Recabal, A., Fernandez, P., Lopez, S., Barahona, M. J., Ordenes, P., Palma, A., Elizondo-Vega, R., Farkas, C., Uribe, A., Caprile, T., Saez, J. C., & Garcia-Robles, M. A. (2021). The FGF2-induced tanycyte proliferation involves a connexin 43 hemichannel/purinergic-dependent pathway. *J Neurochem*, *156*(2), 182-199. https://doi.org/10.1111/jnc.15188

Reigada, D., Navarro-Ruiz, R. M., Caballero-Lopez, M. J., Del Aguila, A., Munoz-Galdeano, T., Maza, R. M., & Nieto-Diaz, M. (2017). Diadenosine tetraphosphate (Ap4A) inhibits ATP-induced excitotoxicity: a neuroprotective strategy for traumatic spinal cord injury treatment. *Purinergic Signal*, *13*(1), 75-87. https://doi.org/10.1007/s11302-016-9541-4

Reimer, M. M., Sorensen, I., Kuscha, V., Frank, R. E., Liu, C., Becker, C. G., & Becker, T. (2008). Motor neuron regeneration in adult zebrafish. *J Neurosci*, *28*(34), 8510-8516. https://doi.org/10.1523/JNEUROSCI.1189-08.2008

Ren, Y., Ao, Y., O'Shea, T. M., Burda, J. E., Bernstein, A. M., Brumm, A. J., Muthusamy, N., Ghashghaei, H. T., Carmichael, S. T., Cheng, L., & Sofroniew, M. V. (2017). Ependymal cell contribution to scar formation after spinal cord injury is minimal, local and dependent on direct ependymal injury. *Sci Rep*, *7*, 41122. https://doi.org/10.1038/srep41122

Ribeiro, F. F., Xapelli, S., Miranda-Lourenco, C., Tanqueiro, S. R., Fonseca-Gomes, J., Diogenes, M. J., Ribeiro, J. A., & Sebastiao, A. M. (2016). Purine nucleosides in neuroregeneration and neuroprotection. *Neuropharmacology*, *104*, 226-242. https://doi.org/10.1016/j.neuropharm.2015.11.006

Rodriguez-Zayas, A. E., Torrado, A. I., & Miranda, J. D. (2010). P2Y2 receptor expression is altered in rats after spinal cord injury. *Int J Dev Neurosci*, *28*(6), 413-421. https://doi.org/10.1016/j.ijdevneu.2010.07.001

Rodriguez-Zayas, A. E., Torrado, A. I., Rosas, O. R., Santiago, J. M., Figueroa, J. D., & Miranda, J. D. (2012). Blockade of P2 nucleotide receptors after spinal cord injury reduced the gliotic response and spared tissue. *J Mol Neurosci*, *46*(1), 167-176. https://doi.org/10.1007/s12031-011-9567-6

Rump, A., Smolander, O. P., Ruutel Boudinot, S., Kanellopoulos, J. M., & Boudinot, P. (2020). Evolutionary Origin of the P2X7 C-ter Region: Capture of an Ancient Ballast Domain by a P2X4-Like Gene in Ancient Jawed Vertebrates. *Front Immunol*, *11*, 113. https://doi.org/10.3389/fimmu.2020.00113

Sabin, K. Z., Jiang, P., Gearhart, M. D., Stewart, R., & Echeverri, K. (2019). AP-1(cFos/JunB)/miR-200a regulate the pro-regenerative glial cell response during axolotl spinal cord regeneration. *Commun Biol*, *2*, 91. https://doi.org/10.1038/s42003-019-0335-4

Shihabuddin, L. S., Horner, P. J., Ray, J., & Gage, F. H. (2000). Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. *J Neurosci*, *20*(23), 8727-8735. https://www.ncbi.nlm.nih.gov/pubmed/11102479

Shinozaki, Y., Shibata, K., Yoshida, K., Shigetomi, E., Gachet, C., Ikenaka, K., Tanaka, K. F., & Koizumi, S. (2017). Transformation of Astrocytes to a Neuroprotective Phenotype by Microglia via P2Y1 Receptor Downregulation. *Cell Rep*, *19*(6), 1151-1164. https://doi.org/10.1016/j.celrep.2017.04.047

Shruster, A., Ben-Zur, T., Melamed, E., & Offen, D. (2012). Wnt signaling enhances neurogenesis and improves neurological function after focal ischemic injury. *PLoS One*, *7*(7), e40843. https://doi.org/10.1371/journal.pone.0040843

Sieger, D., Moritz, C., Ziegenhals, T., Prykhozhij, S., & Peri, F. (2012). Long-range Ca2+ waves transmit brain-damage signals to microglia. *Dev Cell*, *22*(6), 1138-1148. https://doi.org/10.1016/j.devcel.2012.04.012

Sirbulescu, R. F., Ilies, I., & Zupanc, G. K. (2009). Structural and functional regeneration after spinal cord injury in the weakly electric teleost fish, Apteronotus leptorhynchus. *J Comp*

Physiol A Neuroethol Sens Neural Behav Physiol, *195*(7), 699-714. https://doi.org/10.1007/s00359-009-0445-4

Stefani, J., Tschesnokowa, O., Parrilla, M., Robaye, B., Boeynaems, J. M., Acker-Palmer, A., Zimmermann, H., & Gampe, K. (2018). Disruption of the Microglial ADP Receptor P2Y13 Enhances Adult Hippocampal Neurogenesis. *Front Cell Neurosci*, *12*, 134. https://doi.org/10.3389/fncel.2018.00134

Stella, N., Estelles, A., Siciliano, J., Tence, M., Desagher, S., Piomelli, D., Glowinski, J., & Premont, J. (1997). Interleukin-1 enhances the ATP-evoked release of arachidonic acid from mouse astrocytes. *J Neurosci*, *17*(9), 2939-2946. https://www.ncbi.nlm.nih.gov/pubmed/9096130

Strand, N. S., Hoi, K. K., Phan, T. M. T., Ray, C. A., Berndt, J. D., & Moon, R. T. (2016). Wnt/beta-catenin signaling promotes regeneration after adult zebrafish spinal cord injury. *Biochem Biophys Res Commun*, *477*(4), 952-956. https://doi.org/10.1016/j.bbrc.2016.07.006

Su, W. F., Wu, F., Jin, Z. H., Gu, Y., Chen, Y. T., Fei, Y., Chen, H., Wang, Y. X., Xing, L. Y., Zhao, Y. Y., Yuan, Y., Tang, X., & Chen, G. (2019). Overexpression of P2X4 receptor in Schwann cells promotes motor and sensory functional recovery and remyelination via BDNF secretion after nerve injury. *Glia*, *67*(1), 78-90. https://doi.org/10.1002/glia.23527

Suadicani, S. O., Brosnan, C. F., & Scemes, E. (2006). P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca2+ signaling. *J Neurosci*, *26*(5), 1378-1385. https://doi.org/10.1523/JNEUROSCI.3902-05.2006

Takeda, A., Atobe, Y., Kadota, T., Goris, R. C., & Funakoshi, K. (2015). Axonal regeneration through the fibrous scar in lesioned goldfish spinal cord. *Neuroscience*, *284*, 134-152. https://doi.org/10.1016/j.neuroscience.2014.09.066

Takeda, A., Nakano, M., Goris, R. C., & Funakoshi, K. (2008). Adult neurogenesis with 5-HT expression in lesioned goldfish spinal cord. *Neuroscience*, *151*(4), 1132-1141. https://doi.org/10.1016/j.neuroscience.2007.10.059

Takeda, A., Okada, S., & Funakoshi, K. (2017). Chondroitin sulfates do not impede axonal regeneration in goldfish spinal cord. *Brain Res*, *1673*, 23-29. https://doi.org/10.1016/j.brainres.2017.08.004

Tsao, H. K., Chiu, P. H., & Sun, S. H. (2013). PKC-dependent ERK phosphorylation is essential for P2X7 receptor-mediated neuronal differentiation of neural progenitor cells. *Cell Death Dis*, *4*, e751. https://doi.org/10.1038/cddis.2013.274

Tsarouchas, T. M., Wehner, D., Cavone, L., Munir, T., Keatinge, M., Lambertus, M., Underhill, A., Barrett, T., Kassapis, E., Ogryzko, N., Feng, Y., van Ham, T. J., Becker, T., & Becker, C. G. (2018). Dynamic control of proinflammatory cytokines Il-1beta and Tnf-alpha by macrophages in zebrafish spinal cord regeneration. *Nat Commun*, *9*(1), 4670. https://doi.org/10.1038/s41467-018-07036-w

Tseng, A. S., Adams, D. S., Qiu, D., Koustubhan, P., & Levin, M. (2007). Apoptosis is required during early stages of tail regeneration in Xenopus laevis. *Dev Biol*, *301*(1), 62-69. https://doi.org/10.1016/j.ydbio.2006.10.048

Uda, Y., Xu, S., Matsumura, T., & Takei, Y. (2016). P2Y4 Nucleotide Receptor in Neuronal Precursors Induces Glutamatergic Subtype Markers in Their Descendant Neurons. *Stem Cell Reports*, *6*(4), 474-482. https://doi.org/10.1016/j.stemcr.2016.02.007

Vasudevan, D., Liu, Y. C., Barrios, J. P., Wheeler, M. K., Douglass, A. D., & Dorsky, R. I. (2021). Regenerated interneurons integrate into locomotor circuitry following spinal cord injury. *Exp Neurol*, *342*, 113737. https://doi.org/10.1016/j.expneurol.2021.113737

Vitalo, A. G., Sirbulescu, R. F., Ilies, I., & Zupanc, G. K. (2016). Absence of gliosis in a teleost model of spinal cord regeneration. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol*, *202*(6), 445-456. https://doi.org/10.1007/s00359-016-1089-9

Wang, J., Yang, J., Liu, P., Bi, X., Li, C., & Zhu, K. (2012). NAD induces astrocyte calcium flux and cell death by ART2 and P2X7 pathway. *Am J Pathol*, *181*(3), 746-752. https://doi.org/10.1016/j.ajpath.2012.05.019

Wang, X., Arcuino, G., Takano, T., Lin, J., Peng, W. G., Wan, P., Li, P., Xu, Q., Liu, Q. S., Goldman, S. A., & Nedergaard, M. (2004). P2X7 receptor inhibition improves recovery after spinal cord injury. *Nat Med*, *10*(8), 821-827. https://doi.org/10.1038/nm1082

Wanner, I. B., Anderson, M. A., Song, B., Levine, J., Fernandez, A., Gray-Thompson, Z., Ao, Y., & Sofroniew, M. V. (2013). Glial scar borders are formed by newly proliferated, elongated astrocytes that interact to corral inflammatory and fibrotic cells via STAT3-dependent mechanisms after spinal cord injury. *J Neurosci*, *33*(31), 12870-12886. https://doi.org/10.1523/JNEUROSCI.2121-13.2013

Wehner, D., Tsarouchas, T. M., Michael, A., Haase, C., Weidinger, G., Reimer, M. M., Becker, T., & Becker, C. G. (2017). Wnt signaling controls pro-regenerative Collagen XII in functional spinal cord regeneration in zebrafish. *Nat Commun*, *8*(1), 126. https://doi.org/10.1038/s41467-017-00143-0

Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A. C., & Reynolds, B. A. (1996). Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J Neurosci*, *16*(23), 7599-7609. https://www.ncbi.nlm.nih.gov/pubmed/8922416

Weissman, T. A., Riquelme, P. A., Ivic, L., Flint, A. C., & Kriegstein, A. R. (2004). Calcium waves propagate through radial glial cells and modulate proliferation in the developing neocortex. *Neuron*, *43*(5), 647-661. https://doi.org/10.1016/j.neuron.2004.08.015

Wu, D., Lee, S., Luo, J., Xia, H., Gushchina, S., Richardson, P. M., Yeh, J., Krugel, U., Franke, H., Zhang, Y., & Bo, X. (2018). Intraneural Injection of ATP Stimulates Regeneration of Primary Sensory Axons in the Spinal Cord. *J Neurosci*, *38*(6), 1351-1365. https://doi.org/10.1523/JNEUROSCI.1660-17.2017

Xing, L., Anbarchian, T., Tsai, J. M., Plant, G. W., & Nusse, R. (2018). Wnt/beta-catenin signaling regulates ependymal cell development and adult homeostasis. *Proc Natl Acad Sci U S A*, *115*(26), E5954-E5962. https://doi.org/10.1073/pnas.1803297115

Xu, F., Yang, J., Lu, F., Liu, R., Zheng, J., Zhang, J., Cui, W., Wang, C., Zhou, W., Wang, Q., Chen, X., & Chen, J. (2018). Fast Green FCF Alleviates Pain Hypersensitivity and Down-Regulates the Levels of Spinal P2X4 Expression and Pro-inflammatory Cytokines in a Rodent Inflammatory Pain Model. *Front Pharmacol*, *9*, 534. https://doi.org/10.3389/fphar.2018.00534

Xu, J., Cheng, S., Jiao, Z., Zhao, Z., Cai, Z., Su, N., Liu, B., Zhou, Z., & Li, Y. (2019). Fire Needle Acupuncture Regulates Wnt/ERK Multiple Pathways to Promote Neural Stem Cells to Differentiate into Neurons in Rats with Spinal Cord Injury. *CNS Neurol Disord Drug Targets*, *18*(3), 245-255. https://doi.org/10.2174/1871527318666190204111701

Zhu, Y., Soderblom, C., Krishnan, V., Ashbaugh, J., Bethea, J. R., & Lee, J. K. (2015). Hematogenous macrophage depletion reduces the fibrotic scar and increases axonal

growth after spinal cord injury. *Neurobiol Dis*, *74*, 114-125. https://doi.org/10.1016/j.nbd.2014.10.024

Zukor, K. A., Kent, D. T., & Odelberg, S. J. (2011). Meningeal cells and glia establish a permissive environment for axon regeneration after spinal cord injury in newts. *Neural Dev*, *6*, 1. https://doi.org/10.1186/1749-8104-6-1

Zupanc, G. K. H. (2019). Stem-Cell-Driven Growth and Regrowth of the Adult Spinal Cord in Teleost Fish. *Dev Neurobiol*, *79*(5), 406-423. https://doi.org/10.1002/dneu.22672

Hypothesis: Purinergic signaling through the $P2X_7$ receptor promotes proliferation and neuronal differentiation of radial glia following spinal cord injury in adult zebrafish

Objectives:

1. Describe cellular expression of $P2X_7$ receptors within the adult zebrafish spinal cord
2. Characterize temporal changes in $P2X_7$ protein expression following spinal cord injury in adult zebrafish
3. Determine the functional effects of $P2X_7$ receptor activation or inhibition on radial glial cell proliferation and neuronal differentiation following spinal cord injury in adult zebrafish

Chapter 2

2.1 Abstract

In contrast to mammals, adult zebrafish undergo successful neural regeneration following spinal cord injury (SCI). Radial glia (RG) lining the zebrafish central canal undergo injury-induced proliferation and subsequent neuronal differentiation to replace damaged cells and restore motor function. However, the molecular mechanisms that underlie these processes remain elusive. Here, we demonstrate that signaling through the evolutionarily conserved purinergic $P2X_7$ receptor is involved. Within the zebrafish spinal cord, $P2X_7$ receptors have widespread distribution with specific localization to neurons and radial glia. At the protein level, the predominant $P2X_7$ receptor isoforms in zebrafish did not include the full-length variant expressed throughout the murine central nervous system, but two truncated splice variants. In response to SCI, protein expression of the 50 kDa isoform became downregulated at 7 dpi and returned to basal levels of expression at 14 and 21 dpi when compared to naïve controls. Meanwhile, expression of the 37 kDa isoform did not change following injury. Pharmacological activation of $P2X_7$ following SCI resulted in a greater number of proliferating cells around the central canal by 7 dpi, while $P2X_7$ inhibition appeared to have no effect. At 14 dpi, these treatments did not have a significant effect on the number of neurons within the injured spinal cord. This data indicates that $P2X_7$ receptor activation is sufficient to induce cellular proliferation, but not a necessary mediator of either proliferation or neurogenesis following SCI in adult zebrafish. Our findings suggest that unlike in humans, $P2X_7$ signaling may not play a maladaptive role following SCI in adult zebrafish.

2.2 Introduction

Spinal cord injury (SCI), characterized by irreversible sensory, motor, and autonomic dysfunction, is an ever-growing global health burden (Ding et al., 2022). Per year, an estimated 3, 675 Canadians and 900, 000 individuals across the world incur a SCI, significantly adding to the 86, 000 Canadian and 20, 600, 000 global cases currently documented (Ding et al., 2022; Noonan et al., 2012). Although substantial progress over the past several decades has been made in elucidating the pathophysiology of this condition, treatment options remain extremely limited (Krueger et al., 2013; Lynch & Cahalan, 2017). This is due to the multifactorial injury response within the central nervous system (CNS) that results in secondary cell death, neuroinflammation, reactive gliosis, and axonal degeneration. Understanding the underlying mechanisms responsible for the maladaptive cellular responses are key to developing therapeutic interventions that limit damage and promote regeneration.

In contrast to mammals, several non-mammalian vertebrate species, such as Osteichthyes (teleost fish), demonstrate a high degree of regenerative ability following injury to the CNS. Indeed, zebrafish (*Danio rerio*) undergo injury-induced CNS neurogenesis following SCI and achieve functional recovery within 6-8 weeks (Becker et al., 1997; Hui et al., 2010; Reimer et al., 2008). This adaptive process is largely driven by radial glia (RG) lining the central canal of the spinal cord. Although the functions of RG within the adult zebrafish spinal cord are not fully understood, in response to injury, these cells undergo a massive proliferative response and can differentiate into distinct neuronal types that are integrated into spinal circuitry (Reimer et al., 2008). While a few signaling pathways, including, but not limited to, sonic hedgehog, dopamine, serotonin, and wingless-related integration site/ß-catenin, have been implicated in regulating this injury response, signals necessary for regulating adult injury-induced neurogenesis have yet to be

fully elucidated (Barreiro-Iglesias et al., 2015; Briona et al., 2015; Reimer et al., 2009; Reimer et al., 2013). Thus, identifying the cellular and molecular signals employed by these species to restore neuron populations and locomotor function following injury may be integral to the development of neurorestorative treatments for mammals.

One evolutionarily conserved signaling pathway that has been extensively explored following SCI in mammals, but not in zebrafish, is the purinergic system. Nucleoside triphosphates and their metabolites regulate a plethora of cellular processes, including proliferation and neurogenesis, within the CNS during development and in adulthood (Burnstock & Ulrich, 2011; Gomez-Villafuertes, 2016; Oliveira et al., 2016; Ribeiro et al., 2016). These ligands have extensive receptor families, including: metabotropic adenosine receptors, A_1, A_{2A}, A_{2B}, A_3; ionotropic nucleotide receptors, $P2X_{1–7}$; and metabotropic nucleotide receptors, $P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$ (Burnstock, 2018). In the context of CNS regeneration, $P2X_7$ receptors are of particular interest. These receptors function as non-selective cation channels activated by high concentrations of extracellular adenosine 5′-triphosphate (ATP). When ATP stimulation is sustained, as in the first few hours following mammalian SCI, certain splice variants of the $P2X_7$ receptor form a reversible, but cytotoxic, ‘macropore’ (Andrejew et al., 2020; De Salis et al., 2022; Di Virgilio et al., 2018; Wang et al., 2004). Other $P2X_7$ splice variants have been shown to mediate proliferation and neuronal differentiation of embryonic and adult neural progenitor cells in mammals (Glaser et al., 2014; Leeson et al., 2019; Leeson et al., 2018; Tsao et al., 2013). Remarkably, while the $P2X_7$ gene is present across all Metazoa, its specific properties and functions appear to differ across vertebrates (Benzaquen et al., 2019; Liang et al., 2015; Rump et al., 2020). For instance, $P2X_7$ receptor stimulation mediates interleukin-1beta (IL-1 β) secretion in rodents, but it does not do so in zebrafish (López-Castejón et al., 2007). Given the varied, yet

significant role of this receptor following mammalian SCI, in this study we focused on the $P2X_7$ receptor and aimed to uncover its potential role in injury-induced RG cell proliferation and neuronal regeneration following SCI in adult zebrafish.

Here, we found that within the adult zebrafish spinal cord, $P2X_7$ receptors are expressed by neurons and radial glia. Strikingly, we failed to detect protein expression of the full length $P2X_7$ receptor, suggesting that the predominant $P2X_7$ receptor isoforms expressed in zebrafish may differ from those expressed in murine species. Treatment with $P2X_7$ agonist, BzATP, significantly increased $PCNA^+$ expression around the central canal at 7 dpi, while $P2X_7$ antagonist JNJ 47965567 had no detectable effect at the same time point. This suggested that $P2X_7$ receptor activation may be sufficient to mediate RG cell proliferation following injury, but not necessary. Meanwhile, treatment with $P2X_7$ agonist or antagonist had no effect on HuC/D^+ expression at 14 dpi, suggesting that $P2X_7$ receptor activation does not play a critical role in neurogenesis. Together, our results suggest that while $P2X_7$ signaling is involved in the zebrafish SCI response, it does not appear to play a major role in mediating injury-induced RG cell activity.

2.3 Materials and Methods

2.3.1 Animals

Zebrafish (*Danio rerio*) aged 3-8 months from the Tupfel Long-Fin (TL) strain were used for all experiments. Male and female fish were kept and bred in a zebrafish facility located within the Life Sciences Centre at McMaster University. All experiments were approved by the McMaster Animal Research Ethics Board (Animal Utilization Protocol 19-08-22).

2.3.2 Spinal Cord Lesion

Prior to any surgical procedure, individual fish were acclimated to an environmental chamber maintained at 28°C in a 14-hour light cycle and fed dry food twice daily for 48 hours. As previously described (Becker et al., 1997), adult fish were anesthetized by immersion in 0.02% aminobenzoic acid ethyl methyl ester (MS222; Sigma-Aldrich, St. Louis, Missouri) in sterile water for ~1 min. The anesthetized fish were then placed on a pre-wet agar plate under a dissection microscope, and a small vertical incision was made at the level of the thoracic spinal cord. The muscle layers were then blunt dissected and carefully separated with forceps, the vertebral column was exposed, and the spinal cord underwent full transection with visual confirmation of completeness. 1 μL of Vetbond tissue adhesive gel was quickly placed on the external incision. Fish were transferred to a new tank with sterile water and recovered in an environmentally controlled chamber again maintained at 28°C and set to a 14-hour light cycle. Fish were housed individually and monitored twice daily to assess physical appearance (eye condition, fin and skin condition, mucus production, spinal deformities, abdomen size), clinical signs (changes in food consumption, respiratory rate, posture in water), and behaviour (hypo or hyperactivity). Fish were euthanized if they displayed grossly abnormal physical appearance,

clinical signs, or behaviour. Fish were not fed for 24 hours post anesthesia, but regular feeding twice daily commenced afterwards and continued until the end of the experiment. Water was changed and assessed for quality daily.

2.3.3 Drug Administration

At distinct intervals, adult zebrafish were treated with various compounds via intraperitoneal injections as described by Barreiro-Iglesias and colleagues (2015). Briefly, adult zebrafish were anesthetized by immersion in 0.02% MS222, removed, and placed on a pre-wet sponge under a dissection scope. The fish then underwent a single intraperitoneal injection of 20 µL saline containing one of the following dissolved compounds: 2'(3')-O-(4-Benzoylbenzoyl) adenosine-5'-triphosphate tri(triethylammonium) salt (BzATP; 100 µM, Abbexa, Cambridge, United Kingdom, Cat# abx076752) or JNJ 47965567 (200 µM, Tocris Bioscience, Bristol, United Kingdom, Cat# 5299). Vehicle injections of saline alone served as controls. Fish in experiments completed at 7 days received an injection at 3 and 6 days post injury (dpi). Fish in experiments completed at 14 days received an injection at 3, 6, 9, and 12 dpi.

2.3.4 Immunohistochemical Procedures

To localize expression of select proteins, adult zebrafish spinal cord tissue was processed for histology. Zebrafish were euthanized by immersion overdose in 1% MS222 in water for 5 minutes and immediately transcardially perfused with 200 µL saline followed by 3000 µL 4% paraformaldehyde (PFA; Sigma-Aldrich, Cat# 158127) dissolved in 0.01 M phosphate buffered saline (PBS). The vertebral column was then removed and placed in 4% PFA overnight before being transferred to a 20% sucrose solution (in 0.01 M PBS) for another 12-24 hours. Following fixation and cryoprotection, the vertebral column was dissected to expose the injured spinal cord,

the injury site was identified, and each rostral and caudal segment was isolated. From each rostral and caudal spinal cord, a section of 600 µm from the injury site was embedded in Tissue Plus O.C.T. Compound Clear (Thermo Fisher Scientific, Waltham, Massachusetts, Cat# SGN4585) and stored in a 7 x 7 mm base bold (Ted Pella, Inc., Redding, California, Cat# 27147-1) that was flash frozen in liquid nitrogen and stored at -80°C. To prepare the sections for cryosectioning, the spinal cord blocks were warmed to -20°C and mounted on a sectioning stage. Frozen spinal cord sections (10 µm thick) were collected on gelatin coated slides using a Leica CM1900 cryostat. Gelatin coated slides were used to minimize detachment of sections from slides. They were pre-made by dipping frosted slides in a 40-50°C solution of 0.5% gelatin 300 bloom and 0.05% chromium potassium sulphate for 1 minute.

For all immunohistological markers used in this study, each set of tissue sections underwent a specific protocol for antigen retrieval prior to immunodetection. For anti-PCNA, GFAP, NeuN, and $P2X_7$ detection, slides were incubated in 10 mM sodium citrate and 0.05% Tween-20 (pH 6) overnight at 60°C. For detection of anti-HuC/D, slides were incubated in 50 mM Tris (pH 8) for 30 minutes at 85°C. Following antigen retrieval, all samples were washed in 0.01 M PBS twice for 10 min and then incubated in 1% BSA (Sigma-Aldrich) and 0.1% Triton X-100 in 0.01 M PBS for 60 min at room temperature (RT) in a humidity chamber. The blocking agent was then removed and replaced with one of the following primary antibodies diluted in 0.1% Triton X-100/0.01 M PBS: PCNA (mouse monoclonal, 1:1000, Abcam, Cambridge, United Kingdom, Cat# ab29, RRID# AB_303394), GFAP (chicken polyclonal, 1:2000, Thermo Fisher Scientific, Cat# PA1-10004, RRID# AB_1074620), NeuN (mouse monoclonal, 1:500, Abcam, Cat# ab104224, RRID# AB_10711040), HuC/D (mouse monoclonal, 5µg/mL, Thermo Fisher Scientific, Cat#A-21271, RRID# AB_221448), or $P2X_7$ (rabbit polyclonal; 1:500,

Alomone Labs, Cat# APR-004, RRID# AB_2040068). Samples were then stored overnight at 4°C. The next day, samples were washed in 0.01 M PBS three times for 15 minutes. The slides were then incubated with one of the following secondary antibody dilutions in 0.1% Triton X-100/0.01 M PBS for 2 hours at RT: donkey anti-rabbit AlexaFluor 568 (1:500, Invitrogen, Waltham, Massachusetts, Cat# A10042, RRID# AB_2534017), fluorescein donkey anti-chicken (FITC; 1:200, Jackson ImmunoResearch, Cat# 703-095-155, RRID# AB_2340356), or fluorescein goat anti-mouse (FITC; 1:100, Jackson ImmunoResearch, Cat# 115-095-166, RRID# AB_2338601). Samples were washed again in 0.01 M PBS three times for 15 minutes. Coverslips were mounted onto slides using 2-3 drops of ProLong Gold antifade reagent with DAPI nuclear stain (Invitrogen, Cat# P36935) and stored at 4°C protected from light exposure. All spinal cord cross sections were imaged at 20X objective magnification using an automated stage on a Zeiss Axio Imager M2 Fluorescent microscope (Carl Zeiss, Oberkochen, Germany), Axiocam 506 camera (Carl Zeiss), and ZEN Blue (Carl Zeiss) acquisition software.

For assessment of $P2X_7^+$ colocalization with various cell types, the sample size was 3 fish per group. Meanwhile, for cell quantification experiments, the sample size was 6-9 fish per treatment group. For all immunohistochemical analysis, 8-10 sections encompassing either 600 µm naïve spinal cord or 600 µm rostral and 600 µm caudal to the lesion site were analyzed per fish (n). Quantification of cells detected in spinal cord transection images was completed using ImageJ with filters for background subtraction using settings for a rolling ball radius of 75 pixels. Manual counts of PCNA+ cells within a 30µm radius of the central canal were completed for each section of tissue. Automated counts for HuC/D^+ cells within the entire cross-section were completed for each section of tissue. HuC/D^+ and $DAPI^+$ channels were made into binary images which were then overlayed. The overlayed image was put through an automated cell counter and

set to exclude any particles smaller than 20 pixels. All cell counts and data analysis were completed by an individual who was blinded to the experimental groups.

2.3.5 Protein Assays and Quantification

Protein samples were prepared from 2 mm sections of adult zebrafish thoracic spinal cord tissue isolated from the following: (1) thoracic spinal cord tissue from naïve fish, (2) tissue within the injury site (3) tissue adjacent to the rostral side of the injury site, and (4) tissue adjacent to the caudal side of the injury site. Tissue samples from each location in injured zebrafish were collected at either 7, 14, or 21 dpi. Sample size ranged from 4-6 adult zebrafish for every time point.

All samples were flash frozen in liquid nitrogen and stored at -80°C before being mechanically homogenized in 20 µL of 1X Brain Extraction Buffer (25 mM HEPES pH 7.3, 150 mM KCL, 8% glycerol, 0.1% NP-40, Roche ULTRA protease inhibitor, and Roche PhoSTOP phosphatase inhibitor) using a Teflon pestle for several minutes (Reynolds et al., 2021). The homogenate was chilled on ice for 15 minutes, prior to centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was removed and transferred to a new microtube. Samples were chilled on ice until use in the DC protein assay (BioRad, Mississauga, Ontario) which was used to standardize protein content used for Western blotting. A standard curve consisting of serial dilutions of bovine serum albumin (BSA; Sigma-Aldrich, Cat# A-7906) was prepared. The standards and samples were prepared per manufacturers instructions (Bio-Rad). Plates were gently shaken for 15 min prior to spectrophotometer (xMark Microplate Absorbance Spectrophotometer, Bio-Rad) readings taken at 750 nm. Samples were flash frozen in liquid nitrogen and returned to storage at -80°C.

Protein samples (10 μg) were loaded on precast polyacrylamide TGX Stain-Free, 4-12% gradient, SDS gels (BioRad) and were separated electrophoretically at 125 V for 45-50 minutes. Each loaded protein sample corresponded to a single fish. Protein extraction from adult mouse hippocampal tissue was loaded as a positive control, while protein extraction from adult naïve zebrafish whole brain tissue was loaded as a cross-gel control. Following electrophoresis, gels were activated by UV exposure for 45 seconds to permit the visualization of total protein loaded within each lane. Proteins were transferred onto polyvinylidenedifluoride (PVDF; BioRad) membranes using the TransBlot Turbo system using the settings 'turbo' > '1 mini-gel' (BioRad). Membranes were then imaged for total protein prior to incubation in 10 mM sodium citrate and 0.05% Tween-20 (pH 6) overnight at 60°C. The next day, membranes were incubated in 5% BSA (Sigma-Aldrich) in 1X Tris-buffered saline solution with Tween-20 for 1 hour and then gently rotated for 3 hours at RT in primary antibody dilution. This included anti-$P2X_7$ (rabbit polyclonal; 1:500, Alomone Labs, Jerusalem, Israel, Cat# APR-004, RRID# AB_2040068). A negative control for the antibody was performed using a blocking peptide specific for the antibody binding site. This included a $P2X_7$ control antigen (Alomone Labs, Cat# BLP-PR004). Membranes were pre-incubated with the blocking peptide overnight at 4°C prior to antibody exposure. Membranes were then incubated in secondary antibody (donkey anti-rabbit horseradish peroxidase, 1:2500, Cytiva Lifescience, Marlborough, Massachusetts, Cat# NA934VS, RRID# AB_772206) for 2 hours, developed in enhanced chemiluminescence (BioRad) substrate for 5 minutes, and imaged using a ChemiDoc system (BioRad). ImageLab 6.1 software (BioRad) was used to quantify the relative densitometry of bands of interest and total protein. Each protein of interest was normalized to the loading control and cross-gel control lanes to account for variation of membrane transfers. Results were expressed as fold change

relative to the naïve control. Error bars are representative of standard deviation (SD). Statistical significance between groups was determined using one-way ANOVA and post-hoc Dunnett test for multiple comparisons.

2.4 Results

2.4.1 $P2X_7$ is expressed by radial glia and neurons within the adult zebrafish spinal cord

In addition to mediating various homeostatic functions, RG are essential for neural regeneration following CNS injury in zebrafish (Briona & Dorsky, 2014). The cell bodies of RG line the central canal of the spinal cord, while their processes extend out to the pial surface. Immunohistological analyses showed that $P2X_7$ colocalizes with glial fibrillary acidic protein (GFAP); a marker for radial glia (Lyons et al., 2003), in the thoracic spinal cord of naïve and injured (7 and 14 dpi) fish (Figure 1A-F). Within the naïve cord, colocalization of $P2X_7^+$ and $GFAP^+$ was primarily detected in cellular processes of RG as opposed to cell bodies (Figure 1B). However, within the spinal cord at 7 and 14 dpi cord, colocalization of $P2X_7^+$ and $GFAP^+$ was detected in both cell bodies and cellular processes (Figure 1D, F).

We next examined expression of $P2X_7$ on neurons within the zebrafish spinal cord following similar conditions. We found strong colocalization of $P2X_7$ with $NeuN^+$ cells, demonstrating $P2X_7$ expression on postmitotic neurons (mature and immature stages, but out of the cell cycle) (Wolf et al., 1996) (Figure 2A-F). In the naïve spinal cord, colocalization of $P2X_7^+$ and $NeuN^+$ was detected in primarily large neurons (Figure 2B). Following 7 and 14 dpi, colocalization of $P2X_7^+$ and $NeuN^+$ was detected in both large neurons and small, newly generated neurons (Figure 2D, F). It is important to note that not all $NeuN^+$ cells colocalized with $P2X_7^+$ (Figure 2B, D, F).

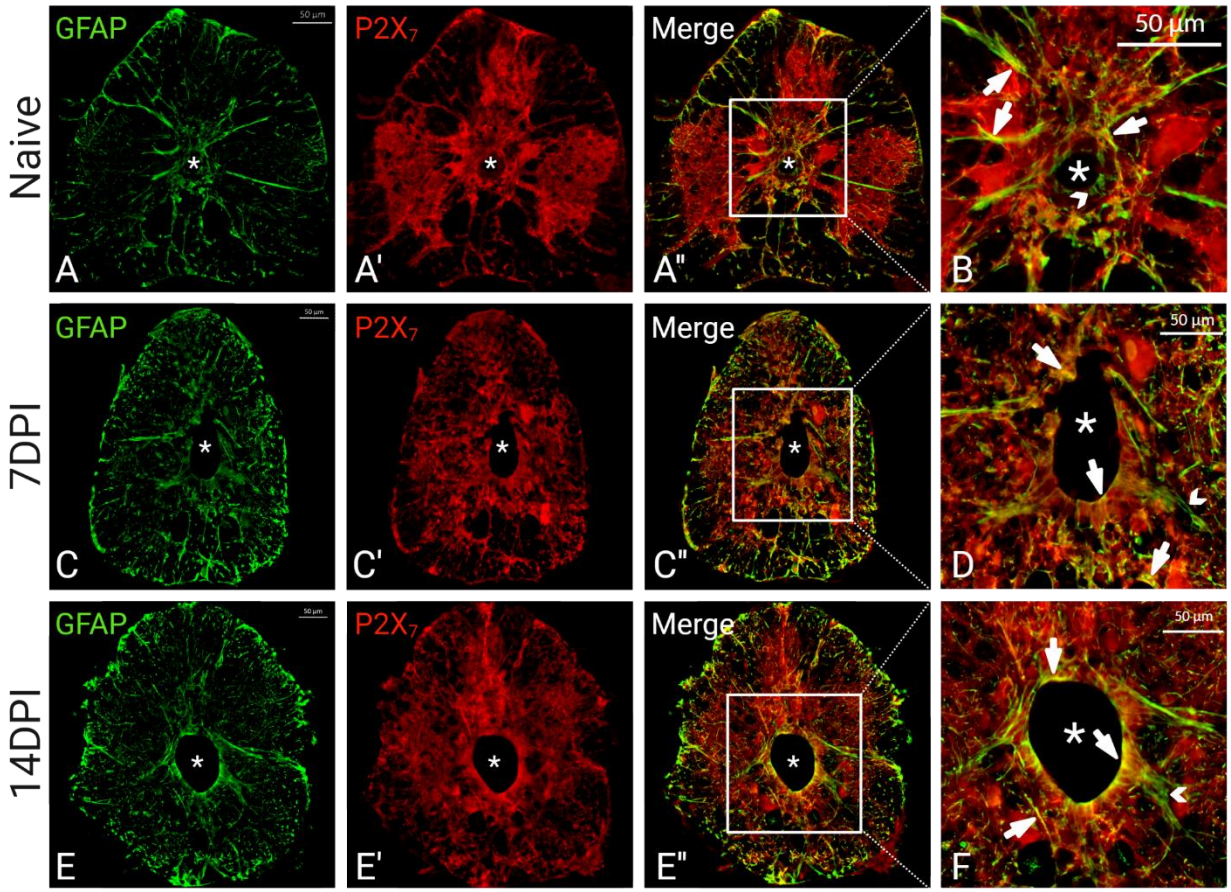

Figure 1. P2X$_7$ receptors are expressed by GFAP$^+$ radial glial cells. Spinal cord cross sections are shown (dorsal is up; asterisk indicates central canal). Representative images of GFAP and P2X$_7$ labelling in naïve fish (A-B), at 7 dpi (C-D), and at 14 dpi (E-F). Colocalization (arrows) is seen in radial processes and cell bodies (B, D, F). Areas where colocalization is not observed have also been denoted (open arrowheads) (B, D, F). n=3/group.

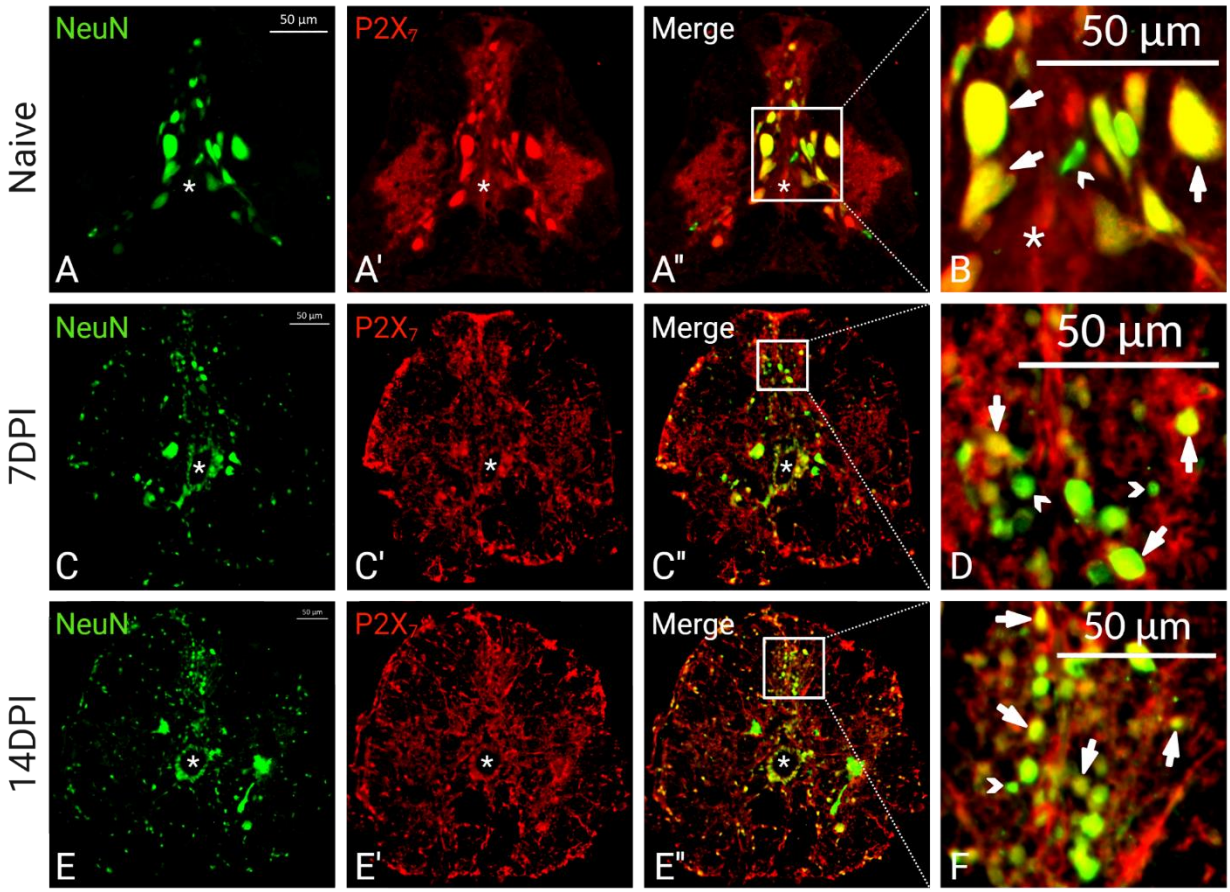

Figure 2. $P2X_7$ receptors are expressed by $NeuN^+$ neurons. Spinal cord cross sections are shown (dorsal is up; asterisk indicates central canal). Representative images of NeuN and $P2X_7$ labelling in naïve fish (A-B), at 7 dpi (C-D), and at 14 dpi (E-F). Colocalization (arrows) is seen in large neuronal cell bodies (A'', B, C'', E'') and in smaller, newly generated neurons (D, F). Neurons that do not colocalize with $P2X_7$ have also been denoted (open arrowheads) (B, D, F). n=3/group.

2.4.2 Differential expression of $P2X_7$ following spinal cord injury in adult zebrafish

In response to mammalian SCI, protein expression of the $P2X_7$ receptor becomes significantly upregulated by 3-7 dpi and remains elevated until at least 30 days (Fan et al., 2020; Gomez-Villafuertes et al., 2015; Kobayashi et al., 2011). To assess temporal changes in $P2X_7$ protein expression following zebrafish SCI, we performed western blots on spinal cord tissue collected from naïve animals and during various time periods of recovery from individuals that sustained spinal cord transection. Injured tissue was collected at: 7 dpi during peak RG cell proliferation; 14 dpi during peak neurogenesis; and 21 dpi during the start of axonal regeneration (Barreiro-Iglesias et al., 2015; Hui et al., 2015; Reimer et al., 2008; Tsarouchas et al., 2018).

Since mammals and zebrafish have highly conserved genes encoding the 'ballast domain', otherwise known as the extended C-terminal tail of the $P2X_7$ receptor, we probed the zebrafish spinal cord with a $P2X_7$ antibody targeting the C-terminus (McCarthy et al., 2019; Rump et al., 2020). This antibody typically detects the full-length 68-85kDa mammalian $P2X_7$ receptor. We demonstrated antibody specificity for $P2X_7$ using mouse hippocampal tissue as a positive control and pre-absorption of a blocking peptide as a negative control (Carvalho et al., 2021; Leeson et al., 2018) (Figure 3A). In zebrafish samples, we identified immunoreactive bands in the 50 kDa and 37 kDa regions; meanwhile, those in the 68-85 kDa region were either completely absent or demonstrated limited staining in just a few individuals. The molecular weights of the proteins we detected correspond to recognized $P2X_7$ splice variants in mammals, namely $P2X_7B$ and $P2X_7J$, that are reported to lack the C-terminus (Cheewatrakoolpong et al., 2005). This suggests that our antibody detected most $P2X_7$ isoforms rather than a select few.

Protein expression of the 50 kDa isoform was consistent with naïve levels at 14 dpi (naïve, n=6; Ro, n=6; In, n=6; Ca, n=6; p= 0.9425) (Figure 3D-D') and at 21 dpi (naïve, n=6; Ro,

n=6; In, n=6; Ca, n=6; p= 0.1351) (Figure 3E-E'). However, protein expression of this isoform was significantly downregulated within the injury site relative to naïve controls at 7 dpi (naïve, n=6; Ro, n=4; In, n=4; Ca, n=4; p = 0.0468) (Figure 3C-C'). Meanwhile, expression of the 37 kDa isoform did not differ at 7, 14, or 21 dpi when compared to naïve controls (Figure 3C, C'', D, D'', E, E'').

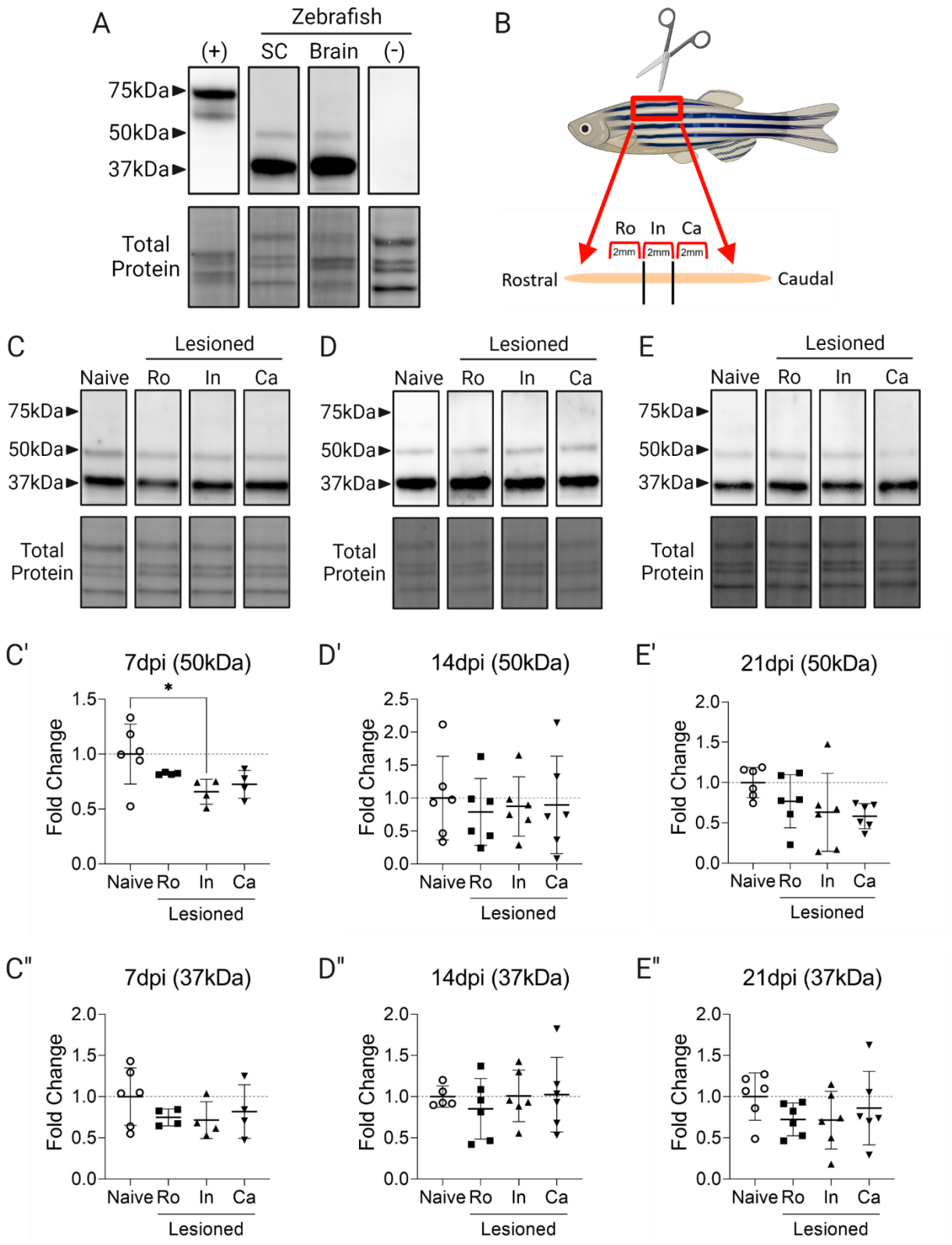
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Figure 3. Temporal changes in $P2X_7$ receptor protein expression following SCI in adult zebrafish. (A) Representative Western blot and corresponding total protein in mouse hippocampal tissue as a positive control (+), naïve zebrafish spinal cord (SC) and brain tissue, and pre-absorption of a blocking peptide as a negative control (-). (B) Schematic representation of lesioned tissue. Representative Western blot and corresponding total protein at 7 dpi (C), 14 dpi (D), and 21 dpi (E). Quantitative analysis of the 50 kDa isoform showed significant downregulation within the injury (In) site at 7 dpi (n=4, one-way ANOVA with Dunnett's post hoc test, $p = 0.0468$) (C') when normalized to naïve tissue (n=6). Protein expression of the 50 kDa isoform returned to basal levels at 14 dpi (D') and 21 dpi (E'). Quantitative analysis of the 37 kDa isoform showed consistent expression at 7 dpi (C''), 14 dpi (D''), and 21 dpi (E'') when normalized to naïve tissue. Data presented as means ± SD. *, significant differences between injury (In) and naïve groups, $p<0.05$.

2.4.3 $P2X_7$ agonism increases injury-induced radial glial cell proliferation around the central canal

During spontaneous regeneration, RG undergo a massive proliferative response at 7 dpi (Hui et al., 2015). To quantify proliferation, we analyzed the number of proliferating cell nuclear antigen ($PCNA^+$) positive cells within the injured spinal cord at 7 dpi (Bologna-Molina et al., 2013). Consistent with previous work by Barreiro and colleagues (2015), we detected a significant increase in the number of $PCNA^+$ cells around the central canal at 7 dpi when compared to naïve controls (Figure 4D). Furthermore, we found that $P2X_7$ receptors are expressed by $PCNA^+$ cells in the naïve zebrafish spinal cord (Figure 4A-A'') and at 7 dpi both rostral (Figure 4B-B'') and caudal (Figure 4C-C'') to the lesion.

To directly test the involvement of $P2X_7$ signaling in mediating RG cell proliferation, we administered intraperitoneal injections of $P2X_7$ agonist BzATP or $P2X_7$ antagonist JNJ 47965567 at 3 and 6 dpi, and then quantified proliferation around the central canal at 7 dpi. Vehicle injections delivered in the same time intervals served as controls. We used a BzATP concentration of 100 μM because this concentration has previously been shown to activate $P2X_7$ receptors in larval zebrafish *in vivo* (Chang et al., 2011). We used a JNJ concentration of 200 μM based on *in vivo* studies in murine species (Bhattacharya et al., 2013; Ly et al., 2020). Both treatments were well tolerated by adult zebrafish with no adverse effects.

Quantification of $PCNA^+$ cells around the central canal indicated that the number of proliferating cells decreased as distance from the lesion increased (Figure 5E, J). When comparing vehicle to BzATP treated fish, we noted that BzATP stimulated cell proliferation around the central canal within the first 400 μm rostral to the lesion (Figure 5E) and the first 300 μm caudal to the lesion (Figure 5J) but had relatively no effect in the distal spinal cord. Thus, for

each sample (n), we limited our counts to spinal sections within the first 400 μm rostral and caudal to the injury site and compared the values between treatment groups. When comparing vehicle to BzATP treated fish, we found a significant increase in $PCNA^+$ cells around the central canal both rostral (Saline, n=8; BzATP, n=8; p=0.0012; Figure 5A, B, D) and caudal (Saline, n=10; BzATP, n=8; p=0.0436; Figure 5F, G, I) to the lesion. Meanwhile, JNJ injections had no effect on proliferative events rostral (JNJ, n=6; p=0.4695; Figure 5C, D) or caudal (JNJ, n=6; p=0.8876; Figure 5H, I) to the lesion.

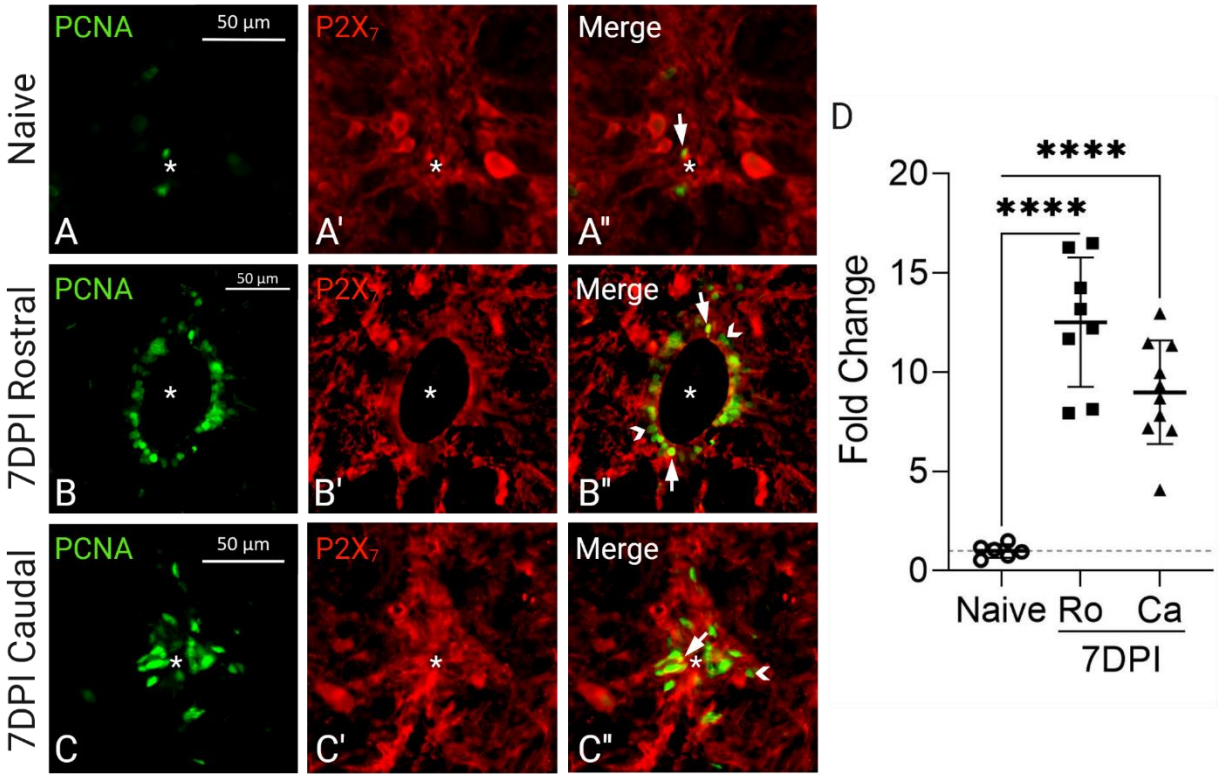


Figure 4. P2X$_7$ receptors are expressed by PCNA$^+$ cells around the central canal. Spinal cord cross sections are shown (dorsal is up; asterisk indicates central canal). Representative images of PCNA and P2X$_7$ labelling in naïve fish (A-A'') and within the 7 dpi rostral (B-B'') and caudal (C-C'') spinal cord. n=3/group. Colocalization of PCNA$^+$ and P2X$_7^+$ is seen in some cells lining the central canal (arrows) but not in others (open arrowhead) (A'', B'', C''). When normalized to naïve controls (n=6), PCNA$^+$ expression around the central canal became significantly upregulated both rostral (n=8, one-way ANOVA with Dunnett's post hoc test, $p<0.0001$) and caudal (n=10, $p<0.0001$) to the lesion (D). Data presented as means ± SD. ****, significant differences between rostral (Ro) and caudal (Ca) to naïve groups, $p < .0001$.

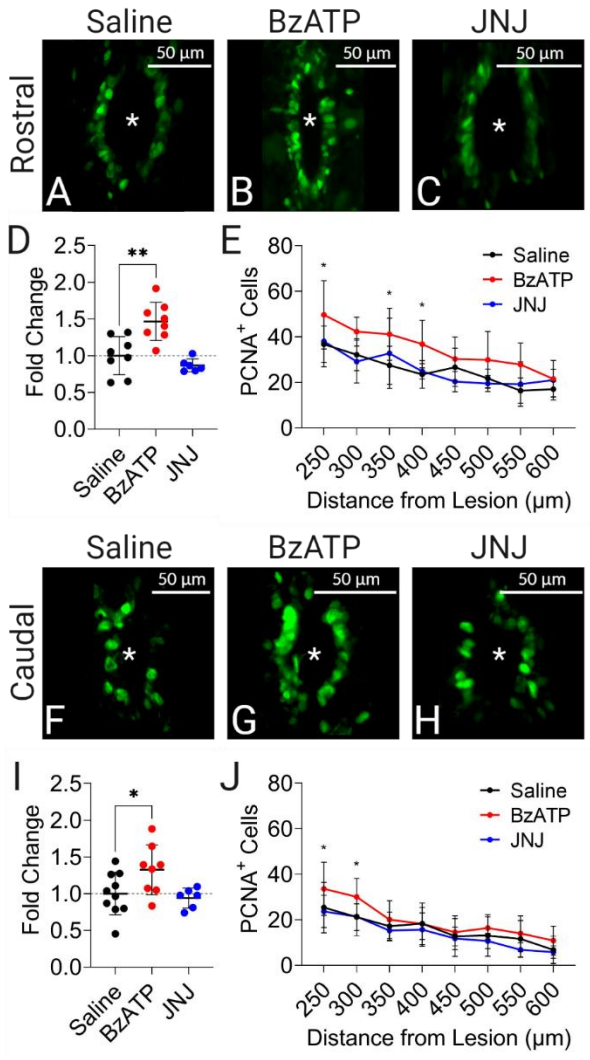


Figure 5. Treatment with BzATP increases PCNA$^+$ expression around the central canal. Spinal cord cross sections are shown (dorsal is up; asterisk indicates central canal). Representative images of PCNA$^+$ expression following treatment with saline (A, F), BzATP (B, G), and JNJ (C, H). Intraperitoneal injections of 100 µM BzATP at 3 and 6 dpi significantly increased the number of PCNA$^+$ cells around the central canal at 250 µm, 350 µm, and at 400 µm rostral to the lesion (E, two-way ANOVA with Dunnett's post hoc test) and at 250 µm and 300 µm caudal to the lesion (J) when compared to intraperitoneal injections of saline alone. Analysis of summative cell counts indicate that BzATP significantly increased PCNA$^+$ expression both rostral (D, saline, n=8; BzATP, n=8; one-way ANOVA with Dunnett's post hoc test, p=0.0012) and caudal (I, saline, n=10; BzATP, n=8; p=0.0436) to the lesion when compared to saline. JNJ had no effect on proliferative events both rostral (D, JNJ, n=6; p=0.4695) and caudal (I, JNJ, n=6; p=0.8876) to the lesion when compared to saline. Data presented as means ± SD. *, significant differences between BzATP and saline $p<0.05$. **, significant differences between BzATP and saline $p<0.01$.

2.4.4 $P2X_7$ agonism or antagonism has no effect on injury-induced neuronal regeneration

During spontaneous regeneration, peak neurogenesis occurs at 14 dpi (Reimer et al., 2008). To assess neuronal regeneration, we used pan-neuronal marker HuC/D (Ghosh & Hui, 2016; Hui et al., 2010). Similar to previous reports, we detected a significant increase in the number of HuC/D^+ cells within the spinal cord at 14 dpi when compared to naïve controls (Figure 6D). We also found that $P2X_7$ receptors are expressed by HuC/D^+ cells in naïve fish (Figure 6A-A'') and at 14 dpi within the spinal cord both rostral (Figure 6B-B'') and caudal (Figure 6C-C'') to the lesion.

We assessed the involvement of $P2X_7$ signaling in mediating neuronal regeneration by administering intraperitoneal injections of $P2X_7$ agonist BzATP or $P2X_7$ antagonist JNJ 47965567 at 3, 6, 9, and 12 dpi, and then analyzed HuC/D^+ expression at 14 dpi. Vehicle injections delivered in the same time frames served as controls. Quantification of HuC/D^+ cells within the injured spinal cord indicated that the number of neurons present decreased slightly as distance from the lesion increased (Figure 7E, J). This effect was more pronounced in the spinal cord tissue that was rostral to the lesion (Figure 7E). When comparing vehicle to BzATP treated fish, we noted a significant decrease in the number of HuC/D^+ expressing cells within the first 250 μm rostral to the lesion (Figure 7E), but no change in the number of HuC/D^+ expressing cells within the caudal cord (Figure 7J). Again, for each sample (n), we limited our counts to spinal sections within the first 400 μm closest to the injury site (as above) and compared these values between treatment groups. When comparing vehicle to BzATP or vehicle to JNJ treated fish, we found there were no significant differences in the total number of HuC/D^+ expressing cells both rostral (Figure 7A-D) and caudal (Figure 7F-I) to the lesion.

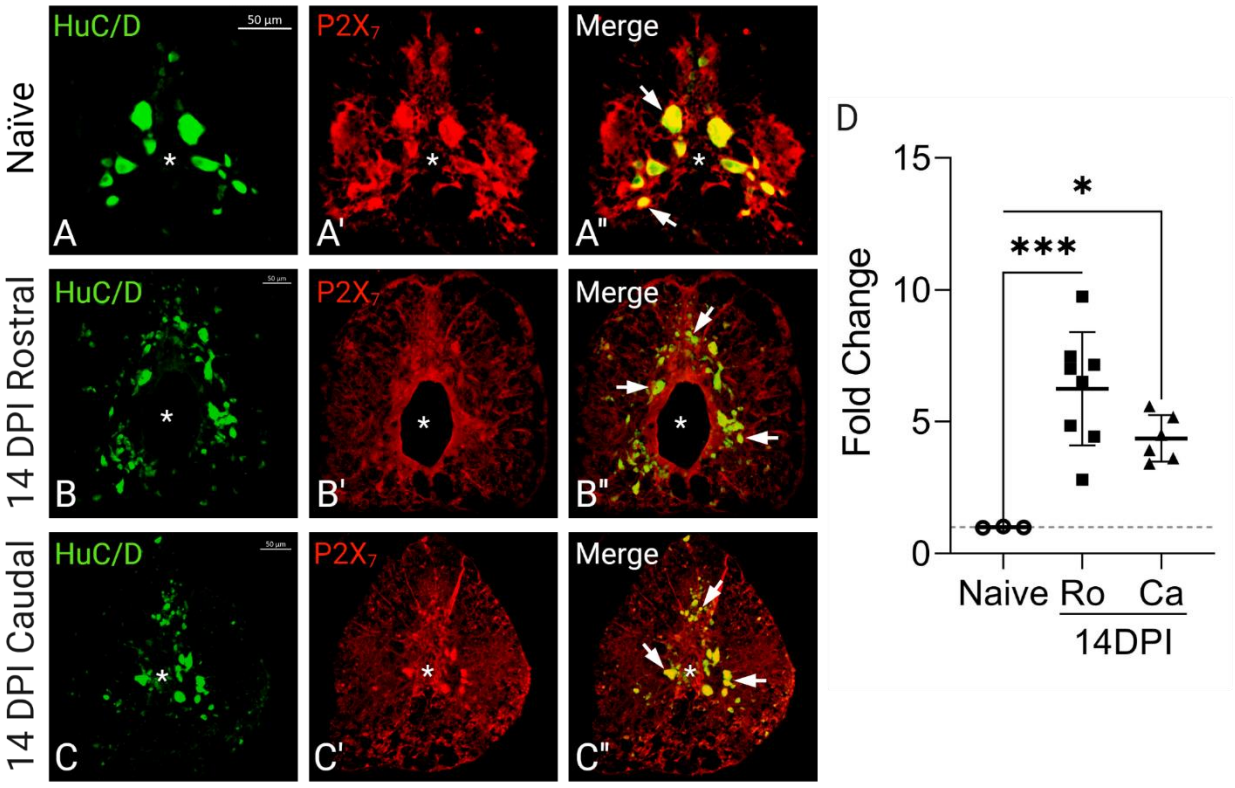


Figure 6. P2X$_7$ receptors are expressed by HuC/D$^+$ cells around the central canal. Spinal cord cross sections are shown (dorsal is up; asterisk indicates central canal). Representative images of HuC/D and P2X$_7$ labelling in naïve fish (A-A'') and within the 14 dpi rostral (B-B'') and caudal (C-C'') spinal cord. n=3/group. Colocalization is seen in small and large HuC/D$^+$ cells (arrows) (A'', B'', C''). When normalized to naïve controls (n=3), HuC/D$^+$ expression around the central canal became significantly upregulated both rostral (n=8, one-way ANOVA with Dunnett's post hoc test, p=0.0005) and caudal (n=6, p=0.0174) to the lesion (D). Data presented as means ± SD. *, significant differences between caudal (Ca) and naïve p<0.05. ***, significant differences between rostral (Ro) and naïve p<0.001.

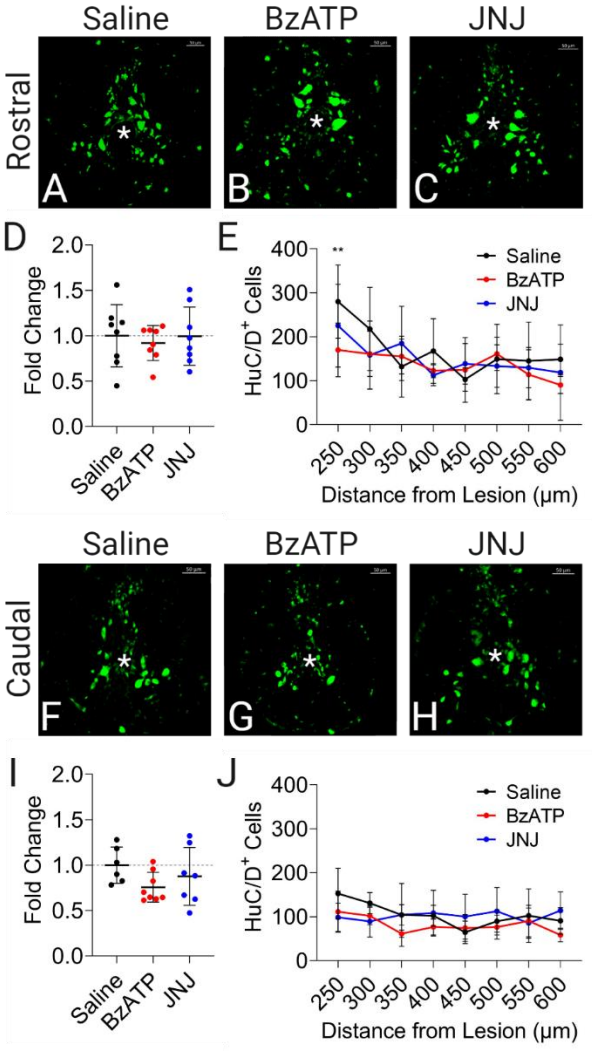


Figure 7. Treatment with BzATP or JNJ have no effect on HuC/D$^+$ expression within the spinal cord. Spinal cord cross sections are shown (dorsal is up; asterisk indicates central canal). Representative images of HuC/D$^+$ expression following treatment with saline (A, F), BzATP (B, G), or JNJ (C, H). Intraperitoneal injections of 100 μM BzATP at 3, 6, 9, and 12 dpi significantly decreased the number of HuC/D$^+$ cells at 14 dpi within the first 250 μm rostral to the lesion (E, two-way ANOVA with Dunnett's post hoc test) but had no effect caudal to the lesion (J) when compared to intraperitoneal injections of saline alone. Analysis of summative cell counts indicates that BzATP or JNJ had no significant effect on the number of HuC/D$^+$ expressing cells both rostral (D, saline, n=8; BzATP, n=8; JNJ, n=8; one-way ANOVA with Dunnett's post hoc test) and caudal (I; saline, n=6; BzATP, n=8; JNJ, n= 7) to the lesion when compared to saline. Data presented at means ± SD. **, significant differences between BzATP and saline $p<0.01$.

2.5 Discussion

The $P2X_7$ receptor has been implicated in a plethora of cellular functions and disease pathologies, including CNS injury. However, its role in adaptive plasticity and modulating injury-induced RG cell proliferation and neuronal regeneration in adult zebrafish is unknown. Here, we show that the $P2X_7$ receptor has widespread distribution throughout the adult zebrafish spinal cord with specific localization to neurons and radial glia. Examination of $P2X_7$ isoform expression revealed that several isoforms were commonly expressed in the zebrafish spinal cord, with the exception of the 68-85 kDa full length mammalian $P2X_7A$ isoform, which appeared to be mostly absent. Pharmacological activation of $P2X_7$ following spinal cord transection in zebrafish resulted in a greater number of proliferating cells around the central canal by 7 dpi, while $P2X_7$ inhibition appeared to have no effect. At 14 dpi, these treatments did not have a significant effect on the number of neurons within the injured spinal cord. This data indicates that $P2X_7$ receptor activation is sufficient to induce cellular proliferation but is not a necessary mediator of either proliferation or neurogenesis following SCI in adult zebrafish.

2.5.1 Cellular Expression of $P2X_7$

Within the mammalian CNS, $P2X_7$ receptors are widely expressed. It is generally accepted that immune cells, oligodendrocytes, oligodendrocyte precursor cells, and astrocytes express functional $P2X_7$ receptors (Fan et al., 2020; He et al., 2017; Khan et al., 2019; Kobayashi et al., 2011; Matute et al., 2007; Peng et al., 2009; Savio et al., 2018; Wang et al., 2009). Expression has also been identified in ependymal cells of the brain and spinal cord, as well as neural progenitors from the subgranular and subventricular zones of the brain (Francistiová et al., 2021; Genzen et al., 2009; Leeson et al., 2018; Marichal et al., 2016). Meanwhile, expression of functional $P2X_7$ receptors in mature neurons appears more controversial with contradictory

evidence supporting both possibilities (Deuchars et al., 2001; Illes et al., 2017; Kaczmarek-Hajek et al., 2018; Khan et al., 2019; Metzger et al., 2017; Miras-Portugal et al., 2017; Wang et al., 2004).

In larval zebrafish, $P2X_7$ mRNA is expressed in the brain and spinal cord, as well as in most other tissues (Appelbaum et al., 2007; Chang et al., 2011). While peripheral immune cells of larval zebrafish have been proposed to possess functional $P2X_7$ receptors, thorough cellular characterization within larval or adult zebrafish CNS tissue has not yet been done (Ogryzko et al., 2014). In our study, we observed widespread distribution of $P2X_7$ expression throughout the thoracic spinal cord of adult zebrafish. Colocalization of $P2X_7$ expression showed that it was primarily expressed by mature and immature neurons, labelled with either $NeuN^+$ or HuC/D^+, radial glia, labelled with $GFAP^+$, and proliferating cells around the central canal, labelled with $PCNA^+$.

2.5.2 $P2X_7$ Splice Variants

The rather diverse portfolio of roles for $P2X_7$ receptors may be attributed to the existence of different splice variants that perform distinct functions (Adinolfi et al., 2018; Burnstock & Knight, 2018). Alternative splicing is highly varied across species and in terms of $P2X_7$, there have been 13 splice variants identified in humans and 5 in mice. In humans, three of the 13 identified splice variants, namely $P2X_7A$ (~68-85 kDa), $P2X_7B$ (~42-50 kDa), and $P2X_7J$ (~29-38 kDa) are particularly relevant to CNS regeneration (Benzaquen et al., 2019; Cheewatrakoolpong et al., 2005; Ollà et al., 2020; Pegoraro et al., 2021). $P2X_7A$ encodes the full-length protein typically associated with mediating cell death via ‘macropore’ formation (Cheewatrakoolpong et al., 2005). In comparison, $P2X_7B$ has a truncated C-terminus which makes it incapable of forming a ‘macropore’ but allows it to retain ion channel activity

(Cheewatrakoolpong et al., 2005). When the two splice variants are co-expressed, $P2X_7B$ can potentiate the activities of $P2X_7A$ (Adinolfi et al., 2010). $P2X_7J$ lacks the second transmembrane domain and intracellular C-terminus tail making it inactive when expressed alone; however, when co-expressed with $P2X_7A$, $P2X_7J$ exerts a dominant negative effect over $P2X_7A$-mediated cytotoxic functions (Feng et al., 2006; Guzman-Aranguez et al., 2017). mRNA analysis reveals that humans have evolved to favor $P2X_7B$ as the predominant isoform over $P2X_7A$ in the brain and spinal cord under homeostatic conditions (Adinolfi et al., 2010; Cheewatrakoolpong et al., 2005).

In murine species, four of the five identified splice variants, namely $P2X_7a$, $P2X_7b$, $P2X_7c$, and $P2X_7d$, are particularly relevant to CNS regeneration. Like human $P2X_7A$, $P2X_7a$ is the full-length, cytotoxic protein. $P2X_7b$ and $P2X_7c$ have truncated C-termini, but $P2X_7c$ is 11 amino acids longer (Masin et al., 2012). The truncation in these splice variants is less severe, with more of the C-terminus being conserved, when compared to human $P2X_7B$ variants. Like $P2X_7B$, $P2X_7b$ cannot form 'macropores'; however, unlike $P2X_7B$, $P2X_7b$ negatively modulates the activity of $P2X_7a$ when the two proteins are co-expressed (Kido et al., 2014; Masin et al., 2012). Interestingly, as more $P2X_7a/b$ heteromers form, total expression of $P2X_7a$ decreases (Masin et al., 2012). Nevertheless, $P2X_7b$ and c are ineffectively trafficked to the plasma membrane, so while some proteins may reach the membrane, most remain intracellular (Masin et al., 2012). Although $P2X_7b$ and c form functional channels, they have lower activity than $P2X_7a$ (Masin et al., 2012). $P2X_7d$ has a truncated extracellular loop and lacks the second transmembrane domain and intracellular C-terminus tail (Benzaquen et al., 2019). Structurally, it is comparable to human $P2X_7J$, but has a more significant truncation. Like $P2X_7b$, this protein does not form 'macropores' and negatively modulates $P2X_7a$ activity when the two proteins are

co-expressed (Kido et al., 2014; Masin et al., 2012). Unlike humans, mRNA analysis in murine species suggests that they have evolved to favor $P2X_7$a as the predominant isoform in the brain, when compared to $P2X_7$b and c (Masin et al., 2012; Nicke et al., 2009). Whether this is also the case in the spinal cord is undocumented.

Different $P2X_7$ splice variants have not yet been identified in zebrafish; however, their existence seems possible. Since mammals and zebrafish have highly conserved genes encoding the extended C-terminus tail, we probed the zebrafish spinal cord with a $P2X_7$ antibody targeting several amino acids within this region (McCarthy et al., 2019; Rump et al., 2020). This antibody has been shown to detect the full length $P2X_7$ receptor in mammals (Leeson et al., 2018). Across all groups, the majority of individual zebrafish did not express the 68-85 kDa protein corresponding to the full length $P2X_7$A receptor in mammals. We did, however, detect strong bands around 50 kDa, matching the molecular weight of human $P2X_7$B and murine $P2X_7$b/c, and 37 kDa, matching the molecular weight of human $P2X_7$J and murine $P2X_7$d (Benzaquen et al., 2019; Sluyter, 2017). Given that we used a C-terminus targeting antibody that cannot detect mammalian $P2X_7$B and $P2X_7$J splice variants, it seems unlikely that the 50 and 37 kDa zebrafish $P2X_7$ splice variants are structurally or functionally homologous to the truncated mammalian $P2X_7$ proteins. Our data demonstrates that zebrafish express alternative splice variations of the $P2X_7$ receptor with conserved C-termini. This suggests that these variants are perhaps more similar in function to mammalian $P2X_7$A receptors. In mammals, only the $P2X_7$A variant expresses the complete C-terminus tail, which enhances channel activity and is necessary for ‘macropore’ formation (Karasawa et al., 2017). Indeed, while ‘macropore’ formation has not be recorded in teleost fish, BzATP has been shown to mediate $P2X_7$ mediated cell death and cell proliferation in larval zebrafish: two functions that can be driven $P2X_7$A activation in mammals

(Adinolfi et al., 2010; Chang et al., 2011; Medrano et al., 2020). Nevertheless, it is important to note that the functions of the zebrafish $P2X_7$ splice variants are unlikely to be identical to those of mammalian $P2X_7A$ receptors. This is because although the C-terminus is conserved, the human $P2X_7$ gene is only 42% similar to that in zebrafish (Sluyter, 2017). These significant structural differences may account for why certain characteristic functions of mammalian $P2X_7$ receptor activation, such as interleukin-1 beta (IL-1β) secretion, are not shared by zebrafish $P2X_7$ receptors (López-Castejón et al., 2007). Thus, our data suggests that zebrafish $P2X_7$ mRNA undergoes alternative splicing to generate distinct proteins that contribute to yet unknown functions of this receptor.

During pathological conditions in humans, such as cancer, Huntington's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis, or epilepsy, $P2X_7$ receptor expression is upregulated (Chong et al., 2010; Giuliani et al., 2014; Jimenez-Pacheco et al., 2016; Martin et al., 2019; Ollà et al., 2020; Pegoraro et al., 2020; Yiangou et al., 2006). Similar findings are seen in murine models (Lara et al., 2020; Territo & Zarrinmayeh, 2021). While changes in $P2X_7$ receptor expression have not been described in response to human SCI, they have been characterized in murine species. Indeed, $P2X_7A$ mRNA and protein levels become significantly upregulated by 3-7 days post SCI and remain elevated until at least 30 days (Fan et al., 2020; Gomez-Villafuertes et al., 2015; Kobayashi et al., 2011). In contrast, we showed that in adult zebrafish, $P2X_7$ protein expression of the 50 kDa isoform was downregulated at 7 days and returned to basal levels of expression at 14 and 21 days, when compared to naïve controls. Meanwhile, $P2X_7$ protein expression of the 37 kDa isoform did not change at 7, 14, or 21 days following SCI when compared to naïve controls. In mammals, the predominant view is that $P2X_7$ upregulation potentiates secondary cell death (Peng et al., 2009; Wang et al., 2004). Given that

the period of injury-induced secondary cell death in fish is quite brief and only lasts for several days, it seems feasible that in these species, $P2X_7$ receptor expression remains at basal levels during the early SCI response so that it may dampen the severity of the secondary cellular injury (Hui et al., 2010; Hui et al., 2014; Ogryzko et al., 2014; Tsarouchas et al., 2018).

2.5.3 $P2X_7$ Mediated Cellular Proliferation and Neuronal Differentiation

Spinal ependymal cells lining the murine central canal are highly differentiated and do not generate other cell types under homeostatic conditions (Stenudd et al., 2022). The exception to this is *in vitro*, or in response to direct injury to the spinal cord. When exposed to these exceptional conditions, a small subpopulation (approximately 8%) of spinal ependymal cells denoted ependymal A (EpA) generate neurons, astrocytes, and oligodendrocytes *in vitro* (Stenudd et al., 2022; Weiss et al., 1996). In response to direct injury of the spinal cord, EpA cells de-differentiate and acquire stem cell properties, such as the ability to self-renew and generate migrating glial, but not neuronal, progeny (Barnabé-Heider et al., 2010; Stenudd et al., 2022). The caveat is that ependymal cell progeny migrate minimal distances and only contribute to approximately 2.1% of newly generated astrocytes (Ren et al., 2017). Meanwhile, if the ependymal layer is not directly damaged during SCI, spinal ependymal cells do not contribute to the SCI response (Ren et al., 2017). Yet, transplantation of an *in vitro* expanded clonal population of spinal ependymal cells into the dentate gyrus of the hippocampus, a neurogenic niche within the mammalian brain that is maintained into adulthood, induced their activation and differentiation into neurons (Shihabuddin et al., 2000). Thus, microenvironmental cues appear to play an integral role in determining the activity and cell fate of endogenous spinal ependymal cells.

Throughout development and in adulthood, radial glia line the central canal of teleost fish and fulfil the homeostatic functions of mammalian ependymal cells and astrocytes (Becker et al., 2018). It has been proposed that RG are ancestral homologs of mammalian radial glia and astrocytes (Jurisch-Yaksi et al., 2020). During mammalian development, radial glia are present throughout the spinal cord where they give rise to new neurons, macroglia, and ependymal cells; however, during early postnatal development, these cells undergo terminal differentiation into astrocytes (Barry & McDermott, 2005; Malatesta et al., 2000; Xing et al., 2018). Following SCI in zebrafish, RG undergo a significant proliferative response and, unlike mammalian EpA cells, can differentiate into neurons or glial cells to repopulate the injured spinal cord (Hui et al., 2015; Stenudd et al., 2022). Prior to our study, the role of $P2X_7$ signaling in mediating injury-induced RG cell activity had not yet been explored.

Multiples lines of evidence suggest that elevated $P2X_7$ receptor expression potentiates cellular proliferation. Indeed, populations of highly proliferative stem or cancer cells express high levels of $P2X_7$ (Francistiová et al., 2021; Giuliani et al., 2014; Pegoraro et al., 2020). While both human $P2X_7$A and B have growth-promoting effects, that of $P2X_7$B appears to be more efficient (Giuliani et al., 2014). In humans, tumors expressing higher levels of $P2X_7$B have greater cell density than those expressing similar levels of $P2X_7$A and B (Giuliani et al., 2014). Treatment of mouse embryonic stem cells with a $P2X_7$ agonist increases proliferation, while treatment with a $P2X_7$ antagonist decreases proliferation (Glaser et al., 2014). Given these findings, one possible explanation for the decrease in expression of the 50 kDa isoform we detected during the peak in RG proliferation may be that this protein, like human $P2X_7$A and $P2X_7$B, is a potent inducer of cellular proliferation. Under homeostatic conditions, this isoform may be involved in maintaining basal rates of proliferation, but to prevent uncontrolled cell

growth, zebrafish downregulate protein expression of the 50 kDa $P2X_7$ isoform during the period of peak RG cell proliferation. This downregulation doesn't adversely affect the rate of proliferation, suggesting that various other signaling pathways simultaneously promote RG re-entry into the cell cycle (Barreiro-Iglesias et al., 2015; Briona et al., 2015; Reimer et al., 2009; Reimer et al., 2013). Since expression of the 50 kDa $P2X_7$ isoform is downregulated in zebrafish at 7 dpi, it may account for the effect of $P2X_7$ agonism, and lack of effect by antagonism, on total $PCNA^+$ expression around the central canal.

The $P2X_7$ receptor may also be involved in regulating cellular differentiation; however, the respective roles of the mammalian $P2X_7$ isoforms in this process remain unclear. When mouse embryonic stem cells and human induced pluripotent stem cells (hiPSCs) are induced to undergo neuronal differentiation, the expression of $P2X_7$ decreases (Francistiová et al., 2021; Glaser et al., 2014). One group reported that while the expression levels of $P2X_7A$ stayed relatively constant, those of $P2X_7B$ decreased (Glaser et al., 2014). Another group reported a decrease in $P2X_7A$ expression, but did not analyze $P2X_7B$ (Francistiová et al., 2021). Thus, it is unclear which $P2X_7$ isoform plays a more predominant role in this process. Treatment of mouse embryonic stem cells with a $P2X_7$ receptor agonist had no effect on neuronal differentiation, while treatment with a $P2X_7$ receptor antagonist increased neuronal differentiation (Glaser et al., 2014). This suggests that $P2X_7$ activation negatively regulates neuronal differentiation. In comparison to neural differentiation, when hiPSCs or mesenchymal stromal cells derived from human subcutaneous adipose tissue (S-ASCs) are induced to differentiate into a non-neuronal cell type, total expression of $P2X_7A$ and $P2X_7B$ is maintained or even increased (Carluccio et al., 2019; Francistiová et al., 2021). One group studying phenotype specification of mouse embryonal carcinoma cells found that $P2X_7$ receptor activation promotes gliogenesis (Yuahasi et

al., 2012). In our study, we detected basal levels of expression of the 50 kDa $P2X_7$ isoform during the period of peak injury-induced neurogenesis (14 dpi). In response to chronic $P2X_7$ receptor agonism or antagonism, we detected no statistically significant changes in the total number of HuC/D^+ cells. Our findings indicate that $P2X_7$ signaling likely does not play a predominant role in neurogenesis following SCI in adult zebrafish. Nevertheless, we did not assess changes in gliogenesis, so it may be possible that treatment with BzATP pushed some RG cells towards a glial fate.

In conclusion, our results suggest that, unlike in humans, $P2X_7$ signaling does not play a maladaptive role following SCI in adult zebrafish. Using immunohistochemical techniques, we detected $P2X_7$ expression on neurons and radial glial cells; however, additional investigation will be required to confirm the identity of select $P2X_7$ isoforms expressed by these cell types. *In vivo* analysis of the effects of $P2X_7$ agonism or antagonism showed that while $P2X_7$ activation is sufficient to promote proliferation, it is not required. Meanwhile, $P2X_7$ signaling did not appear to play a significant role in mediating neurogenesis. Future studies examining the possible effects of $P2X_7$ on differentiation of other cell types within the spinal cord may offer some new insights into injury-induced glial or immune cell regulation. In all, our findings suggest alternative roles of the purinergic system following CNS injury in teleost fish versus mammals.

2.6 References

Adinolfi, E., Cirillo, M., Woltersdorf, R., Falzoni, S., Chiozzi, P., Pellegatti, P., Callegari, M. G., Sandonà, D., Markwardt, F., Schmalzing, G., & Di Virgilio, F. (2010). Trophic activity of a naturally occurring truncated isoform of the P2X7 receptor. *FASEB J*, *24*(9), 3393-3404. https://doi.org/10.1096/fj.09-153601

Adinolfi, E., Giuliani, A. L., De Marchi, E., Pegoraro, A., Orioli, E., & Di Virgilio, F. (2018). The P2X7 receptor: A main player in inflammation. *Biochem Pharmacol*, *151*, 234-244. https://doi.org/10.1016/j.bcp.2017.12.021

Andrejew, R., Oliveira-Giacomelli, Á., Ribeiro, D. E., Glaser, T., Arnaud-Sampaio, V. F., Lameu, C., & Ulrich, H. (2020). The P2X7 Receptor: Central Hub of Brain Diseases. *Front Mol Neurosci*, *13*, 124. https://doi.org/10.3389/fnmol.2020.00124

Appelbaum, L., Skariah, G., Mourrain, P., & Mignot, E. (2007). Comparative expression of p2x receptors and ecto-nucleoside triphosphate diphosphohydrolase 3 in hypocretin and sensory neurons in zebrafish. *Brain Res*, *1174*, 66-75. https://doi.org/10.1016/j.brainres.2007.06.103

Barnabé-Heider, F., Göritz, C., Sabelström, H., Takebayashi, H., Pfrieger, F. W., Meletis, K., & Frisén, J. (2010). Origin of new glial cells in intact and injured adult spinal cord. *Cell Stem Cell*, *7*(4), 470-482. https://doi.org/10.1016/j.stem.2010.07.014

Barreiro-Iglesias, A., Mysiak, K. S., Scott, A. L., Reimer, M. M., Yang, Y., Becker, C. G., & Becker, T. (2015). Serotonin Promotes Development and Regeneration of Spinal Motor Neurons in Zebrafish. *Cell Rep*, *13*(5), 924-932. https://doi.org/10.1016/j.celrep.2015.09.050

Barry, D., & McDermott, K. (2005). Differentiation of radial glia from radial precursor cells and transformation into astrocytes in the developing rat spinal cord. *Glia*, *50*(3), 187-197. https://doi.org/10.1002/glia.20166

Becker, C. G., Becker, T., & Hugnot, J. P. (2018). The spinal ependymal zone as a source of endogenous repair cells across vertebrates. *Prog Neurobiol*, *170*, 67-80. https://doi.org/10.1016/j.pneurobio.2018.04.002

Becker, T., Wullimann, M. F., Becker, C. G., Bernhardt, R. R., & Schachner, M. (1997). Axonal regrowth after spinal cord transection in adult zebrafish. *J Comp Neurol*, *377*(4), 577-595. https://doi.org/10.1002/(sici)1096-9861(19970127)377:4<577::aid-cne8>3.0.co;2-#

Benzaquen, J., Heeke, S., Janho Dit Hreich, S., Douguet, L., Marquette, C. H., Hofman, P., & Vouret-Craviari, V. (2019). Alternative splicing of P2RX7 pre-messenger RNA in health and diseases: Myth or reality? *Biomed J*, *42*(3), 141-154. https://doi.org/10.1016/j.bj.2019.05.007

Bhattacharya, A., Wang, Q., Ao, H., Shoblock, J. R., Lord, B., Aluisio, L., Fraser, I., Nepomuceno, D., Neff, R. A., Welty, N., Lovenberg, T. W., Bonaventure, P., Wickenden, A. D., & Letavic, M. A. (2013). Pharmacological characterization of a novel centrally permeable P2X7 receptor antagonist: JNJ-47965567. *Br J Pharmacol*, *170*(3), 624-640. https://doi.org/10.1111/bph.12314

Bologna-Molina, R., Mosqueda-Taylor, A., Molina-Frechero, N., Mori-Estevez, A. D., & Sánchez-Acuña, G. (2013). Comparison of the value of PCNA and Ki-67 as markers of cell proliferation in ameloblastic tumors. *Med Oral Patol Oral Cir Bucal*, *18*(2), e174-179. https://doi.org/10.4317/medoral.18573

Briona, L. K., & Dorsky, R. I. (2014). Radial glial progenitors repair the zebrafish spinal cord following transection. *Exp Neurol*, *256*, 81-92. https://doi.org/10.1016/j.expneurol.2014.03.017

Briona, L. K., Poulain, F. E., Mosimann, C., & Dorsky, R. I. (2015). Wnt/ss-catenin signaling is required for radial glial neurogenesis following spinal cord injury. *Dev Biol*, *403*(1), 15-21. https://doi.org/10.1016/j.ydbio.2015.03.025

Burnstock, G. (2018). Purine and purinergic receptors. *Brain Neurosci Adv*, *2*, 2398212818817494. https://doi.org/10.1177/2398212818817494

Burnstock, G., & Knight, G. E. (2018). The potential of P2X7 receptors as a therapeutic target, including inflammation and tumour progression. *Purinergic Signal*, *14*(1), 1-18. https://doi.org/10.1007/s11302-017-9593-0

Burnstock, G., & Ulrich, H. (2011). Purinergic signaling in embryonic and stem cell development. *Cell Mol Life Sci*, *68*(8), 1369-1394. https://doi.org/10.1007/s00018-010-0614-1

Carluccio, M., Zuccarini, M., Ziberi, S., Giuliani, P., Morabito, C., Mariggiò, M. A., Lonardo, M. T., Adinolfi, E., Orioli, E., Di Iorio, P., Caciagli, F., & Ciccarelli, R. (2019). Involvement of P2X7 Receptors in the Osteogenic Differentiation of Mesenchymal Stromal/Stem Cells Derived from Human Subcutaneous Adipose Tissue. *Stem Cell Rev Rep*, *15*(4), 574-589. https://doi.org/10.1007/s12015-019-09883-6

Carvalho, K., Martin, E., Ces, A., Sarrazin, N., Lagouge-Roussey, P., Nous, C., Boucherit, L., Youssef, I., Prigent, A., Faivre, E., Eddarkaoui, S., Gauvrit, T., Vieau, D., Boluda, S., Huin, V., Fontaine, B., Buée, L., Delatour, B., Dutar, P., . . . Delarasse, C. (2021). P2X7-deficiency improves plasticity and cognitive abilities in a mouse model of Tauopathy. *Prog Neurobiol*, *206*, 102139. https://doi.org/10.1016/j.pneurobio.2021.102139

Chang, M. Y., Lu, J. K., Tian, Y. C., Chen, Y. C., Hung, C. C., Huang, Y. H., Chen, Y. H., Wu, M. S., Yang, C. W., & Cheng, Y. C. (2011). Inhibition of the P2X7 receptor reduces cystogenesis in PKD. *J Am Soc Nephrol*, *22*(9), 1696-1706. https://doi.org/10.1681/asn.2010070728

Cheewatrakoolpong, B., Gilchrest, H., Anthes, J. C., & Greenfeder, S. (2005). Identification and characterization of splice variants of the human P2X7 ATP channel. *Biochem Biophys Res Commun*, *332*(1), 17-27. https://doi.org/10.1016/j.bbrc.2005.04.087

Chong, J. H., Zheng, G. G., Zhu, X. F., Guo, Y., Wang, L., Ma, C. H., Liu, S. Y., Xu, L. L., Lin, Y. M., & Wu, K. F. (2010). Abnormal expression of P2X family receptors in Chinese pediatric acute leukemias. *Biochem Biophys Res Commun*, *391*(1), 498-504. https://doi.org/10.1016/j.bbrc.2009.11.087

De Salis, S. K. F., Li, L., Chen, Z., Lam, K. W., Skarratt, K. K., Balle, T., & Fuller, S. J. (2022). Alternatively Spliced Isoforms of the P2X7 Receptor: Structure, Function and Disease Associations. *Int J Mol Sci*, *23*(15). https://doi.org/10.3390/ijms23158174

Deuchars, S. A., Atkinson, L., Brooke, R. E., Musa, H., Milligan, C. J., Batten, T. F., Buckley, N. J., Parson, S. H., & Deuchars, J. (2001). Neuronal P2X7 receptors are targeted to presynaptic terminals in the central and peripheral nervous systems. *J Neurosci*, *21*(18), 7143-7152. https://doi.org/10.1523/jneurosci.21-18-07143.2001

Di Virgilio, F., Schmalzing, G., & Markwardt, F. (2018). The Elusive P2X7 Macropore. *Trends Cell Biol*, *28*(5), 392-404. https://doi.org/10.1016/j.tcb.2018.01.005

Ding, W., Hu, S., Wang, P., Kang, H., Peng, R., Dong, Y., & Li, F. (2022). Spinal Cord Injury: The Global Incidence, Prevalence, and Disability From the Global Burden of Disease

Study 2019. *Spine (Phila Pa 1976)*, *47*(21), 1532-1540. https://doi.org/10.1097/brs.0000000000004417

Fan, X., Ma, W., Zhang, Y., & Zhang, L. (2020). P2X7 Receptor (P2X7R) of Microglia Mediates Neuroinflammation by Regulating (NOD)-Like Receptor Protein 3 (NLRP3) Inflammasome-Dependent Inflammation After Spinal Cord Injury. *Med Sci Monit*, *26*, e925491. https://doi.org/10.12659/msm.925491

Feng, Y. H., Li, X., Wang, L., Zhou, L., & Gorodeski, G. I. (2006). A truncated P2X7 receptor variant (P2X7-j) endogenously expressed in cervical cancer cells antagonizes the full-length P2X7 receptor through hetero-oligomerization. *J Biol Chem*, *281*(25), 17228-17237. https://doi.org/10.1074/jbc.M602999200

Francistiová, L., Vörös, K., Lovász, Z., Dinnyés, A., & Kobolák, J. (2021). Detection and Functional Evaluation of the P2X7 Receptor in hiPSC Derived Neurons and Microglia-Like Cells. *Front Mol Neurosci*, *14*, 793769. https://doi.org/10.3389/fnmol.2021.793769

Genzen, J. R., Platel, J. C., Rubio, M. E., & Bordey, A. (2009). Ependymal cells along the lateral ventricle express functional P2X(7) receptors. *Purinergic Signal*, *5*(3), 299-307. https://doi.org/10.1007/s11302-009-9143-5

Ghosh, S., & Hui, S. P. (2016). Regeneration of Zebrafish CNS: Adult Neurogenesis. *Neural Plast*, *2016*, 5815439. https://doi.org/10.1155/2016/5815439

Giuliani, A. L., Colognesi, D., Ricco, T., Roncato, C., Capece, M., Amoroso, F., Wang, Q. G., De Marchi, E., Gartland, A., Di Virgilio, F., & Adinolfi, E. (2014). Trophic activity of human P2X7 receptor isoforms A and B in osteosarcoma. *PLoS One*, *9*(9), e107224. https://doi.org/10.1371/journal.pone.0107224

Glaser, T., de Oliveira, S. L., Cheffer, A., Beco, R., Martins, P., Fornazari, M., Lameu, C., Junior, H. M., Coutinho-Silva, R., & Ulrich, H. (2014). Modulation of mouse embryonic stem cell proliferation and neural differentiation by the P2X7 receptor. *PLoS One*, *9*(5), e96281. https://doi.org/10.1371/journal.pone.0096281

Gomez-Villafuertes, R. (2016). Contribution of purinergic receptors to spinal cord injury repair: stem cell-based neuroregeneration. *Neural Regen Res*, *11*(3), 418-419. https://doi.org/10.4103/1673-5374.179049

Gomez-Villafuertes, R., Rodriguez-Jimenez, F. J., Alastrue-Agudo, A., Stojkovic, M., Miras-Portugal, M. T., & Moreno-Manzano, V. (2015). Purinergic Receptors in Spinal Cord-Derived Ependymal Stem/Progenitor Cells and Their Potential Role in Cell-Based Therapy for Spinal Cord Injury. *Cell Transplant*, *24*(8), 1493-1509. https://doi.org/10.3727/096368914X682828

Guzman-Aranguez, A., Pérez de Lara, M. J., & Pintor, J. (2017). Hyperosmotic stress induces ATP release and changes in P2X7 receptor levels in human corneal and conjunctival epithelial cells. *Purinergic Signal*, *13*(2), 249-258. https://doi.org/10.1007/s11302-017-9556-5

He, Y., Taylor, N., Fourgeaud, L., & Bhattacharya, A. (2017). The role of microglial P2X7: modulation of cell death and cytokine release. *J Neuroinflammation*, *14*(1), 135. https://doi.org/10.1186/s12974-017-0904-8

Hui, S. P., Dutta, A., & Ghosh, S. (2010). Cellular response after crush injury in adult zebrafish spinal cord. *Dev Dyn*, *239*(11), 2962-2979. https://doi.org/10.1002/dvdy.22438

Hui, S. P., Nag, T. C., & Ghosh, S. (2015). Characterization of Proliferating Neural Progenitors after Spinal Cord Injury in Adult Zebrafish. *PLoS One*, *10*(12), e0143595. https://doi.org/10.1371/journal.pone.0143595

Hui, S. P., Sengupta, D., Lee, S. G., Sen, T., Kundu, S., Mathavan, S., & Ghosh, S. (2014). Genome wide expression profiling during spinal cord regeneration identifies comprehensive cellular responses in zebrafish. *PLoS One*, *9*(1), e84212. https://doi.org/10.1371/journal.pone.0084212

Illes, P., Khan, T. M., & Rubini, P. (2017). Neuronal P2X7 Receptors Revisited: Do They Really Exist? *J Neurosci*, *37*(30), 7049-7062. https://doi.org/10.1523/jneurosci.3103-16.2017

Jimenez-Pacheco, A., Diaz-Hernandez, M., Arribas-Blázquez, M., Sanz-Rodriguez, A., Olivos-Oré, L. A., Artalejo, A. R., Alves, M., Letavic, M., Miras-Portugal, M. T., Conroy, R. M., Delanty, N., Farrell, M. A., O'Brien, D. F., Bhattacharya, A., Engel, T., & Henshall, D. C. (2016). Transient P2X7 Receptor Antagonism Produces Lasting Reductions in Spontaneous Seizures and Gliosis in Experimental Temporal Lobe Epilepsy. *J Neurosci*, *36*(22), 5920-5932. https://doi.org/10.1523/jneurosci.4009-15.2016

Jurisch-Yaksi, N., Yaksi, E., & Kizil, C. (2020). Radial glia in the zebrafish brain: Functional, structural, and physiological comparison with the mammalian glia. *Glia*, *68*(12), 2451-2470. https://doi.org/10.1002/glia.23849

Kaczmarek-Hajek, K., Zhang, J., Kopp, R., Grosche, A., Rissiek, B., Saul, A., Bruzzone, S., Engel, T., Jooss, T., Krautloher, A., Schuster, S., Magnus, T., Stadelmann, C., Sirko, S., Koch-Nolte, F., Eulenburg, V., & Nicke, A. (2018). Re-evaluation of neuronal P2X7 expression using novel mouse models and a P2X7-specific nanobody. *Elife*, *7*. https://doi.org/10.7554/eLife.36217

Karasawa, A., Michalski, K., Mikhelzon, P., & Kawate, T. (2017). The P2X7 receptor forms a dye-permeable pore independent of its intracellular domain but dependent on membrane lipid composition. *Elife*, *6*. https://doi.org/10.7554/eLife.31186

Khan, M. T., Deussing, J., Tang, Y., & Illes, P. (2019). Astrocytic rather than neuronal P2X7 receptors modulate the function of the tri-synaptic network in the rodent hippocampus. *Brain Res Bull*, *151*, 164-173. https://doi.org/10.1016/j.brainresbull.2018.07.016

Kido, Y., Kawahara, C., Terai, Y., Ohishi, A., Kobayashi, S., Hayakawa, M., Kamatsuka, Y., Nishida, K., & Nagasawa, K. (2014). Regulation of activity of P2X7 receptor by its splice variants in cultured mouse astrocytes. *Glia*, *62*(3), 440-451. https://doi.org/10.1002/glia.22615

Kobayashi, K., Takahashi, E., Miyagawa, Y., Yamanaka, H., & Noguchi, K. (2011). Induction of the P2X7 receptor in spinal microglia in a neuropathic pain model. *Neurosci Lett*, *504*(1), 57-61. https://doi.org/10.1016/j.neulet.2011.08.058

Krueger, H., Noonan, V. K., Trenaman, L. M., Joshi, P., & Rivers, C. S. (2013). The economic burden of traumatic spinal cord injury in Canada. *Chronic Dis Inj Can*, *33*(3), 113-122.

Lara, R., Adinolfi, E., Harwood, C. A., Philpott, M., Barden, J. A., Di Virgilio, F., & McNulty, S. (2020). P2X7 in Cancer: From Molecular Mechanisms to Therapeutics. *Front Pharmacol*, *11*, 793. https://doi.org/10.3389/fphar.2020.00793

Leeson, H. C., Chan-Ling, T., Lovelace, M. D., Brownlie, J. C., Gu, B. J., & Weible, M. W. (2019). P2X7 receptor signaling during adult hippocampal neurogenesis. *Neural Regen Res*, *14*(10), 1684-1694. https://doi.org/10.4103/1673-5374.257510

Leeson, H. C., Kasherman, M. A., Chan-Ling, T., Lovelace, M. D., Brownlie, J. C., Toppinen, K. M., Gu, B. J., & Weible, M. W., 2nd. (2018). P2X7 Receptors Regulate Phagocytosis and Proliferation in Adult Hippocampal and SVZ Neural Progenitor Cells: Implications for Inflammation in Neurogenesis. *Stem Cells*, *36*(11), 1764-1777. https://doi.org/10.1002/stem.2894

Liang, X., Samways, D. S., Wolf, K., Bowles, E. A., Richards, J. P., Bruno, J., Dutertre, S., DiPaolo, R. J., & Egan, T. M. (2015). Quantifying Ca2+ current and permeability in ATP-gated P2X7 receptors. *J Biol Chem*, *290*(12), 7930-7942. https://doi.org/10.1074/jbc.M114.627810

López-Castejón, G., Young, M. T., Meseguer, J., Surprenant, A., & Mulero, V. (2007). Characterization of ATP-gated P2X7 receptors in fish provides new insights into the mechanism of release of the leaderless cytokine interleukin-1 beta. *Mol Immunol*, *44*(6), 1286-1299. https://doi.org/10.1016/j.molimm.2006.05.015

Ly, D., Dongol, A., Cuthbertson, P., Guy, T. V., Geraghty, N. J., Sophocleous, R. A., Sin, L., Turner, B. J., Watson, D., Yerbury, J. J., & Sluyter, R. (2020). The P2X7 receptor antagonist JNJ-47965567 administered thrice weekly from disease onset does not alter progression of amyotrophic lateral sclerosis in SOD1(G93A) mice. *Purinergic Signal*, *16*(1), 109-122. https://doi.org/10.1007/s11302-020-09692-4

Lynch, J., & Cahalan, R. (2017). The impact of spinal cord injury on the quality of life of primary family caregivers: a literature review. *Spinal Cord*, *55*(11), 964-978. https://doi.org/10.1038/sc.2017.56

Lyons, D. A., Guy, A. T., & Clarke, J. D. (2003). Monitoring neural progenitor fate through multiple rounds of division in an intact vertebrate brain. *Development*, *130*(15), 3427-3436. https://doi.org/10.1242/dev.00569

Malatesta, P., Hartfuss, E., & Gotz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development*, *127*(24), 5253-5263. https://www.ncbi.nlm.nih.gov/pubmed/11076748

Marichal, N., Fabbiani, G., Trujillo-Cenoz, O., & Russo, R. E. (2016). Purinergic signalling in a latent stem cell niche of the rat spinal cord. *Purinergic Signal*, *12*(2), 331-341. https://doi.org/10.1007/s11302-016-9507-6

Martin, E., Amar, M., Dalle, C., Youssef, I., Boucher, C., Le Duigou, C., Brückner, M., Prigent, A., Sazdovitch, V., Halle, A., Kanellopoulos, J. M., Fontaine, B., Delatour, B., & Delarasse, C. (2019). New role of P2X7 receptor in an Alzheimer's disease mouse model. *Mol Psychiatry*, *24*(1), 108-125. https://doi.org/10.1038/s41380-018-0108-3

Masin, M., Young, C., Lim, K., Barnes, S. J., Xu, X. J., Marschall, V., Brutkowski, W., Mooney, E. R., Gorecki, D. C., & Murrell-Lagnado, R. (2012). Expression, assembly and function of novel C-terminal truncated variants of the mouse P2X7 receptor: re-evaluation of P2X7 knockouts. *Br J Pharmacol*, *165*(4), 978-993. https://doi.org/10.1111/j.1476-5381.2011.01624.x

Matute, C., Torre, I., Pérez-Cerdá, F., Pérez-Samartín, A., Alberdi, E., Etxebarria, E., Arranz, A. M., Ravid, R., Rodríguez-Antigüedad, A., Sánchez-Gómez, M., & Domercq, M. (2007). P2X(7) receptor blockade prevents ATP excitotoxicity in oligodendrocytes and ameliorates experimental autoimmune encephalomyelitis. *J Neurosci*, *27*(35), 9525-9533. https://doi.org/10.1523/jneurosci.0579-07.2007

McCarthy, A. E., Yoshioka, C., & Mansoor, S. E. (2019). Full-Length P2X(7) Structures Reveal How Palmitoylation Prevents Channel Desensitization. *Cell*, *179*(3), 659-670.e613. https://doi.org/10.1016/j.cell.2019.09.017

Medrano, M. P., Pisera-Fuster, A., Bernabeu, R. O., & Faillace, M. P. (2020). P2X7 and A(2A) receptor endogenous activation protects against neuronal death caused by CoCl(2) - induced photoreceptor toxicity in the zebrafish retina. *J Comp Neurol*, *528*(12), 2000-2020. https://doi.org/10.1002/cne.24869

Metzger, M. W., Walser, S. M., Aprile-Garcia, F., Dedic, N., Chen, A., Holsboer, F., Arzt, E., Wurst, W., & Deussing, J. M. (2017). Genetically dissecting P2rx7 expression within the central nervous system using conditional humanized mice. *Purinergic Signal*, *13*(2), 153-170. https://doi.org/10.1007/s11302-016-9546-z

Miras-Portugal, M. T., Sebastián-Serrano, Á., de Diego García, L., & Díaz-Hernández, M. (2017). Neuronal P2X7 Receptor: Involvement in Neuronal Physiology and Pathology. *J Neurosci*, *37*(30), 7063-7072. https://doi.org/10.1523/jneurosci.3104-16.2017

Nicke, A., Kuan, Y. H., Masin, M., Rettinger, J., Marquez-Klaka, B., Bender, O., Górecki, D. C., Murrell-Lagnado, R. D., & Soto, F. (2009). A functional P2X7 splice variant with an alternative transmembrane domain 1 escapes gene inactivation in P2X7 knock-out mice. *J Biol Chem*, *284*(38), 25813-25822. https://doi.org/10.1074/jbc.M109.033134

Noonan, V. K., Fingas, M., Farry, A., Baxter, D., Singh, A., Fehlings, M. G., & Dvorak, M. F. (2012). Incidence and prevalence of spinal cord injury in Canada: a national perspective. *Neuroepidemiology*, *38*(4), 219-226. https://doi.org/10.1159/000336014

Ogryzko, N. V., Hoggett, E. E., Solaymani-Kohal, S., Tazzyman, S., Chico, T. J., Renshaw, S. A., & Wilson, H. L. (2014). Zebrafish tissue injury causes upregulation of interleukin-1 and caspase-dependent amplification of the inflammatory response. *Dis Model Mech*, *7*(2), 259-264. https://doi.org/10.1242/dmm.013029

Oliveira, A., Illes, P., & Ulrich, H. (2016). Purinergic receptors in embryonic and adult neurogenesis. *Neuropharmacology*, *104*, 272-281. https://doi.org/10.1016/j.neuropharm.2015.10.008

Ollà, I., Santos-Galindo, M., Elorza, A., & Lucas, J. J. (2020). P2X7 Receptor Upregulation in Huntington's Disease Brains. *Front Mol Neurosci*, *13*, 567430. https://doi.org/10.3389/fnmol.2020.567430

Pegoraro, A., De Marchi, E., & Adinolfi, E. (2021). P2X7 Variants in Oncogenesis. *Cells*, *10*(1). https://doi.org/10.3390/cells10010189

Pegoraro, A., Orioli, E., De Marchi, E., Salvestrini, V., Milani, A., Di Virgilio, F., Curti, A., & Adinolfi, E. (2020). Differential sensitivity of acute myeloid leukemia cells to daunorubicin depends on P2X7A versus P2X7B receptor expression. *Cell Death Dis*, *11*(10), 876. https://doi.org/10.1038/s41419-020-03058-9

Peng, W., Cotrina, M. L., Han, X., Yu, H., Bekar, L., Blum, L., Takano, T., Tian, G. F., Goldman, S. A., & Nedergaard, M. (2009). Systemic administration of an antagonist of the ATP-sensitive receptor P2X7 improves recovery after spinal cord injury. *Proc Natl Acad Sci U S A*, *106*(30), 12489-12493. https://doi.org/10.1073/pnas.0902531106

Reimer, M. M., Kuscha, V., Wyatt, C., Sörensen, I., Frank, R. E., Knüwer, M., Becker, T., & Becker, C. G. (2009). Sonic hedgehog is a polarized signal for motor neuron regeneration in adult zebrafish. *J Neurosci*, *29*(48), 15073-15082. https://doi.org/10.1523/jneurosci.4748-09.2009

Reimer, M. M., Norris, A., Ohnmacht, J., Patani, R., Zhong, Z., Dias, T. B., Kuscha, V., Scott, A. L., Chen, Y. C., Rozov, S., Frazer, S. L., Wyatt, C., Higashijima, S., Patton, E. E., Panula, P., Chandran, S., Becker, T., & Becker, C. G. (2013). Dopamine from the brain promotes spinal motor neuron generation during development and adult regeneration. *Dev Cell*, *25*(5), 478-491. https://doi.org/10.1016/j.devcel.2013.04.012

Reimer, M. M., Sorensen, I., Kuscha, V., Frank, R. E., Liu, C., Becker, C. G., & Becker, T. (2008). Motor neuron regeneration in adult zebrafish. *J Neurosci*, *28*(34), 8510-8516. https://doi.org/10.1523/JNEUROSCI.1189-08.2008

Ren, Y., Ao, Y., O'Shea, T. M., Burda, J. E., Bernstein, A. M., Brumm, A. J., Muthusamy, N., Ghashghaei, H. T., Carmichael, S. T., Cheng, L., & Sofroniew, M. V. (2017). Ependymal cell contribution to scar formation after spinal cord injury is minimal, local and dependent on direct ependymal injury. *Sci Rep*, *7*, 41122. https://doi.org/10.1038/srep41122

Reynolds, K. E., Wong, C. R., & Scott, A. L. (2021). Astrocyte-mediated purinergic signaling is upregulated in a mouse model of Fragile X syndrome. *Glia*, *69*(7), 1816-1832. https://doi.org/10.1002/glia.23997

Ribeiro, F. F., Xapelli, S., Miranda-Lourenco, C., Tanqueiro, S. R., Fonseca-Gomes, J., Diogenes, M. J., Ribeiro, J. A., & Sebastiao, A. M. (2016). Purine nucleosides in neuroregeneration and neuroprotection. *Neuropharmacology*, *104*, 226-242. https://doi.org/10.1016/j.neuropharm.2015.11.006

Rump, A., Smolander, O. P., Ruutel Boudinot, S., Kanellopoulos, J. M., & Boudinot, P. (2020). Evolutionary Origin of the P2X7 C-ter Region: Capture of an Ancient Ballast Domain by a P2X4-Like Gene in Ancient Jawed Vertebrates. *Front Immunol*, *11*, 113. https://doi.org/10.3389/fimmu.2020.00113

Savio, L. E. B., de Andrade Mello, P., da Silva, C. G., & Coutinho-Silva, R. (2018). The P2X7 Receptor in Inflammatory Diseases: Angel or Demon? *Front Pharmacol*, *9*, 52. https://doi.org/10.3389/fphar.2018.00052

Shihabuddin, L. S., Horner, P. J., Ray, J., & Gage, F. H. (2000). Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. *J Neurosci*, *20*(23), 8727-8735. https://www.ncbi.nlm.nih.gov/pubmed/11102479

Sluyter, R. (2017). The P2X7 Receptor. *Adv Exp Med Biol*, *1051*, 17-53. https://doi.org/10.1007/5584_2017_59

Stenudd, M., Sabelström, H., Llorens-Bobadilla, E., Zamboni, M., Blom, H., Brismar, H., Zhang, S., Basak, O., Clevers, H., Göritz, C., Barnabé-Heider, F., & Frisén, J. (2022). Identification of a discrete subpopulation of spinal cord ependymal cells with neural stem cell properties. *Cell Rep*, *38*(9), 110440. https://doi.org/10.1016/j.celrep.2022.110440

Territo, P. R., & Zarrinmayeh, H. (2021). P2X(7) Receptors in Neurodegeneration: Potential Therapeutic Applications From Basic to Clinical Approaches. *Front Cell Neurosci*, *15*, 617036. https://doi.org/10.3389/fncel.2021.617036

Tsao, H. K., Chiu, P. H., & Sun, S. H. (2013). PKC-dependent ERK phosphorylation is essential for P2X7 receptor-mediated neuronal differentiation of neural progenitor cells. *Cell Death Dis*, *4*, e751. https://doi.org/10.1038/cddis.2013.274

Tsarouchas, T. M., Wehner, D., Cavone, L., Munir, T., Keatinge, M., Lambertus, M., Underhill, A., Barrett, T., Kassapis, E., Ogryzko, N., Feng, Y., van Ham, T. J., Becker, T., & Becker, C. G. (2018). Dynamic control of proinflammatory cytokines Il-1beta and Tnf-alpha by macrophages in zebrafish spinal cord regeneration. *Nat Commun*, *9*(1), 4670. https://doi.org/10.1038/s41467-018-07036-w

Wang, L. Y., Cai, W. Q., Chen, P. H., Deng, Q. Y., & Zhao, C. M. (2009). Downregulation of P2X7 receptor expression in rat oligodendrocyte precursor cells after hypoxia ischemia. *Glia*, *57*(3), 307-319. https://doi.org/10.1002/glia.20758

Wang, X., Arcuino, G., Takano, T., Lin, J., Peng, W. G., Wan, P., Li, P., Xu, Q., Liu, Q. S., Goldman, S. A., & Nedergaard, M. (2004). P2X7 receptor inhibition improves recovery after spinal cord injury. *Nat Med*, *10*(8), 821-827. https://doi.org/10.1038/nm1082

Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A. C., & Reynolds, B. A. (1996). Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J Neurosci*, *16*(23), 7599-7609. https://www.ncbi.nlm.nih.gov/pubmed/8922416

Wolf, H. K., Buslei, R., Schmidt-Kastner, R., Schmidt-Kastner, P. K., Pietsch, T., Wiestler, O. D., & Blümcke, I. (1996). NeuN: a useful neuronal marker for diagnostic histopathology. *J Histochem Cytochem*, *44*(10), 1167-1171. https://doi.org/10.1177/44.10.8813082

Xing, L., Anbarchian, T., Tsai, J. M., Plant, G. W., & Nusse, R. (2018). Wnt/beta-catenin signaling regulates ependymal cell development and adult homeostasis. *Proc Natl Acad Sci U S A*, *115*(26), E5954-E5962. https://doi.org/10.1073/pnas.1803297115

Yiangou, Y., Facer, P., Durrenberger, P., Chessell, I. P., Naylor, A., Bountra, C., Banati, R. R., & Anand, P. (2006). COX-2, CB2 and P2X7-immunoreactivities are increased in activated microglial cells/macrophages of multiple sclerosis and amyotrophic lateral sclerosis spinal cord. *BMC Neurol*, *6*, 12. https://doi.org/10.1186/1471-2377-6-12

Yuahasi, K. K., Demasi, M. A., Tamajusuku, A. S., Lenz, G., Sogayar, M. C., Fornazari, M., Lameu, C., Nascimento, I. C., Glaser, T., Schwindt, T. T., Negraes, P. D., & Ulrich, H. (2012). Regulation of neurogenesis and gliogenesis of retinoic acid-induced P19 embryonal carcinoma cells by P2X2 and P2X7 receptors studied by RNA interference. *Int J Dev Neurosci*, *30*(2), 91-97. https://doi.org/10.1016/j.ijdevneu.2011.12.010

Chapter 3

3. Discussion

Mammalian spinal cord injury (SCI) is an extremely debilitating neurological condition causing significant and irreversible physiological and psychological damage. Following the primary mechanical injury, a maladaptive secondary cellular injury ensues that is characterized by widespread neuroinflammation, significant cellular death, reactive gliosis, and axonal degeneration. In comparison, select non-mammalian vertebrates, namely zebrafish (*Danio rerio*), display high regenerative ability in adulthood. In these species, the secondary cellular injury is dampened, successful neural regeneration occurs, and functional recovery is achieved. In our study, we identified a role for the purinergic $P2X_7$ receptor in the zebrafish SCI response. Specifically, we found that receptor activation is sufficient but not necessary to promote injury-induced radial glial (RG) cell proliferation. Among the purinergic receptors, $P2X_7$ is the most widely investigated in mammals but its role within the central nervous system (CNS) is also the most controversial, with both neuroprotective and neurotoxic roles being identified. Here, we evaluated $P2X_7$ receptor activation under homeostatic versus pathological conditions and variations in receptor structure and function in zebrafish.

3.1 $P2X_7$ Receptor Function

The members of the adenosine triphosphate (ATP) gated $P2X_{1-7}$ receptor family share many structural similarities. All full length P2X receptors have two transmembrane domains, an extracellular loop, and intracellular N- and C-termini (North, 2002). Among its family members, $P2X_7$ receptors are unique in that they have a significantly longer intracellular C-terminus tail. Three ATP binding sites are present at each of the three subunit interfaces of full length P2X receptors (Hattori & Gouaux, 2012). Most P2X receptors, including $P2X_7$, require all three

binding sites to be occupied for channel gating (Grimes & Young, 2015; Hattori & Gouaux, 2012; Stelmashenko et al., 2012). In response to ligand binding, these channels open to facilitate potassium efflux, and sodium and calcium influx. In addition to the orthosteric $P2X_7$ binding sites, distinct allosteric binding sites have also been identified (Karasawa & Kawate, 2016). Various endogenous and exogenous allosteric modulators that can positively or negatively regulate $P2X_7$ function exist, including, peptides, amyloid beta, antibiotics, plant derivatives, and several receptor agonists and antagonists (Bidula et al., 2019; Elssner et al., 2004; Karasawa et al., 2017; Martínez-Cuesta et al., 2020; Moura et al., 2015; Sanz et al., 2009; Stokes et al., 2020). In this work, we used BzATP, a competitive $P2X_7$ receptor agonist, which binds at the orthosteric sites, and JNJ 47965567, a non-competitive antagonist, which binds at the allosteric sites (Jiang et al., 2021; Karasawa & Kawate, 2016). Interestingly, upon extracellular ATP binding and channel opening, the allosteric sites narrow (Karasawa & Kawate, 2016). This implies that when extracellular ATP is bound, the allosteric sites are not accessible to receptor antagonists such as JNJ 47965567. This suggests that when extracellular ATP concentrations are elevated, such as during the first several hours following mammalian SCI, treatment with JNJ 47965567 will not effectively prevent channel activation (Wang et al., 2004). While individual P2X receptors can co-assemble to form homo- and hetero-trimeric ion channels, $P2X_7$ receptors typically form homotrimers; nevertheless, formation of heterotrimers with $P2X_7/P2X_4$ may also occur (Antonio et al., 2014; Schneider et al., 2017; Sluyter & Stokes, 2011).

Unlike the other six members of the P2X family, which are activated by ATP concentrations within the nanomolar to low micromolar range, $P2X_7$ is activated by ATP concentrations in the hundreds of micromolar range (Martínez-Cuesta et al., 2020; McCarthy et al., 2019). Under physiological conditions, ectonucleotidase activity keeps extracellular ATP

concentrations in the nanomolar range (Ohnishi et al., 2021; Pellegatti et al., 2008; Zimmermann, 2000). CD39 (NTPDase-1) metabolizes ATP into ADP and AMP, while CD73 (ecto-5′-nucleotidase) metabolizes AMP into adenosine (Antonioli et al., 2013). At the same time, intracellular ATP concentrations are in the millimolar range (Ohnishi et al., 2021; Pellegatti et al., 2008). This suggests that the bulk of ATP is almost exclusively cytosolic in healthy tissues and extracellular ATP concentrations are typically not sufficient for receptor activation under homeostatic conditions. Meanwhile, in response to cellular injury or disease, extracellular concentrations of ATP become elevated (Li et al., 2017; Pellegatti et al., 2008; Wang et al., 2004; Wilmes et al., 2022). ATP can be released from cells through two major mechanisms. The first is lytic, or non-regulated ATP release, which typically occurs when cells are damaged or dying (Grygorczyk et al., 2021). The second is non-lytic, or regulated ATP release, which may occur through ATP-binding cassette (ABC) transporters, pannexin and connexin hemichannels, calcium homeostasis modulator 1 (CALHM1), volume-regulated anion channels (VRACs), maxi-anion channels (MACs), cytosolic vesicles, and even through $P2X_7$ receptors (Di Virgilio, Boeynaems, et al., 2009; Pellegatti et al., 2005; Suadicani et al., 2006; Vultaggio-Poma et al., 2020). In addition, once in the extracellular space, ATP can act as a stimulus for additional ATP release by local cells: a process called ATP-induced ATP release (Anderson et al., 2004; Dou et al., 2012; Kobayakawa et al., 2019). Thus, during pathological conditions, extracellular ATP concentrations can quickly rise to levels that are sufficient for $P2X_7$ receptor activation.

For several decades, the prevailing dogma was that sustained stimulation with ATP gradually increases the permeability of $P2X_7$ receptors so that these cation channels ‘dilate’ to become non-selective ‘macropores’ (Cockcroft & Gomperts, 1979; Virginio et al., 1999). Recently, it was demonstrated that gradual channel dilation does not occur and instead, a channel

pore permeable to small biological cations and large synthetic cations like N-methyl-D-glucosamine ($NMDG^+$) opens within milliseconds of ATP binding (Harkat et al., 2017; Karasawa et al., 2017). Ion flux is determined by cation size, as the flow of small sodium (Na^+) ions through the $P2X_7$ channel is much faster than that of large $NMDG^+$ ions (Harkat et al., 2017). It has been proposed that the delayed flux of fluorescent dyes with very high molecular weights, such as YO-PRO-1 and ethidium bromide, could be because these dyes need "to sample more conformations and orientations before a successful permeation can occur" (Harkat et al., 2017). However, others argue that the minimal cross section of YO-PRO-1, the larger of the two dyes, is at least 2 Å larger than the open channel diameter of the full-length rat $P2X_7$ receptor, so passage of this fluorescent dye through the channel is not entirely feasible (Batista Napotnik & Miklavčič, 2018; Dunning et al., 2021). Nevertheless, ethidium bromide uptake occurs in response to $P2X_7$ receptor stimulation with high micromolar concentrations of ATP (Leeson et al., 2018). So, an alternative mechanism is that $P2X_7$ receptors may functionally couple with other pore forming channels, such as Ca^{2+}-activated chloride (Cl^-) channels, to mediate uptake of large fluorescent dyes (Dunning et al., 2021; Janks et al., 2019).

$P2X_7$ receptors mediate diverse cellular processes like unprogrammed cell death, programmed cell death, cellular proliferation, and phagocytosis (Adinolfi et al., 2005; Bidula et al., 2019; Leeson et al., 2018). The concentration of extracellular ATP used to stimulate the receptor and the duration of the stimulus appear to determine, at least in part, which specific process occurs. When extracellular ATP reaches pathological concentrations, $P2X_7$ receptor overstimulation results in uncontrolled Ca^{2+} influx into the cytoplasm and mitochondria, and subsequent cell death (Di Virgilio, Ferrari, et al., 2009). Stimulation with extracellular concentrations of ATP around 3-5 millimolar induces $P2X_7$-mediated osmotic lysis via cell

swelling and rupture (Bidula et al., 2019). Lytic cell death often results in additional ATP release and exacerbation of the injury or disease (Bidula et al., 2019). In comparison, under physiological conditions, when extracellular ATP is present but not in the high micromolar range, basal $P2X_7$ receptor activity can moderately increase intracellular Ca^{2+} concentrations. This stimulates an increase in intramitochondrial Ca^{2+} concentrations which facilitates more efficient oxidative phosphorylation, resulting in enhanced ATP production (Di Virgilio, Ferrari, et al., 2009). An increase in intracellular ATP consequently facilitates cell survival and growth (Di Virgilio, Ferrari, et al., 2009). Curiously, while some studies report that $P2X_7$ receptor stimulation with non-pathological concentrations of ATP stimulates cell proliferation, others argue that it inhibits this process (Bianco et al., 2006; Glaser et al., 2014; Leeson et al., 2018; Tsao et al., 2013; Zhang et al., 2019). Alternatively, additional groups have found that stimulation with sub-lethal extracellular concentrations of ATP around 500 micromolar can facilitate programmed cell death (Bidula et al., 2019). These contradictory findings may be explained by the existence of different $P2X_7$ receptor splice variants with distinct functions.

It is interesting to note that in the absence of extracellular ATP, $P2X_7$ receptors on astrocytes or adult neural progenitor cells from the hippocampus and subventricular zone of the rodent brain can facilitate phagocytosis *in vitro* (Leeson et al., 2018; Yamamoto et al., 2013). Phagocytosis is mediated by the closed (unbound) state of the $P2X_7$ receptor. Interestingly, certain non-competitive $P2X_7$ receptor antagonists like AZ10606120 block fluorescent dye uptake without affecting $P2X_7$ mediated phagocytic ability of human monocytes (Ou et al., 2018). Given that JNJ-47965567, the non-competitive $P2X_7$ receptor antagonist used in this study, likely binds the same site, it seems feasible that $P2X_7$ receptor inhibition by this compound similarly did not inhibit endogenous phagocytic ability (Ly et al., 2020). This is important

because CNS injuries and neurodegenerative diseases are typically associated with cellular degeneration and cell death, which create considerable cellular debris. In cases like these, and perhaps following SCI in adult zebrafish, treatment with $P2X_7$ receptor antagonists may dampen secondary cell death by inhibiting $P2X_7$ mediated cytotoxic cell death while simultaneously enhancing debris clearance by promoting $P2X_7$ mediated phagocytic activity. A fascinating next for the current study would be to assess how treatment with BzATP or JNJ-47965567 affected $P2X_7$ mediated phagocytosis.

While our study specifically analyzed the effect of $P2X_7$ receptor activation and inhibition on RG cell proliferation and neuronal differentiation following SCI in adult zebrafish, it is likely that treatment with BzATP or JNJ 47965567 affected various other cell types and cellular processes. In addition to cell death, proliferation, and phagocytosis, $P2X_7$ receptors are also involved in mediating immune cell activation, reactive gliosis, axonal regeneration, and gliogenesis (Stefanova & Scott, 2022). Further exploration of such off-target effects is required.

3.2 $P2X_7$ Splice Variants

Although some similarities exist, alternative splicing is highly varied across species. Out of the thirteen splice variants identified in humans, only nine ($P2X_7$A-J) have been studied in detail, and of these, only three ($P2X_7$A, B, and J) are expressed under physiological conditions (Figure 1) (Benzaquen et al., 2019). Human $P2X_7$C, E, and G code for a protein that is presumed to be too short to form a channel receptor, while $P2X_7$D, F, and H lack the N-terminus sequence required for insertion into the cell membrane (Benzaquen et al., 2019). In murine species, five splice variants ($P2X_7$a-d, and k) that can be expressed under physiological conditions have been identified and studied (Figure 1) (Benzaquen et al., 2019; Sluyter, 2017).

The full-length protein, $P2X_7A$ in humans and $P2X_7a$ in murine species, retains its canonical role as an ion channel and is also capable of taking up large molecules like fluorescent dyes (Cheewatrakoolpong et al., 2005; Di Virgilio et al., 2018; Leeson et al., 2018). It is interesting to note that murine species have an additional splice variant, $P2X_7k$, that is quite similar to $P2X_7a$, but with a differently spliced N-terminus and some variation in the first transmembrane domain (Benzaquen et al., 2019). This variant is more sensitive to ATP than $P2X_7a$ and has a greater propensity to take up large ions like $NMDG^+$ (Nicke et al., 2009). Part of $P2X_7k$'s enhanced activity has been explained by the presence of a second mode of receptor activation via ADP-ribosylation, which does not activate $P2X_7a$ (Schwarz et al., 2012). mRNA analysis has revealed that while $P2X_7k$ is lowly expressed within most mouse tissues, including the brain and spinal cord, it is generally not the predominant isoform (Nicke et al., 2009). Humans have one C-terminus truncated variant: $P2X_7B$, while murine species have two: $P2X_7b$, also called $P2X_7$ 13B or $P2X_7v3$, and $P2X_7c$, also called $P2X_713C$ or $P2X_7v2$ (Masin et al., 2012; Sluyter, 2017). While these splice variants retain their roles as ion channels, they do not typically take up large molecules (Cheewatrakoolpong et al., 2005; Sluyter, 2017). It has recently been demonstrated that it isn't the lack of a C-terminus tail that prevents formation of a large pore in these splice variants (Karasawa et al., 2017). Instead, the presence of a C-terminus tail counteracts the inhibitory effect direct cholesterol binding to the $P2X_7$ transmembrane domain has on channel activity (Karasawa et al., 2017). When cholesterol is absent from the plasma membrane channel activity, as indicated by Ca^{2+} and YO-PRO-1 uptake, of C-terminus truncated $P2X_7$ variants is comparable to that of $P2X_7A$ (Karasawa et al., 2017). Therefore, the absence of a C-terminus appears to reduce channel activity. Curiously, when $P2X_7B$ is co-expressed with $P2X_7A$ in humans, all known activities of $P2X_7A$ are potentiated, including large pore formation

and cytotoxic cell death (Adinolfi et al., 2010). In comparison, $P2X_7b$ negatively modulates the activity of $P2X_7a$ when the two proteins are co-expressed in mice (Kido et al., 2014; Masin et al., 2012). A variant that lacks the second transmembrane domain is expressed in humans ($P2X_7J$) and in murine species ($P2X_7d$, also called $P2X_7v4$) (Sluyter, 2017). When expressed alone, $P2X_7J$ is inactive; however, when co-expressed with $P2X_7A$, $P2X_7J$ exerts a dominant negative effect over $P2X_7A$ mediated cytotoxic functions in humans (Feng et al., 2006; Guzman-Aranguez et al., 2017). Similarly, $P2X_7d$ negatively modulates $P2X_7a$ activity when the two proteins are co-expressed in murine species (Kido et al., 2014; Masin et al., 2012).

Prior to our study, different $P2X_7$ receptor splice variants in zebrafish had not yet been identified. We detected two truncated proteins matching the molecular weight of human $P2X_7B$ and $P2X_7J$ (Benzaquen et al., 2019; Sluyter, 2017). Our data indicates that these alternative splice variations each have a partially conserved C-terminus. This suggests that these variants are perhaps more similar in function to mammalian $P2X_7A$/a receptors than the mammalian C-terminal truncated proteins.

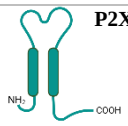
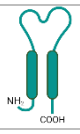
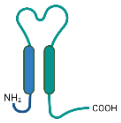
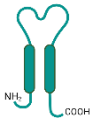
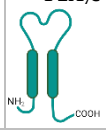
Human			**$P2X_7A$** 	**$P2X_7B$** 		**$P2X_7J$** 
	Structure					
		N-terminus	Conserved	Conserved		Conserved
		Transmembrane Domain 1	Conserved	Conserved		Conserved
		Extracellular Loop	Conserved	Conserved		Truncated
		Transmembrane Domain 2	Conserved	Conserved		Deleted
		C-terminus	Conserved	Truncated		Deleted
	Function					
		Ca^{2+} Signaling Efficiency	High	Low		*Not functional alone
		Permeable to Large Dyes & Organic Cations	Yes	No		No
		Necrosis	Yes	(?)		*Not functional alone
		Apoptosis	Yes	(?)		*Not functional alone
		Proliferation	Yes	Yes *greater than $P2X_7A$		*Not functional alone
		Phagocytosis	Yes			*Not functional alone
		Interaction with Other Isoforms		Hetero-oligomerizes with $P2X_7A$ to potentiate $P2X_7A$-mediated functions		Hetero-oligomerizes with $P2X_7A$ to prevent $P2X_7A$-mediated functions
Murine			**$P2X_7a$** 	**$P2X_7k$** 	**$P2X_7b$** 	**$P2X_7c$** 	**$P2X_7d$** 
	Structure						
		N-terminus	Conserved	Alternatively Spliced	Conserved	Conserved	Conserved
		Transmembrane Domain 1	Conserved	Alternatively Spliced	Conserved	Conserved	Conserved
		Extracellular Loop	Conserved	Conserved	Conserved	Conserved	Truncated *less conserved than $P2X_7J$
		Transmembrane Domain 2	Conserved	Conserved	Conserved	Conserved	Deleted
		C-terminus	Conserved	Conserved	Truncated *more conserved than $P2X_7B$	Truncated *more conserved than $P2X_7b$	Deleted
	Function						
		Ca^{2+} Signaling Efficiency	High	High **>$P2X_7a$	Low	Low	*Not functional alone
		Permeable to Large Dyes & Organic Cations	Yes	Yes **>$P2X_7a$	No	No	No
		Necrosis	Yes	Yes	(?)	(?)	*Not functional alone
		Apoptosis	Yes	Yes	(?)	(?)	*Not functional alone
		Proliferation	Yes	(?)	(?)	(?)	*Not functional alone
		Phagocytosis	Yes	(?)	(?)	(?)	*Not functional alone
		Interaction with Other Isoforms		Does not oligomerize with $P2X_7a$	Hetero-oligomerizes with $P2X_7a$ to prevent $P2X_7a$-mediated functions		Hetero-oligomerizes with $P2X_7a$ to prevent $P2X_7a$-mediated functions

Figure 1. Human versus murine $P2X_7$ receptor splice variant structure and function. Known structure and function of splice variants is listed. Unknown function is expressed as '(?)'.

3.3 Variations in Zebrafish $P2X_7$ Structure and Function

While the human $P2X_7$ gene shares 80-81% similarity with murine species when comparing genomic organization and chromosomal localization, this value decreases to 42% with zebrafish (Sluyter, 2017). Despite these differences, it appears that the two main features that make $P2X_7$ receptors unique from the rest of their family are genetically conserved between species. First, both mammalian and zebrafish $P2X_7$ receptors do not desensitize (López-Castejón et al., 2007). Indeed, zebrafish $P2X_7$ receptor stimulation with high concentrations of ATP generates a similar current in both shape and amplitude to that generated by rodent $P2X_7$ receptor stimulation (Kucenas et al., 2003). However, slight nuances exist. One study individually expressed the genes encoding the full length $P2X_7$ receptor of several species, including, humans, mice, rats, and zebrafish in HEK293 cells (Liang et al., 2015). They found that in HEK293 cells expressing zebrafish $P2X_7$ receptors, Ca^{2+} contributed significantly more to the BzATP-gated current of $P2X_7$ than it did in any other species (Liang et al., 2015). Curiously, highly proliferative human chronic lymphocytic leukemia (CLL) cells generate a larger increase in cytoplasmic Ca^{2+} concentrations in response to $P2X_7$ receptor agonism when compared to healthy lymphocytes (Adinolfi et al., 2002). These results suggest that zebrafish $P2X_7$ receptors may be more heavily involved in regulating Ca^{2+} mediated functions like cell growth or apoptosis when compared to mammalian $P2X_7$ receptors in healthy tissue. Second, both species have highly conserved genes encoding the extended C-terminus tail (McCarthy et al., 2019; Rump et al., 2020). Given that the C-terminus tail enhances channel activity and is necessary for large fluorescent dye uptake in mammals, it seems that zebrafish possess the genetic code to express a $P2X_7$ receptor that may be somewhat similar in function to mammalian $P2X_7A$. However, the exact function of such a receptor cannot yet be ascertained and will likely not be

identical to that in mammals. Indeed, $P2X_7$ receptor stimulation mediates interleukin-1 beta (IL-1β) secretion in rodents, but it does not do so in zebrafish (López-Castejón et al., 2007). These functional differences may be because other components of the $P2X_7$ receptor, like the transmembrane domains, do not share strong sequence homology between mammals and fish species (Rump et al., 2020).

Regardless, some $P2X_7$-mediated functions appear to be conserved between mammals and fish. Two relatively recent studies in larval zebrafish have identified $P2X_7$ signaling as a mediator of cell death and cell proliferation (Chang et al., 2011; Medrano et al., 2020). The first group studied retinal degeneration and found that an injection of BzATP (400 µM) into the vitreous cavity resulted in photoreceptor cell death (Medrano et al., 2020). Unfortunately, whether this occurred through programmed or lytic cell death was not determined. Meanwhile, in a zebrafish model of polycystic kidney disease, authors found that treatment with BzATP (100 µM) enhanced renal cyst formation and growth (Chang et al., 2011). This was similar to our findings that treatment with BzATP (100 µM) enhanced RG cell proliferation around the central canal at 7 days following spinal cord injury in adult zebrafish. Although quite limited in number, studies such as these have begun to shed light on how $P2X_7$ signaling functions in regeneratively competent species. They suggest that like in mammals, the function of zebrafish $P2X_7$ receptors is determined by the concentration of extracellular ATP used to stimulate the receptor, by the duration of the stimulus, and perhaps, by what specific splice variant is expressed.

3.5 Conclusion

Purines, pyrimidines, and their metabolites are evolutionarily conserved signaling molecules critically involved in regulating various cellular processes across vertebrates. Of

particular importance to CNS injury and regeneration is the purinergic $P2X_7$ receptor. Among its many roles, this multifaceted protein mediates cellular proliferation and conversely, cell death. Understanding how function varies across the distinct $P2X_7$ receptor splice variants between mammalian and non-mammalian species may be integral to the development of novel interventions for CNS injuries that limit the cytotoxic effects of $P2X_7$ receptors and instead promote their trophic and phagocytic roles. While our work has begun to elucidate the role of $P2X_7$ signaling in mediating RG cell proliferation and neuronal regeneration following SCI in adult zebrafish, how this complex receptor regulates other aspects of the secondary cellular injury, like cell death, the immune response, reactive gliosis, gliogenesis, and axonal regeneration remains unknown and merits future investigation.

3.6 References

Adinolfi, E., Callegari, M. G., Ferrari, D., Bolognesi, C., Minelli, M., Wieckowski, M. R., Pinton, P., Rizzuto, R., & Di Virgilio, F. (2005). Basal activation of the P2X7 ATP receptor elevates mitochondrial calcium and potential, increases cellular ATP levels, and promotes serum-independent growth. *Mol Biol Cell*, *16*(7), 3260-3272. https://doi.org/10.1091/mbc.e04-11-1025

Adinolfi, E., Cirillo, M., Woltersdorf, R., Falzoni, S., Chiozzi, P., Pellegatti, P., Callegari, M. G., Sandonà, D., Markwardt, F., Schmalzing, G., & Di Virgilio, F. (2010). Trophic activity of a naturally occurring truncated isoform of the P2X7 receptor. *FASEB J*, *24*(9), 3393-3404. https://doi.org/10.1096/fj.09-153601

Adinolfi, E., Melchiorri, L., Falzoni, S., Chiozzi, P., Morelli, A., Tieghi, A., Cuneo, A., Castoldi, G., Di Virgilio, F., & Baricordi, O. R. (2002). P2X7 receptor expression in evolutive and indolent forms of chronic B lymphocytic leukemia. *Blood*, *99*(2), 706-708. https://doi.org/10.1182/blood.v99.2.706

Anderson, C. M., Bergher, J. P., & Swanson, R. A. (2004). ATP-induced ATP release from astrocytes. *J Neurochem*, *88*(1), 246-256. https://doi.org/10.1111/j.1471-4159.2004.02204.x

Antonio, L. S., Stewart, A. P., Varanda, W. A., & Edwardson, J. M. (2014). Identification of P2X2/P2X4/P2X6 heterotrimeric receptors using atomic force microscopy (AFM) imaging. *FEBS Lett*, *588*(12), 2125-2128. https://doi.org/10.1016/j.febslet.2014.04.048

Antonioli, L., Pacher, P., Vizi, E. S., & Haskó, G. (2013). CD39 and CD73 in immunity and inflammation. *Trends Mol Med*, *19*(6), 355-367. https://doi.org/10.1016/j.molmed.2013.03.005

Batista Napotnik, T., & Miklavčič, D. (2018). In vitro electroporation detection methods - An overview. *Bioelectrochemistry*, *120*, 166-182. https://doi.org/10.1016/j.bioelechem.2017.12.005

Benzaquen, J., Heeke, S., Janho Dit Hreich, S., Douguet, L., Marquette, C. H., Hofman, P., & Vouret-Craviari, V. (2019). Alternative splicing of P2RX7 pre-messenger RNA in health and diseases: Myth or reality? *Biomed J*, *42*(3), 141-154. https://doi.org/10.1016/j.bj.2019.05.007

Bianco, F., Ceruti, S., Colombo, A., Fumagalli, M., Ferrari, D., Pizzirani, C., Matteoli, M., Di Virgilio, F., Abbracchio, M. P., & Verderio, C. (2006). A role for P2X7 in microglial proliferation. *J Neurochem*, *99*(3), 745-758. https://doi.org/10.1111/j.1471-4159.2006.04101.x

Bidula, S., Dhuna, K., Helliwell, R., & Stokes, L. (2019). Positive allosteric modulation of P2X7 promotes apoptotic cell death over lytic cell death responses in macrophages. *Cell Death Dis*, *10*(12), 882. https://doi.org/10.1038/s41419-019-2110-3

Chang, M. Y., Lu, J. K., Tian, Y. C., Chen, Y. C., Hung, C. C., Huang, Y. H., Chen, Y. H., Wu, M. S., Yang, C. W., & Cheng, Y. C. (2011). Inhibition of the P2X7 receptor reduces cystogenesis in PKD. *J Am Soc Nephrol*, *22*(9), 1696-1706. https://doi.org/10.1681/asn.2010070728

Cheewatrakoolpong, B., Gilchrest, H., Anthes, J. C., & Greenfeder, S. (2005). Identification and characterization of splice variants of the human P2X7 ATP channel. *Biochem Biophys Res Commun*, *332*(1), 17-27. https://doi.org/10.1016/j.bbrc.2005.04.087

Cockcroft, S., & Gomperts, B. D. (1979). ATP induces nucleotide permeability in rat mast cells. *Nature*, *279*(5713), 541-542. https://doi.org/10.1038/279541a0

Di Virgilio, F., Boeynaems, J. M., & Robson, S. C. (2009). Extracellular nucleotides as negative modulators of immunity. *Curr Opin Pharmacol*, *9*(4), 507-513. https://doi.org/10.1016/j.coph.2009.06.021

Di Virgilio, F., Ferrari, D., & Adinolfi, E. (2009). P2X(7): a growth-promoting receptor-implications for cancer. *Purinergic Signal*, *5*(2), 251-256. https://doi.org/10.1007/s11302-009-9145-3

Di Virgilio, F., Schmalzing, G., & Markwardt, F. (2018). The Elusive P2X7 Macropore. *Trends Cell Biol*, *28*(5), 392-404. https://doi.org/10.1016/j.tcb.2018.01.005

Dou, Y., Wu, H. J., Li, H. Q., Qin, S., Wang, Y. E., Li, J., Lou, H. F., Chen, Z., Li, X. M., Luo, Q. M., & Duan, S. (2012). Microglial migration mediated by ATP-induced ATP release from lysosomes. *Cell Res*, *22*(6), 1022-1033. https://doi.org/10.1038/cr.2012.10

Dunning, K., Martz, A., Peralta, F. A., Cevoli, F., Boué-Grabot, E., Compan, V., Gautherat, F., Wolf, P., Chataigneau, T., & Grutter, T. (2021). P2X7 Receptors and TMEM16 Channels Are Functionally Coupled with Implications for Macropore Formation and Current Facilitation. *Int J Mol Sci*, *22*(12). https://doi.org/10.3390/ijms22126542

Elssner, A., Duncan, M., Gavrilin, M., & Wewers, M. D. (2004). A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1 beta processing and release. *J Immunol*, *172*(8), 4987-4994. https://doi.org/10.4049/jimmunol.172.8.4987

Feng, Y. H., Li, X., Wang, L., Zhou, L., & Gorodeski, G. I. (2006). A truncated P2X7 receptor variant (P2X7-j) endogenously expressed in cervical cancer cells antagonizes the full-length P2X7 receptor through hetero-oligomerization. *J Biol Chem*, *281*(25), 17228-17237. https://doi.org/10.1074/jbc.M602999200

Glaser, T., de Oliveira, S. L., Cheffer, A., Beco, R., Martins, P., Fornazari, M., Lameu, C., Junior, H. M., Coutinho-Silva, R., & Ulrich, H. (2014). Modulation of mouse embryonic stem cell proliferation and neural differentiation by the P2X7 receptor. *PLoS One*, *9*(5), e96281. https://doi.org/10.1371/journal.pone.0096281

Grimes, L., & Young, M. T. (2015). Purinergic P2X receptors: structural and functional features depicted by X-ray and molecular modelling studies. *Curr Med Chem*, *22*(7), 783-798. https://doi.org/10.2174/0929867321999141212131457

Grygorczyk, R., Boudreault, F., Ponomarchuk, O., Tan, J. J., Furuya, K., Goldgewicht, J., Kenfack, F. D., & Yu, F. (2021). Lytic Release of Cellular ATP: Physiological Relevance and Therapeutic Applications. *Life (Basel)*, *11*(7). https://doi.org/10.3390/life11070700

Guzman-Aranguez, A., Pérez de Lara, M. J., & Pintor, J. (2017). Hyperosmotic stress induces ATP release and changes in P2X7 receptor levels in human corneal and conjunctival epithelial cells. *Purinergic Signal*, *13*(2), 249-258. https://doi.org/10.1007/s11302-017-9556-5

Harkat, M., Peverini, L., Cerdan, A. H., Dunning, K., Beudez, J., Martz, A., Calimet, N., Specht, A., Cecchini, M., Chataigneau, T., & Grutter, T. (2017). On the permeation of large organic cations through the pore of ATP-gated P2X receptors. *Proc Natl Acad Sci U S A*, *114*(19), E3786-e3795. https://doi.org/10.1073/pnas.1701379114

Hattori, M., & Gouaux, E. (2012). Molecular mechanism of ATP binding and ion channel activation in P2X receptors. *Nature*, *485*(7397), 207-212. https://doi.org/10.1038/nature11010

Janks, L., Sprague, R. S., & Egan, T. M. (2019). ATP-Gated P2X7 Receptors Require Chloride Channels To Promote Inflammation in Human Macrophages. *J Immunol*, *202*(3), 883-898. https://doi.org/10.4049/jimmunol.1801101

Jiang, L. H., Caseley, E. A., Muench, S. P., & Roger, S. (2021). Structural basis for the functional properties of the P2X7 receptor for extracellular ATP. *Purinergic Signal*, *17*(3), 331-344. https://doi.org/10.1007/s11302-021-09790-x

Karasawa, A., & Kawate, T. (2016). Structural basis for subtype-specific inhibition of the P2X7 receptor. *Elife*, *5*. https://doi.org/10.7554/eLife.22153

Karasawa, A., Michalski, K., Mikhelzon, P., & Kawate, T. (2017). The P2X7 receptor forms a dye-permeable pore independent of its intracellular domain but dependent on membrane lipid composition. *Elife*, *6*. https://doi.org/10.7554/eLife.31186

Kido, Y., Kawahara, C., Terai, Y., Ohishi, A., Kobayashi, S., Hayakawa, M., Kamatsuka, Y., Nishida, K., & Nagasawa, K. (2014). Regulation of activity of P2X7 receptor by its splice variants in cultured mouse astrocytes. *Glia*, *62*(3), 440-451. https://doi.org/10.1002/glia.22615

Kobayakawa, K., Ohkawa, Y., Yoshizaki, S., Tamaru, T., Saito, T., Kijima, K., Yokota, K., Hara, M., Kubota, K., Matsumoto, Y., Harimaya, K., Ozato, K., Masuda, T., Tsuda, M., Tamura, T., Inoue, K., Edgerton, V. R., Iwamoto, Y., Nakashima, Y., & Okada, S. (2019). Macrophage centripetal migration drives spontaneous healing process after spinal cord injury. *Sci Adv*, *5*(5), eaav5086. https://doi.org/10.1126/sciadv.aav5086

Kucenas, S., Li, Z., Cox, J. A., Egan, T. M., & Voigt, M. M. (2003). Molecular characterization of the zebrafish P2X receptor subunit gene family. *Neuroscience*, *121*(4), 935-945. https://doi.org/10.1016/s0306-4522(03)00566-9

Leeson, H. C., Kasherman, M. A., Chan-Ling, T., Lovelace, M. D., Brownlie, J. C., Toppinen, K. M., Gu, B. J., & Weible, M. W., 2nd. (2018). P2X7 Receptors Regulate Phagocytosis and Proliferation in Adult Hippocampal and SVZ Neural Progenitor Cells: Implications for Inflammation in Neurogenesis. *Stem Cells*, *36*(11), 1764-1777. https://doi.org/10.1002/stem.2894

Li, J., Li, X., Jiang, X., Yang, M., Yang, R., Burnstock, G., Xiang, Z., & Yuan, H. (2017). Microvesicles shed from microglia activated by the P2X7-p38 pathway are involved in neuropathic pain induced by spinal nerve ligation in rats. *Purinergic Signal*, *13*(1), 13-26. https://doi.org/10.1007/s11302-016-9537-0

Liang, X., Samways, D. S., Wolf, K., Bowles, E. A., Richards, J. P., Bruno, J., Dutertre, S., DiPaolo, R. J., & Egan, T. M. (2015). Quantifying Ca2+ current and permeability in ATP-gated P2X7 receptors. *J Biol Chem*, *290*(12), 7930-7942. https://doi.org/10.1074/jbc.M114.627810

López-Castejón, G., Young, M. T., Meseguer, J., Surprenant, A., & Mulero, V. (2007). Characterization of ATP-gated P2X7 receptors in fish provides new insights into the mechanism of release of the leaderless cytokine interleukin-1 beta. *Mol Immunol*, *44*(6), 1286-1299. https://doi.org/10.1016/j.molimm.2006.05.015

Ly, D., Dongol, A., Cuthbertson, P., Guy, T. V., Geraghty, N. J., Sophocleous, R. A., Sin, L., Turner, B. J., Watson, D., Yerbury, J. J., & Sluyter, R. (2020). The P2X7 receptor antagonist JNJ-47965567 administered thrice weekly from disease onset does not alter progression of amyotrophic lateral sclerosis in SOD1(G93A) mice. *Purinergic Signal*, *16*(1), 109-122. https://doi.org/10.1007/s11302-020-09692-4

Martínez-Cuesta, M., Blanch-Ruiz, M. A., Ortega-Luna, R., Sánchez-López, A., & Álvarez, Á. (2020). Structural and Functional Basis for Understanding the Biological Significance of P2X7 Receptor. *Int J Mol Sci*, *21*(22). https://doi.org/10.3390/ijms21228454

Masin, M., Young, C., Lim, K., Barnes, S. J., Xu, X. J., Marschall, V., Brutkowski, W., Mooney, E. R., Gorecki, D. C., & Murrell-Lagnado, R. (2012). Expression, assembly and function of novel C-terminal truncated variants of the mouse P2X7 receptor: re-evaluation of P2X7 knockouts. *Br J Pharmacol*, *165*(4), 978-993. https://doi.org/10.1111/j.1476-5381.2011.01624.x

McCarthy, A. E., Yoshioka, C., & Mansoor, S. E. (2019). Full-Length P2X(7) Structures Reveal How Palmitoylation Prevents Channel Desensitization. *Cell*, *179*(3), 659-670.e613. https://doi.org/10.1016/j.cell.2019.09.017

Medrano, M. P., Pisera-Fuster, A., Bernabeu, R. O., & Faillace, M. P. (2020). P2X7 and A(2A) receptor endogenous activation protects against neuronal death caused by CoCl(2) - induced photoreceptor toxicity in the zebrafish retina. *J Comp Neurol*, *528*(12), 2000-2020. https://doi.org/10.1002/cne.24869

Moura, G., Lucena, S. V., Lima, M. A., Nascimento, F. D., Gesteira, T. F., Nader, H. B., Paredes-Gamero, E. J., & Tersariol, I. (2015). Post-translational allosteric activation of the P2X7 receptor through glycosaminoglycan chains of CD44 proteoglycans. *Cell Death Discov*, *1*, 15005. https://doi.org/10.1038/cddiscovery.2015.5

Nicke, A., Kuan, Y. H., Masin, M., Rettinger, J., Marquez-Klaka, B., Bender, O., Górecki, D. C., Murrell-Lagnado, R. D., & Soto, F. (2009). A functional P2X7 splice variant with an alternative transmembrane domain 1 escapes gene inactivation in P2X7 knock-out mice. *J Biol Chem*, *284*(38), 25813-25822. https://doi.org/10.1074/jbc.M109.033134

North, R. A. (2002). Molecular physiology of P2X receptors. *Physiol Rev*, *82*(4), 1013-1067. https://doi.org/10.1152/physrev.00015.2002

Ohnishi, Y., Yamamoto, M., Sugiura, Y., Setoyama, D., & Kishima, H. (2021). Rostro-caudal different energy metabolism leading to differences in degeneration in spinal cord injury. *Brain Commun*, *3*(2), fcab058. https://doi.org/10.1093/braincomms/fcab058

Ou, A., Gu, B. J., & Wiley, J. S. (2018). The scavenger activity of the human P2X7 receptor differs from P2X7 pore function by insensitivity to antagonists, genetic variation and sodium concentration: Relevance to inflammatory brain diseases. *Biochim Biophys Acta Mol Basis Dis*, *1864*(4 Pt A), 1051-1059. https://doi.org/10.1016/j.bbadis.2018.01.012

Pellegatti, P., Falzoni, S., Pinton, P., Rizzuto, R., & Di Virgilio, F. (2005). A novel recombinant plasma membrane-targeted luciferase reveals a new pathway for ATP secretion. *Mol Biol Cell*, *16*(8), 3659-3665. https://doi.org/10.1091/mbc.e05-03-0222

Pellegatti, P., Raffaghello, L., Bianchi, G., Piccardi, F., Pistoia, V., & Di Virgilio, F. (2008). Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. *PLoS One*, *3*(7), e2599. https://doi.org/10.1371/journal.pone.0002599

Rump, A., Smolander, O. P., Ruutel Boudinot, S., Kanellopoulos, J. M., & Boudinot, P. (2020). Evolutionary Origin of the P2X7 C-ter Region: Capture of an Ancient Ballast Domain by a P2X4-Like Gene in Ancient Jawed Vertebrates. *Front Immunol*, *11*, 113. https://doi.org/10.3389/fimmu.2020.00113

Sanz, J. M., Chiozzi, P., Ferrari, D., Colaianna, M., Idzko, M., Falzoni, S., Fellin, R., Trabace, L., & Di Virgilio, F. (2009). Activation of microglia by amyloid {beta} requires P2X7 receptor expression. *J Immunol*, *182*(7), 4378-4385. https://doi.org/10.4049/jimmunol.0803612

Schneider, M., Prudic, K., Pippel, A., Klapperstück, M., Braam, U., Müller, C. E., Schmalzing, G., & Markwardt, F. (2017). Interaction of Purinergic P2X4 and P2X7 Receptor Subunits. *Front Pharmacol*, *8*, 860. https://doi.org/10.3389/fphar.2017.00860

Schwarz, N., Drouot, L., Nicke, A., Fliegert, R., Boyer, O., Guse, A. H., Haag, F., Adriouch, S., & Koch-Nolte, F. (2012). Alternative splicing of the N-terminal cytosolic and transmembrane domains of P2X7 controls gating of the ion channel by ADP-ribosylation. *PLoS One*, *7*(7), e41269. https://doi.org/10.1371/journal.pone.0041269

Sluyter, R. (2017). The P2X7 Receptor. *Adv Exp Med Biol*, *1051*, 17-53. https://doi.org/10.1007/5584_2017_59

Sluyter, R., & Stokes, L. (2011). Significance of P2X7 receptor variants to human health and disease. *Recent Pat DNA Gene Seq*, *5*(1), 41-54. https://doi.org/10.2174/187221511794839219

Stefanova, E. E., & Scott, A. L. (2022). Purinergic signaling systems across comparative models of spinal cord injury. *Neural Regen Res*, *17*(11), 2391-2398. https://doi.org/10.4103/1673-5374.338993

Stelmashenko, O., Lalo, U., Yang, Y., Bragg, L., North, R. A., & Compan, V. (2012). Activation of trimeric P2X2 receptors by fewer than three ATP molecules. *Mol Pharmacol*, *82*(4), 760-766. https://doi.org/10.1124/mol.112.080903

Stokes, L., Bidula, S., Bibič, L., & Allum, E. (2020). To Inhibit or Enhance? Is There a Benefit to Positive Allosteric Modulation of P2X Receptors? *Front Pharmacol*, *11*, 627. https://doi.org/10.3389/fphar.2020.00627

Suadicani, S. O., Brosnan, C. F., & Scemes, E. (2006). P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca2+ signaling. *J Neurosci*, *26*(5), 1378-1385. https://doi.org/10.1523/JNEUROSCI.3902-05.2006

Tsao, H. K., Chiu, P. H., & Sun, S. H. (2013). PKC-dependent ERK phosphorylation is essential for P2X7 receptor-mediated neuronal differentiation of neural progenitor cells. *Cell Death Dis*, *4*, e751. https://doi.org/10.1038/cddis.2013.274

Virginio, C., MacKenzie, A., North, R. A., & Surprenant, A. (1999). Kinetics of cell lysis, dye uptake and permeability changes in cells expressing the rat P2X7 receptor. *J Physiol*, *519 Pt 2*(Pt 2), 335-346. https://doi.org/10.1111/j.1469-7793.1999.0335m.x

Vultaggio-Poma, V., Sarti, A. C., & Di Virgilio, F. (2020). Extracellular ATP: A Feasible Target for Cancer Therapy. *Cells*, *9*(11). https://doi.org/10.3390/cells9112496

Wang, X., Arcuino, G., Takano, T., Lin, J., Peng, W. G., Wan, P., Li, P., Xu, Q., Liu, Q. S., Goldman, S. A., & Nedergaard, M. (2004). P2X7 receptor inhibition improves recovery after spinal cord injury. *Nat Med*, *10*(8), 821-827. https://doi.org/10.1038/nm1082

Wilmes, M., Pinto Espinoza, C., Ludewig, P., Stabernack, J., Liesz, A., Nicke, A., Gelderblom, M., Gerloff, C., Falzoni, S., Tolosa, E., Di Virgilio, F., Rissiek, B., Plesnilla, N., Koch-Nolte, F., & Magnus, T. (2022). Blocking P2X7 by intracerebroventricular injection of P2X7-specific nanobodies reduces stroke lesions. *J Neuroinflammation*, *19*(1), 256. https://doi.org/10.1186/s12974-022-02601-z

Yamamoto, M., Kamatsuka, Y., Ohishi, A., Nishida, K., & Nagasawa, K. (2013). P2X7 receptors regulate engulfing activity of non-stimulated resting astrocytes. *Biochem Biophys Res Commun*, *439*(1), 90-95. https://doi.org/10.1016/j.bbrc.2013.08.022

Zhang, Y., Cheng, H., Li, W., Wu, H., & Yang, Y. (2019). Highly-expressed P2X7 receptor promotes growth and metastasis of human HOS/MNNG osteosarcoma cells via

PI3K/Akt/GSK3β/β-catenin and mTOR/HIF1α/VEGF signaling. *Int J Cancer*, *145*(4), 1068-1082. https://doi.org/10.1002/ijc.32207

Zimmermann, H. (2000). Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol*, *362*(4-5), 299-309. https://doi.org/10.1007/s002100000309