Neuroprotection induced by the adenosine A_1-receptor and IL-6: Do they use the same mechanisms?

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Summary:

In many chronic neurodegenerative diseases, as well as in acute conditions, there is excess of glutamate which results in a toxic environment for neurons. This toxicity is responsible for the neuronal cell death found in these diseases. Substances like adenosine and Interleukin-6 (IL-6) have been shown to induce protection against this glutamate toxicity. Adenosine exerts this neuroprotective effect via the adenosine A_1-receptor. However the mechanisms by which IL-6 induces this neuroprotection are still unclear. A connection between the two seemed likely because they were found in many of the same conditions. It has been shown that IL-6 is released from astrocytes by activation of the adenosine A_{2b}-receptor. In other studies is has been shown that IL-6 upregulates the adenosine A_1-receptor. It is also shown that without the upregulation of the A_1-receptor by IL-6 the receptor has no neuroprotective properties via the A_1-receptor. This could mean that the adenosine protection effect needs IL-6. These facts all support a link between adenosine and IL-6 neuroprotection. It could be that the neuroprotective properties assigned to IL-6 could all be due to it upregulating the adenosine A_1-receptor. Also it could be that IL-6 uses the same mechanisms for its protection. Although the mechanisms by which IL-6 induces protection are not clear the release of IL-6 is not only dependent on adenosine. The $P2Y_1$-receptor on the astrocyte can also release IL-6 when its ligand ATP binds to it. All this information could eventually be used in a treatment for the conditions glutamate toxicity causes neuronal death.

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Introduction:

Many chronic neurodegenerative diseases such as Alzheimer's disease, Multiple sclerosis (MS), Huntington's disease and Amyotrophic Lateral Sclerosis (ALS) have neuronal death by an excess of glutamate in common (Choi 1988a). This excess of glutamate has a toxic effect in the neurons. Glutamate toxicity can also be found in acute conditions such as hypoglycaemia, seizures, stroke, ischemia or trauma (Choi 1988b).

Under normal conditions, glutamate concentration can be increased up to 1mM in the synaptic cleft, which is rapidly decreased in the lapse of milliseconds. However when there is an excess of glutamate around the synaptic cleft, the neuron undergoes apoptosis. Apoptosis occurs when receptors for glutamate such as the Nmethyl- D-aspartate (NMDA)- receptor and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)- receptor are over activated. Pathologically high levels of glutamate allow high levels of calcium ions to enter the cell via their receptors (Manev et al. 1989; Choi 1988b). Ca^{2+} influx into cells activates a number of enzymes, including phospholipases, endonucleases, and proteases. Resulting in the opening of the mitochondrial permeability transition pore in the cytosol. This pore opens when the organelles absorb too much calcium. Opening of the pore can cause mitochondria to swell and release proteins thus leading to apoptosis and can also cause mitochondria to release more calcium.

Under pathological conditions like ischemia and seizure the energy supply in the brain decreases. In this case ATP is metabolized into adenosine and transported out of the cell. Adenosine is essential for energy consumption and energy supply. In order to maintain a stable cellular energy content, adenosine can suppress neuronal firing (reduce energy consumption) and increase the cerebral blood flow (Haas and Selbach 2000). By these actions adenosine can induce protection of neuronal cells during these pathological conditions.

Inadequate ATP production resulting from brain trauma can eliminate electrochemical gradients of certain ions. Glutamate transporters require the maintenance of these ion gradients in order to remove glutamate from the extracellular space. The loss of ion gradients results not only in the halting of glutamate uptake, but also in the reversal of the transporters, causing them to release glutamate and aspartate into the extracellular space. This results in a build-up of glutamate and further damaging activation of glutamate receptors (Siegel 1999).

Glutamate activates several classes of metabotropic receptors and three major types of ionotropic receptor. These later receptors are ligand gated ionic channels permeable to Na^{+} and K^{+} and, depending on the subtype, also permeable to Ca^{2+}. AMPA-receptors are largely impermeable to Ca^{2+} and participate in most forms of fast synaptic transmission. The NMDA-receptor is only receptor activated under certain conditions. One of these conditions is an excess of glutamate. The NMDA-receptor has three main features (fig.1):

1. High permeability to Ca^{2+} ions
2. Voltage-dependent lock by Mg^{2+} ions
3. Slow gating kinetics

These features make NMDA-receptors, under normal conditions, suitable for mediating plastic changes in the brain. An example of such plastic changes is long term potentiation. This is a phenomenon seen in brain slices and *in vivo* and believed to model basic mechanisms of memory formation (Danysz and Parsons 2003).

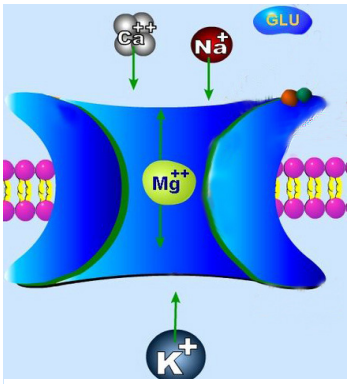


Figure 1 NMDA-receptor. The Mg^{2+} blockade in the channel. On the extracellular domain the binding sites for agonists such as glutamate. The Mg^{2+} blocade prevents ions such as Ca^{2+} and Na^{+} from coming in and K^{+} from going out though its channel.

When there is too much glutamate the brain has its own defensive system. In pathological conditions ATP is hydrolyzed.The hydrolysis of ATP produces adenosine. When adenosine diffuses outside the cell the extracellular adenosine is shown to have neuroprotective properties and may be a feedback mechanism to counteract the excess of glutamate. The most important receptor by which adenosine mediates this neuroprotection is the adenosine A_1-receptor. This receptor is shown to induce neuroprotection in many different ways. It has a neuroprotective effect during seizures (Angelatou et al. 1991), it down regulates the NMDA-receptor (de.Mendonca A. et al. 1995; Sebastiao et al. 2000), stabilises the membrane potential (Gerber and Gahwiler 1994; Trussell and Jackson 1985) and causes inhibition of glutamate release in the presynaptic neuron (Barrie and Nicholls 1993; Masino et al. 2002). Also the adenosine A_{2b}-receptor plays a role in neuroprotection. This receptor may have a role in the secretion of IL-6, which is also been found to have neuroprotective properties (Hama et al. 1989). In recent studies it was hypothesized that because the A_1-receptor and IL-6 were released under the same conditions that they might be related (Biber et al. 2008).

In this thesis there will be discussed what is known about the mechanisms by which adenosine induces neuroprotection via the A_1-receptor and if these are the same mechanisms by which IL-6 induces neuroprotection. I expect that at least part of the IL-6 protection pathway is due to its link with the adenosine neuroprotection pathway.

Adenosine induced neuroprotection:

Basic function:

Adenosine is present in all tissues of mammalian organisms. It is formed within cells as a result of hydrolysis of ATP through the action of ecto-5'-nucleotidase. The formation of adenosine therefore depends upon ATP breakdown and synthesis. In the extracellular compartment, the level of adenosine is dependent upon the direct hydrolysis of ATP into adenosine. ATP

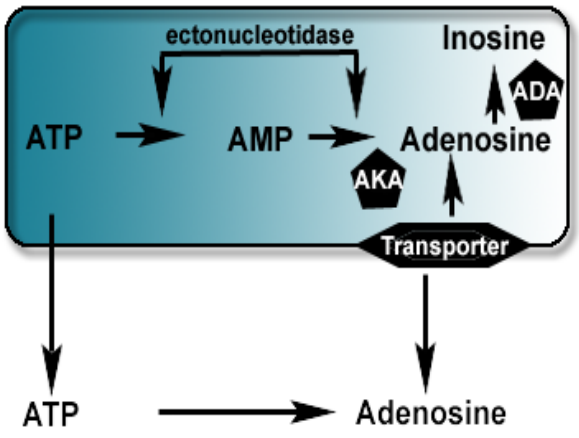


Figure 2 The mechanism by which ATP is metabolized into adenosine by enzymes. AKA is adenosine kinase and converts AMP into adenosine and ADA is adenosine deaminase which metabolizes adenosine into Inosine.

is released from both neurons and glial cells (Wardas 2002). Extracellularly adenosine concentrations are normally kept in equilibrium by specific reuptake mechanisms working via bidirectional transporters (Zimmermann H. and Braun N. 1995); (fig.2).

Two intracellular enzymes are important to adenosine metabolism: adenosine deaminase (ADA) and adenosine kinase (AKA). ADA has a high capacity for adenosine. It removes an amine group from adenosine and produces inosine,. Inosine is then metabolised by purine nucleoside phosphorylase to hypoxanthine and ribose-1-phosfate. Hypoxanthine is either oxidized by xanthine oxidase to xanthine, or converted to inosine mono-phosfate by hypoxanthine-guanine phosphotibosyl tranferase (HGPRT) for purine salvage. HGPRT activity is high in the brain while xanthine oxidase is very low in neurons and glial cells. AKA phosphorylates adenosine to AMP which can be phosphorylated into ADP and then to ATP. AKA has a low capacity for adenosine (Zamzow et al. 2008).

Adenosine exerts its effect via its receptors. Adenosine receptors are present on axon terminals of excitatory neurons whose putative neurotransmitter is glutamate (Goodman and Weigle 1983; Wojcik and Neff 1983). These receptors are located on the cell membrane and they belong to the G-protein coupled class of receptors. The different types of adenosine receptors are A_1, A_{2a}, A_{2b}, and A_3 (Wardas 2002). A_1-receptors are widely distributed in the brain and are present on neurons and glial cells. The highest expression of the A_1-receptor has been found in the cortex, cerebellum, thalamus and hippocampus (Wardas 2002). In the hippocampus a large amount of A1-receptors are located on the intrinsic neurons and the receptor is found in great densities in the CA_1 area (Corradetti et al. 1984). The A_1-receptor is responsible for the protection of neuronal cells against damage. The A_1-receptor exerts this effect in different mechanism both presynaptically and postsynaptically.

Other forms of adenosine protection have been found. Such as the fact that adenosine inhibits free radicals protecting the cells from hyper oxidation (de Mendonca A. et al. 2000). Next to that, adenosine is a crucial factor in the control of cerebral circulation (de Mendonca A. et al. 2000), and causes vasodilatation of cerebral arteries resulting in a reduction of the negative consequences seen in ischemia (Muramatsu et al. 1980; Wardas 2002). In addition, adenosine may prevent leukocytes sticking to endothelial cells in blood vessels and thereby help control second seizures (Cronstein et al. 1986). Moreover, the drop in body temperature seen with adenosine analogues should contribute to the neuroprotection (de Mendonca A. et al. 2000).

Adenosine protection mechanism:

Adenosine mediates neuroprotection via some different pathways both presynaptically and postsynaptically. The effect of the adenosine A_1-receptor has been examined in many different ways with many different agonists and antagonists. The direct acting adenosine A_1-receptor agonist, such as N^6- cyclopentyladenosine (CPA) and 2-chloro-N6-cyclopentyladenosine (CCPA), are known to mediate this neuroprotection. It inhibits the glutamate release from the synaptic terminal and depresses the electrophysiological response in hippocampal slices (Corradetti et al. 1984).

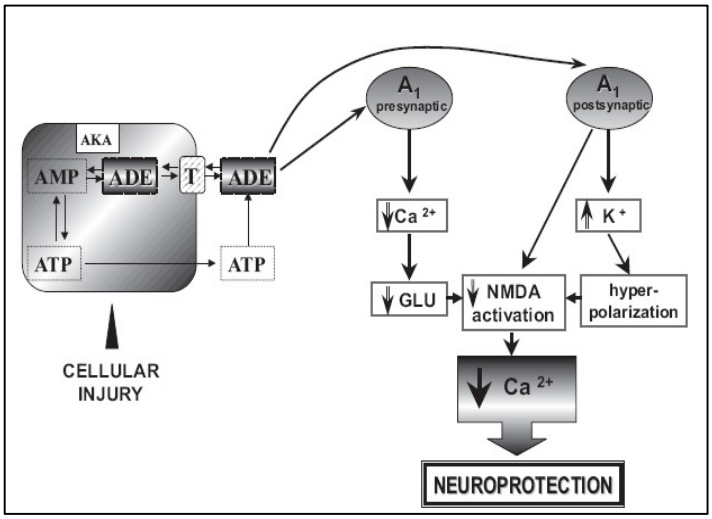


Figure 3 modified from (Wardas 2002). The cellular injury like stroke or glutamate toxicity result in extracellular adenosine. This is binds to both presynaptic and postsynaptic A_1-receptors that have various effects. Presynaptically it inhibits calcium influx and reduces glutamate release. Postsynaptically it opens potassium channels and induces hyperpolarization. Less glutamate and hyperpolarization results in less activation of the NMDA-receptor which leads to less calcium influx. This decreases the apoptosis and protects the neurons.

The released adenosine that acts on the presynaptic adenosine A_1-recpetor may reduce the influx of Ca^{2+} through voltage-dependent calcium channels and thus may inhibit the release of glutamate which result in a reduction of its excitatory effect on the postsynaptic membrane (Braun et al. 1998; Lee and Lowenkopf 1993); (fig.3).By inhibiting the release of glutamate, adenosine reduces the NMDA-receptor activation and inhibits the NMDA mediated influx of CA^{2+} (fig.3) which is the main reason why there is apoptosis in these cells (Braun et al. 1998; Klotz et al. 1999). Also postsynaptically A_1-receptor activates K^+-channels and hyperpolarizes the cell. This makes it harder for the cell to be stimulated (Braun et al. 1998; Klotz et al. 1999). In addition the A_1-receptor can postsynaptically stabilize the Mg^{2+} blockade on the NMDA-receptor (fig.3). It has been shown that both adenosine and A_1-receptor agonists (CHA, CPA, CCPA, CADO, R-PIA) reduced the neuronal damage by excitotoxicity whereas antagonists (CPT, DPCPX) caused more cell death (Wardas 2002). Indicating that the A1-receptor is responsible for the neuroprotection. Interestingly, chronic treatment with agonists (Von Lubitz et al. 1994) along with antagonists, in separate experiments, show an inversion of the effects. For example the antagonist caffeine when chronically applied, showed an increase in neuronal survival (Rudolphi et al. 1989; Rudolphi and Schubert 1997; Von Lubitz et al. 1994). This inversion may be the result of an upregulation of the A_1-receptor in response to the antagonist, and in the case of the agonist there is desentization and downregulation when the antagonist is chronically applied (de Mendonca A. et al. 2000).

The inhibitory action of adenosine on neurotransmitter release may enable adenosine to act as an anti-epileptic agent (Dragunow and Goddard 1984). This possible anti-epileptic action can be exerted through the central adenosine A_1-receptor (Angelatou et al. 1993) as sited in (Angelatou et al. 1991; Barraco et al. 1984; Dichter and Ayala 1987; Kostopoulos 2009). The possibility of an anti-epileptic action of adenosine was examined by inducing chemical seizures and looking at the effect on the adenosine A_1-receptor. The Pentylenetetrazole (PTZ) induction for seizures results in the upregulation of A_1-receptor. In addition, the PTZ induced seizure latency changes with daily successive PTZ injection in a dose dependent manner and the pattern of this change in latency seems to be correlated in time to changes in adenosine A_1-receptor density in the cortex and cerebellum (Angelatou et al. 1991). In the normal human temporal cortex, the adenosine receptors are equally distributed in the six layers. In epileptic patients the density is also equally distributed. In these patients the A_1-receptor is upregulated in these regions. The upregulation of adenosine A_1-receptors might be due to neuronal hyperactivity of the epileptic tissue (Angelatou et al. 1993).

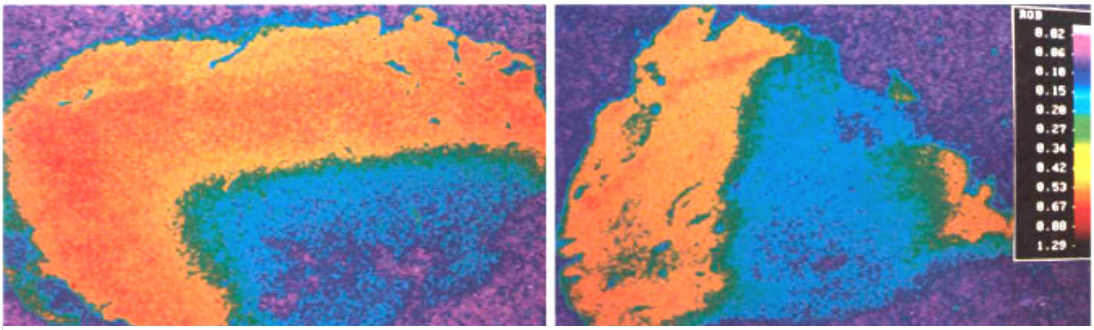
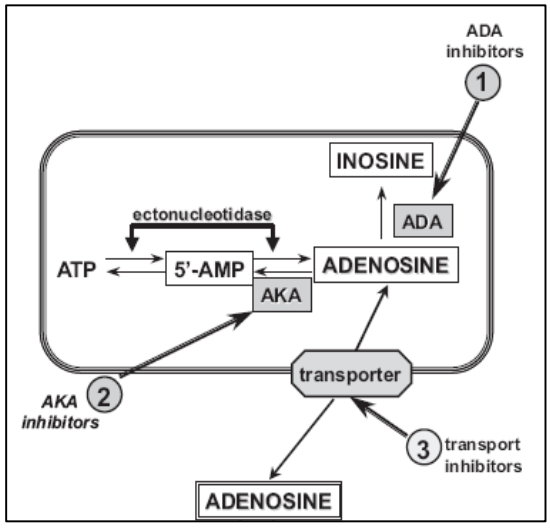


Figure 3 (Angelatou et al. 1993) Computer-generated, color-coded autoradiographic images showing the distribution of A_1 adenosine receptors as detected by quantitativeautoradiography, using the ligand [3H]CHA (12 nM), in temporal cortical tissue obtained from epileptic and non-epileptic patients. In the left image it shows the patients tissue and in the right image, the control patients without epilepsy). The color bar displayed next to the "control section' indicates graduation of relative optical density (ROD), which is converted to density values of A_1- receptors (fmoles [3H]CHA/mg tissue). The epileptic patients show a increase in binding the ligand and thus an increase in the amount of A_1-recptor.

Figure 4 modified from (Wardas 2002) The possible areas where pharmaceutical drugs may be effective in increasing the extracellular adenosine. Adenosine kinase (AKA) inhibitors for inhibiting adenosine being metabolized into AMP. Adenosine deaminase (ADA) inhibitors fro preventing adenosine to be converted into inosine and transporter inhibitors for inhibiting adenosine re-uptake.



Substances that increase the extracellular level of adenosine could also be protective against ischemia and seizures. For instance substances that prevent degradation of adenosine, such as ADA and AKA inhibitors, or substances that prevent their transport back into the cell (Wardas 2002) (fig.4). For instance 2-deoxycoformycin inhibits ADA preventing

histological changes in the hippocampus by decreasing the infarct area and neuronal degradation (Gidday et al. 1995; Lin and Phillis 1992; Phillis and O'Regan 1989) as seen in (Wardas 2002). The same effect has been observed with EHNA, also an ADA inhibitor, in retina ischemia (Larsen and Osborne 1996). Furthermore, AKA inhibitors have been seen to provide neuroprotection (Jiang et al. 1997; Miller et al. 1996; Phillis and Smith-Barbour 1993; Tatlisumak et al. 1998) as seen in (Wardas 2002). Additionally the adenosine transporter might be blocked in order to prevent the re-uptake of adenosine. However, most transporters for adenosine are bidirectional and inhibition of these transporters will also prevent adenosine from exiting the cell (Fredholm B.B. 1998; Thorn and Jarvis 1996; Wardas 2002; Zimmermann H. and Braun N. 1995). Likewise, adenosine can be amplified using positive allosteric modulation of adenosine binding to A_1-receptors. This is a long recognized manner to control the protein function. Modulators bind to regulatory sites that are different from the active site on the protein. This results in conformational changes that may influence protein function. Allosteric modulation with PD 81723 attenuated the weight loss of the effected cerebral hemisphere in a focal ischemia (de Mendonca A. et al. 2000).

But adenosine is not the only substance that can induce neuroprotection. Next to the protection seen by adenosine, interleukin-6 (IL-6) has been found to show neuroprotection against many of the same conditions as adenosine.

IL-6 neuroprotection:

Basic function:

IL-6 is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine. Although cytokines were believed to be solely involved in regulation of the immune response in the brain and periphery, now it has been obvious that these cytokines have a neuroprotective property (Carlson et al. 1999; Hindley et al. 1994; Stoll et al. 2000) as seen in (M.C.Wittendorp 2004). IL-6 was first thought to be harmful because it was found in many diseases and in some cases is seems to be neurodegenerative , but now it seems as though the increased levels may indicate an intrinsic response to counteract brain damage (M.C.Wittendorp 2004; Gadient and Otten 1997; Jankowsky and Patterson 2001). Brain damage in general activates glial cells in the brain, mostly microglia and astrocytes. These activated microglia and astrocytes promote neuronal survival by supporting damaged neurons (Bruce-Keller 1999; M.C.Wittendorp 2004). They release factors aimed to protect neurons from brain damage such as IL-6 (M.C.Wittendorp 2004).

IL-6 has been found in many of the same neurodegenerative diseases as adenosine. IL-6 is present at low levels in physiological conditions but increases dramatically during chronic and acute disorders including Alzheimer's disease, Parkinson's disease, MS, trauma, and ischemia (Gadient and Otten 1997)as sited in (Ali et al. 2000; Benveniste 1998; Frei et al. 1991; Hautecoeur et al. 1997; Kossmann et al. 1996; Maimone et al. 1997).

It has been shown that a chronic inflammatory process is part of the Alzheimer's pathology (Rogers et al. 1996). This is supported by the fact that prolonged treatment with non-steroidal anti-inflammatory drugs led to a reduced risk at developing

Alzheimer's disease (Breitner et al. 1994; Breitner et al. 1995)**.** And, in Parkinson's disease IL-6 is markedly elevated in the nigrostriatal dopaminergic region of Parkinson's disease patients (Mogi et al. 1994). Also, Parkinson's patients show elevated levels of IL-6 in their cerebrospinal fluid. But as in Alzheimer's disease the mechanisms remain unclear. IL-6 also dramatically rises in the CNS in acute conditions like brain damage (Kiefer et al. 1993; Kossmann et al. 1996; Woodroofe et al. 1991) and stroke (Beamer et al. 1995; Fassbender et al. 1994)**.**

IL-6 is expressed by both neuron and glial cells and when it is secreted IL-6 promotes the survival of neurons (Hama et al. 1989; Pizzi et al. 2004). IL-6 acts through a receptor complex composed of the membrane bound IL-6 receptor and the gp130 protein, which is expressed in almost every cell type. Il-6 needs the gp130 protein because it has no transducing activity on its own. Only when the IL-6 receptor forms a receptor complex with the gp130 protein is it able to activate a transduction pathway. The membrane bound IL-6 receptors are expressed by both glial and neuronal cells (Gahring et al. 1996; Schneider et al. 1998; Schobitz et al. 1992; Tchelingerian et al. 1993). The gp130 protein is a transducing subunit used by the entire IL-6-type cytokine family (Elson et al. 2000; Keller et al. 1996a). The binding of IL-6 to its receptor induces homo-dimerization of the gp130 protein, leading to an activation of the Janus Kinase signal transducer and activator of transcription (JAK/STAT3) signalling pathway. This causes tyrosine phosphorylation of STAT3 and its translocation to the nucleus (Heinrich et al. 2003c)(fig.6).

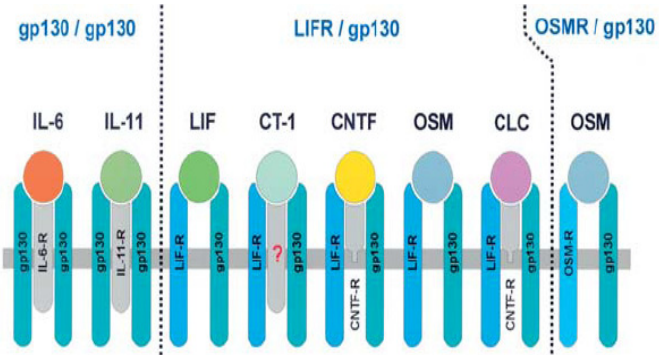


Figure 5 IL-6-type receptor complexes (Heinrich et al. 2003a). The IL-6 type cytokines each have a different receptor complex but they all have a gp130 protein. IL-6 and IL-11 have two gp130 proteins while the others have next to their own receptor and gp130 also another protein.

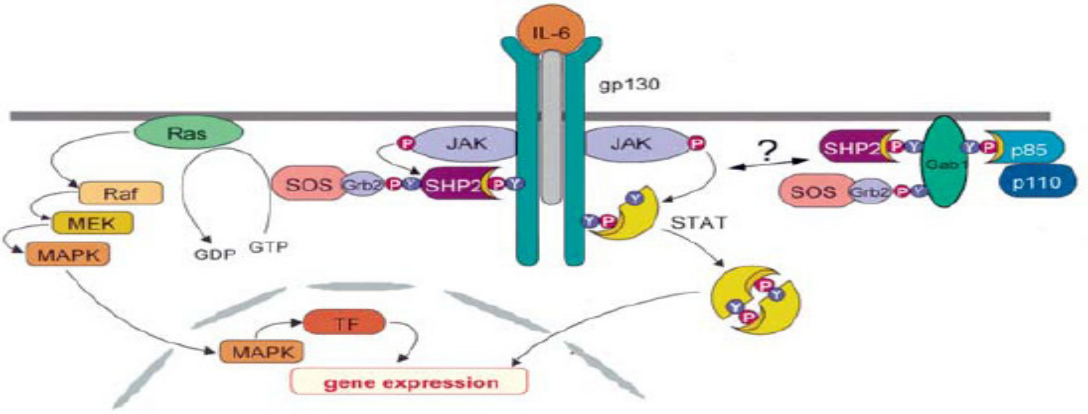


Figure 6 IL-6 signal transduction (Heinrich et al. 2003b). The two major pathways by which IL-6 induces transcription. The JAK signaling via transcription factors (TF) and the STAT signaling.

Next to the membrane bound receptors there is a soluble form of IL-6-receptor (sIL-6R)(Marz et al. 1999; Novick et al. 1989; Novick et al. 1990). When this receptor is linked to IL-6 (Keller et al. 1996) the receptor induces dimerization of gp130. This process is called transsignaling. The soluble form of the IL-6 receptor can be made by either IL-6-receptors shedding from the membrane, or by alternatively splicing of the mRNA for the receptor (Knupfer and Preiss 2008) (fig.7).Because of this, the effect of IL-6 is greatly increased because now IL-6 can activate cells that do not have the membrane bound receptor by directly interacting with the gp130 protein (Pizzi et al. 2004).

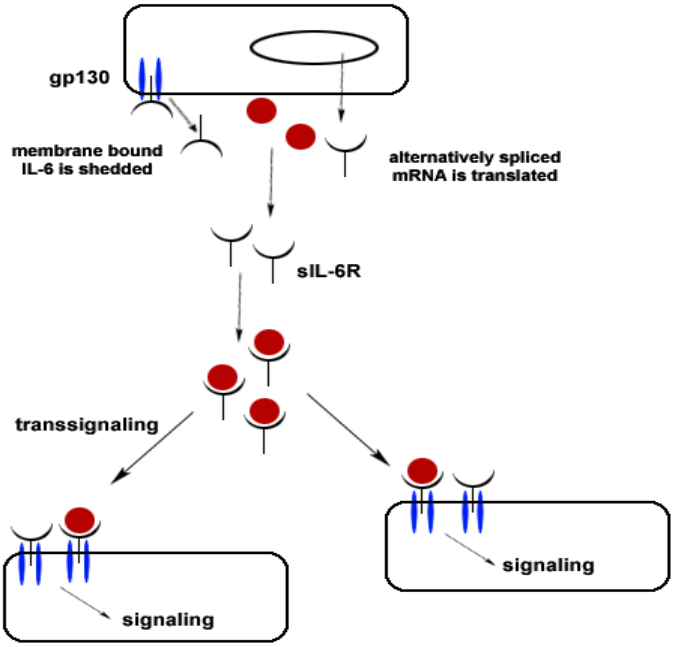


Figure 7 transsingaling of sIL-6R. modified from (Gadient and Otten 1997; Knupfer and Preiss 2008) The soluble form of the IL-6 receptor is either shedded or translated in the cell, then it is secreted. Then it binds to IL-6 and it attaches to the membrane and forms a receptor complex with the gp130 protein. This induces signaling.

IL-6 protection mechanism:

In recent studies IL-6 has been shown to have neuroprotective properties. The mechanisms by which this neuroprotection is carried out are complicated and unclear, since injury to the CNS leads to an inflammatory response from microglia, astrocytes, and brain macrophages ((Wang et al. 2009). This complexity is confirmed by the bidirectional effect of IL-6. This bidirectional effect is both dose and time-dependent. IL-6 concentrations up to 500 ng show neuroprotection however, higher concentrations induce neurodegeneration (Loddick et

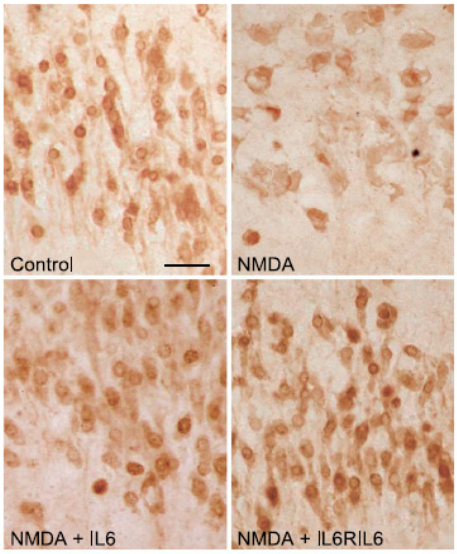


Figure 8 cell survival after NMDA excitotoxicity (Pizzi et al. 2004) neuron immunoreactivity in the CA1 region of hippocampal slices exposed NMDA with or without IL6RIL6 and IL-6 .both Il-6 and IL-6RIL-6 showed a rescue of neurons.

al. 1998)**.** The time-dependent effect is apparent in the fact that chronic application of IL-6 has seen to be neurodegenerative. However in several studies, a protective effect has been shown. IL-6 prevented cerebellar granule neurons from the decrease in neuronal vitality, the increase in apoptotic neurons, and the overload of intracellular Ca^{2+} induced by glutamate and NMDA. NMDA being a NMDA-receptor agonist mimicking glutamate toxicity, suggesting that IL-6 has neuroprotective properties (Wang et al. 2009). An excessive activation of the NMDA receptors by either glutamate or NMDA has been shown to be a cause of neurodegeneration (Beal 1992; Pizzi et al. 2004; Whetsell, Jr. 1996). IL-6 promotes cell survival when cells are incubated with the excess of NMDA. Cell survival is higher when you stimulate cells with IL-6 and with IL-6R+IL-6, a chimeric protein obtained by fusion of the coding sequence of the naturally occurring sIL-6R and IL-6 (Chebath et al. 1997; Pizzi et al. 2004; Pizzi et al. 2004) (fig. 8).

The protection mechanisms are not as clear as in the adenosine mechanism because only recently has this effect of IL-6 been discovered. In spite of these uncertainties it has been suggested that IL-6 protect neurons by activation of by both JAK/STAT3 and RAS/MAPK pathways (Wang et al. 2009) (fig. 6). These pathways have been shown to inhibit the NMDA-induced decrease of neuronal vitality and inhibit the enhancement of caspase-3 activation. The latter being a marker for apoptosis (Wang et al. 2009). The fact that IL-6 protects neurons is further strengthened by the effect of an anti-gp130 antibody which when applied attenuated the neuroprotective effects of IL-6 (Wang et al. 2007)**.**

Adenosine A_{2b}-receptor releases IL-6:

As previously discussed, adenosine is released into the extracellular space (Rudolphi et al. 1992) during glutamate toxicity. Adenosine receptors are expressed on both neurons and glial cells.(Fredholm et al. 1994). Because glial cells are the main source of cytokines in the brain (Aloisi et al. 1992) and because levels of both cytokines and adenosine are increased following glutamate toxicity, IL-6 release from glial cells might be regulated by adenosine. Astrocytes are known to produce IL-6 in cell cultures. IL-6 is released at the same time as when glutamate and adenosine are released into the extracellular space under pathological conditions. The release of those substances under the same conditions suggest that there might be a link between IL-6 and adenosine against this glutamate toxicity. Glutamate and adenosine analogue 2-chloroadenosine (2CA) were added to primary cultured neurons and the IL-6 concentration was measured with ELISA (Schwaninger et al. 1997a). First it was determined if IL-6 was released by 2CA from microglia or from astrocytes. But in

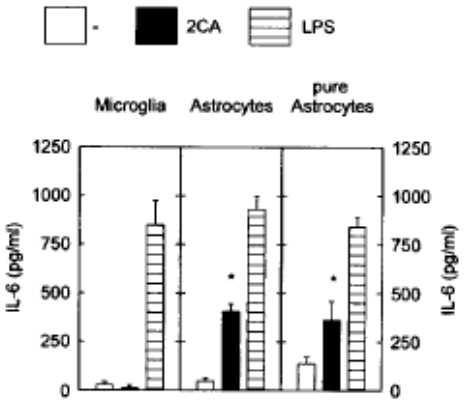


Figure 9 (Schwaninger et al. 1997b) Secretion of IL-6 from primary glial cells in the presence of the adenosine analogue 2CA or LPS. In parallel experiments microglia, astrocytes, or purified astrocytes were stimulated.

microglia it was LPS that stimulated release of IL-6 and only on astrocytes had 2CA an significant effect (Schwaninger et al. 1997d)(fig. 9).

To test which adenosine receptor was responsible for the release of IL-6 from astrocytes several non-selective and selective agonists were added. The A_1-receptor agonist CPA, the selective agonist for the A_{2a}-receptor CGS-21680, and the non-selective agonist 2CA were added to astrocytes and IL-6 concentration were determined. Only 2CA showed a significant increase in IL-6, indicating that the A_1 or A_{2a}-receptor was not responsible. However, the non-selective agonist did show a response so there is an adenosine receptor involved. In parallel Fiebich et al 1996 examined the effect of CPA, NECA and some antagonist. Only NECA had a significant effect. NECA was added to cell culture and the expression of IL-6 protein was determined (Fiebich et al. 1996b) (fig.10). This further strengthened the hypothesis that occupation of an adenosine receptor is responsible for the synthesis and release of IL-6.

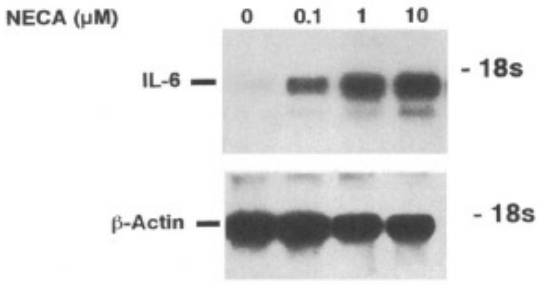


Figure 10 NECA stimulation and Il-6 expression (Fiebich et al. 1996a). Northern Blot experiment. The northern blot was hybridized with a cDNA probe coding for IL-6 or for β-actin. NECA increases the expression of IL-6 in a dose dependent manner.

To further examine the receptors involved, antagonists were added. The A_1-receptor antagonist DPCPX only inhibits IL-6 secretion at concentrations of 1µM at which it is known that DPCPX binds the A_{2b}-receptor (Yakel et al. 1993). These results suggest that the A_{2b}-receptor occupancy induces IL-6 secretion in primary cultured neurons (Schwaninger et al. 1997c). Because the A_{2b}-receptor is a low affinity receptor, it will only be activated if the concentrations of adenosine are high such as during brain injury (Feoktistov and Biaggioni 1997; Stevens et al. 2002).

Not only *in vitro,* but also *in vivo* has the effect of the A_{2b}-receptor on the release of IL-6 been found. Using microdialysis, the IL-6 concentration was measured in the presence of NECA. In animals treated with NECA there was a significant increase of the IL-6 concentration, after 60 min of when the NECA was administered. Adding DPCPX in a concentration of 10 µmom/L had no effect on the NECA induced release of IL-6. Whereas MRS 1706, an A_{2b}-receptor antagonist, counteracted the effect of NECA on IL-6 release (Vazquez et al. 2008).

Another study showed that the $P2Y_1$-receptor may also play a part in the release of IL-6 (Fujita et al. 2009). The $P2Y_1$-receptor is a purigenic receptor that can bind ATP. ATP, next to being a supply of energy, is one of the principle mechanisms underlying neuron to astrocyte communication and is important for the normal brain function (Fujita et al. 2009). ATP released from neurons coordinately activates astrocytes through the mobilization of their internal Ca^{2+} stores which triggers the release of chemical

transmitters from astrocytes. This causes feedback regulation of synaptic activity (Fujita et al. 2009).

In addition to this, ATP binds to the $P2Y_1$-receptor to inhibit presynaptic release of glutamate and to stimulate the release of GABA from hippocampal neurons (Fujita et al. 2009). The inhibition of glutamate and release of GABA is in and on itself, a protective mechanism. Furthermore ATP has been shown to accelerate recovery from hypoxic and hypoglycemic perturbation of hippocampal neurotransmission (Aihara et al. 2002). This protection can directly act on the neurons or through the indirect action via the release of intermediate molecules from astrocytes such as IL-6. The neuroprotection brought on by the $P2Y_1$-receptor stimulated astrocytes is probably mediated by the release of IL-6 (Fujita et al. 2009). This hypothesis is further supported by the fact that the $P2Y_1$-receptor agonist, 2MeSADP, dramatically increased the IL-6 concentration in a time and concentration dependent manner. When a anti-IL-6-antibody was applied the $P2Y_1$-receptor mediated neuroprotection was abolished.

The downstream mechanism by which IL-6 induces this protection is still unclear. However in PC12-cells, protection of IL-6 against the neurotoxin, 6-hydroxydopamine, was carried out by activating a free radical mechanism. This lead to the proposal that IL-6 can increase the activity of antioxidative proteins including catalase. IL-6 also protects neurons from 4-hydroxy nonemal-envoken cytotoxicity by increasing the intracellular levels of glutathione (Aihara et al. 2002; Nakajima et al. 2002). Generally is has been accepted that the enzyme catalase and GSH participate in the cellular defenses against H_2O_2 toxicity (oxidative stress). The fact that IL-6 needs 12 hours to induce this protection indicates that IL-6 might need *de novo* synthesis of proteins to protect neurons (Fujita et al. 2009).

A_1-receptor and IL-6:

So it has been shown that both adenosine and IL-6 have neuroprotective properties in the same kind of conditions, and that IL-6 is synthesized and released from astrocytes by the adenosine A_{2b}-receptor (Fiebich et al. 1996b). These two findings led to the hypothesis that the neuroprotective effect of IL-6 might be due to its link to adenosine. (Biber et al. 2008; Biber et al. 2001) hypothesized that IL-6 might be responsible for the upregulation of the adenosine A_1-receptor. They have shown that IL-6 up regulates the mRNA transcripts of the adenosine A_1-receptor (Biber et al. 2001). This upregulation may in turn promote neuronal survival. That upregulation promotes neuronal survival has been confirmed by the fact that upregulation of the adenosine A_1-receptor by chronic treatment with A_1-receptor antagonists increased the neuroprotective effect of adenosine (Rudolphi et al. 1992). Furthermore stimulation with IL-6 of cultured astrocytes induces a concentration and

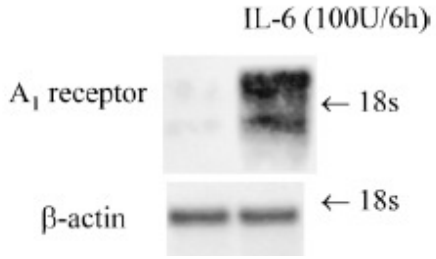


Figure 10 Northern blot analysis of polyA RNA from cultured cortical astrocytes Stimulation with IL-6 led to an upregulation of adenosine A_1 receptor mRNA expression. The control hybridisations were for β-actin.

time-dependent upregulation of adenosine A_1- receptor mRNA (Biber et al. 2001). This is accompanied in astrocytes by an increase in A_1- receptor mediated signalling via the phosphoinositide-dependent pathway.

It has been suggested that IL-6 upregulates the A_1-receptor and thus counteracting the desentization and down-regulation caused by adenosine and its agonists (Biber et al. 2001; de Mendonca A. et al. 2000). This upregulation was apparent in cultured rat cortical astrocytes and organotypic slice cultures from rat cortex (Biber et al. 2001). Stimulation of A_{2b}- receptors with high concentration of adenosine, accumulated under pathological conditions, could at least partially be responsible for the large increase in the synthesis of IL-6 in the brain observed under these conditions. And in turn IL-6 could be partially responsible for the upregulation of the A_1-receptor mRNA. The upregulation of the receptor mRNA is in parallel with the observed increase in the receptor protein (Biber et al. 2001). Later they have shown that the protein is indeed upregulated when there are large concentrations of IL-6 (Biber et al. 2008) (fig 11).

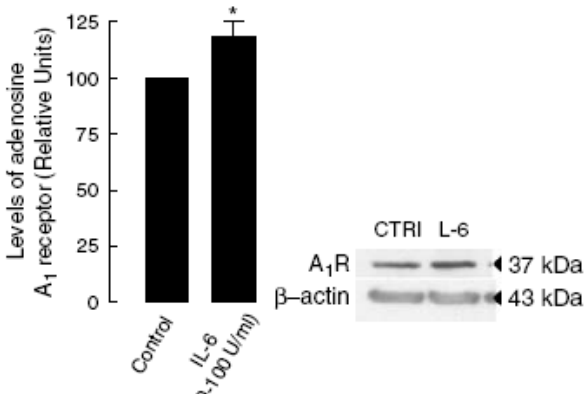


Figure 11 (Biber et al. 2008) Western blot analysis of adenosine A_1 receptor expression in nerve terminals prepared from slices hippocampal slices kept for 6–8 h in the absence or in the presence of IL-6.

Moreover by upregulating the adenosine A_1-receptor, IL-6 enhances the expression and inhibitory actions of presynaptic adenosine A_1-receptors on glutaminergic transmission (Biber et al. 2008).Whether or not IL-6 was linked with the adenosine A_1 receptor upregulation was confirmed when the A_1-receptor activity was blocked with DPCPX. The blockade had no effect on the glutamate induced death, but completely abolished the neuroprotective effect of IL-6 (Biber et al. 2008) (fig. 12. The same effect is seen when the A_1-receptor agonist CPA is added. The neuronal survival with IL-6 is higher and is enhanced with CPA in a dose dependent manner. CPA alone had no effect (fig. 13). This may indicate that without the upregulation of the receptor, the receptors are too few in number or too desensitized to cause an effect when the agonist is added.

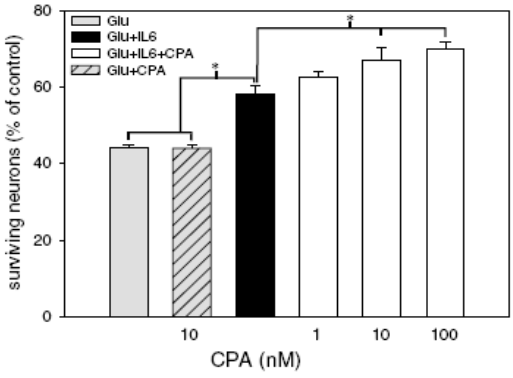


Figure 12 (Biber et al. 2008) Pre-treatment with IL-6 (10 U/ml 24 h) protected neurons from excitotoxicity induced by glutamate at various concentrations (50 mM, 25 mM, and 10 mM). Treatment with 100 nM DPCPX (15 min before glutamate treatment) completely abolished the protective effect of IL-6 treatment but did not influence the effect of glutamate without IL-6 pre-treatment.

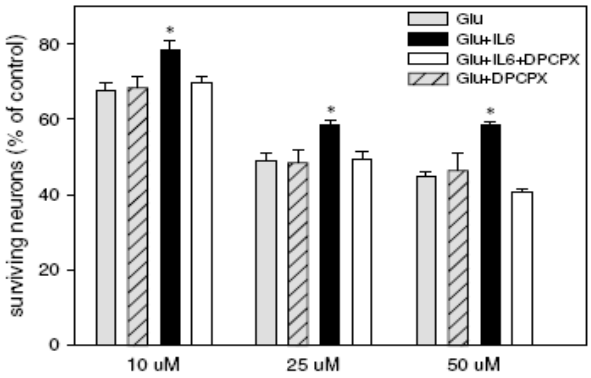


Figure 13 (Biber et al. 2008) Application of N_6-cyclopentyladenosine (CPA, 15 min before glutamate treatment) at several concentrations (1, 10, and 100 nM) significantly increased neuronal survival after glutamate treatment (50 mM) only when neurons had been pre-treated with IL-6. CPA (10 nM) without IL-6 did not affect glutamate-induced neurotoxicity.

The upregulation of A_1-receptor was already observed in seizures (Angelatou et al. 1993). In light of these new findings it was examined whether or not the seizure-induced upregulation of adenosine was also IL-6 dependent. Now it has been shown that there is no upregulation of A_1-receptor in IL-6 knock-out mice (Biber et al. 2008). To examine this, two PTZ injections on two consecutive days were given. The first injection showed a mild seizure score in both animals. While the second seizure score had no significant effect on the seizure score in the wild type mice, the IL-6 mice showed a significant increase in seizure score (fig. 14). The IL-6 knock-out mice showed a significant decrease in A_1-receptor compared to the wild type (Biber et al. 2008). This indicates that in seizures the upregulation of the A_1-receptor is also IL-6 dependent.

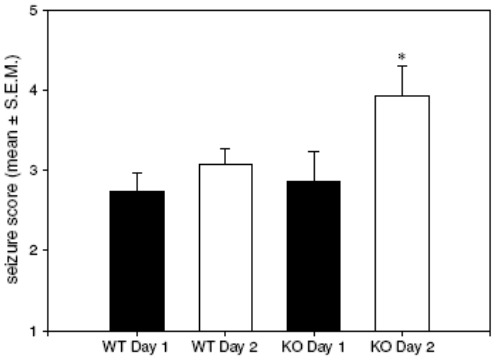


Figure 14 (Biber et al. 2008) Differences in seizure scores between wild-type (WT) and IL-6-deficient mice (KO). There was no difference in seizure scores between WT and IL-6-deficient mice after the first PTZ injection. Similar seizure scores were also detected in WT mice after the second PTZ injection round, whereas IL-6 deficient mice showed a significant increase in seizure score on the second day.

Discussion:

This study tried to find out what mechanisms are involved in neuroprotection. The generally accepted adenosine and IL-6 protection pathways were my main interest. Because both seemed to have neuroprotective properties in the same kind of diseases my question was whether or not the two mechanisms had the same basic mechanisms. The protection of adenosine on the cellular level is induced by the A_1-receptor which is present both presynaptically and postsynaptically. This receptor, downregulates NMDA-receptor activation, inhibits calcium influx thus inhibiting glutamate release and it stimulates potassium influx which hyperpolarizes the cell. Also some secondary neuroprotective effects of adenosine have been found, such as inhibition of adhesion of leukocytes, lowering of body temperature and vasodilatation have been observed as a protective mechanism by adenosine. It has been shown that the upregulation of the

adenosine A_1-receptor results in an increase in A_1-receptor mediated neuroprotection. Upregulation of the receptor can be achieved in many ways. One of these ways is by preconditioning. Moderate seizures will upregelate the receptor so it will be able to protect against an upcoming seizure. Next to seizures upregulating the receptor, the receptor can be upregulated by chronic application of a receptor antagonist. Recently it has been shown that the receptor could also be upregulated by IL-6. It could be that during seizures, when IL-6 is also released, IL-6 is the cause for the upregulation of the A_1-receptor. The upregulation that has been found in previous studies might also be IL-6 mediated. IL-6 was thought to have some neuroprotective properties of its own. Such as protective properties against NMDA toxicity and hypoxic stress. Many mysteries remain about the downstream mechanism by which IL-6 induces this neuroprotection. Part of this could be because IL-6 might not have a protective mechanism other than the upregulation of the adenosine A_1-receptor. However there are some indications that the JAK/STAT3 pathways and the RAS/MAPK pathways are involved. In hypoxic stress IL-6 might activate synthesis of the enzyme catalase and GSH which have been shown to protect cells against hypoxic stress. The fact that it takes IL-6 12 hours to induce these effects might indicate that IL-6 needs *de novo* synthesis of protein. Amongst these proteins could be the adenosine A_1-receptor protein. Additionally without the upregulation of IL-6, the adenosine A_1-receptor shows no neuroprotection, probably because the receptor is too few in numbers, or is desensitized to induce protection. The link between IL-6 and the adenosine protection mechanism is further strengthened by the fact that IL-6 is released from astrocytes by the occupation of the adenosine A_{2b}-receptor. Also IL-6 is released by astrocytes when ATP binds to the $P2Y_1$-receptor on astrocytes. This could indicate that the release and function of IL-6 in the brain is highly intertwined with ATP and its derivatives.

In light of these findings new therapies might be obtained. Directly injecting adenosine is not an option, for it has too many effects in the brain and the periphery. However as discussed previously, substances that increase the extracellular concentration of adenosine in the brain might be an answer. But there are still many obstacles to overcome. Chronic application of IL-6 has shown to be neurodegenerative and we still do not know what the long term effect of chronic IL-6 is in relation to the adenosine A_1-receptor. The relation between IL-6 and the adenosine A_1-receptor could be a reason why treatment with anti-inflammatory medication reduces the risk of Alzheimer's disease. Likewise, the neurodegenerative effect found in other studies could be dose or time-dependent. In addition to this problem, chronic application of adenosine and adenosine A_1-receptor agonists have shown to downregulate and desensitize the receptor. When these findings can be translated into a therapy, it would still be a problem for chronic diseases like Alzheimer's disease and Parkinson's disease, for chronic treatment might have the opposite effect. In acute cases these findings might be an answer. However, the induction of the mechanism takes hours because it relies on *de novo* synthesis of proteins. In acute cases the patient usually already arrives at the hospital a few hours after the seizure. If the drug is then applied, most of the damage will already have been done. However, treatment will protect patients from a second seizure by upregulating the adenosine A_1-receptor. But this protection is short-lived for after 24 hours the effect of IL-6 on the A_1-

receptor is gone and the receptor is downregulated again. It is clear that much research is still needed before this can be used as a therapy.

In conclusion the actions of adenosine and IL-6 might be linked but are not limited to each other. Adenosine has other IL-6 independent pathways and IL-6 might be released into the brain in different ways, such as the $P2Y_1$-receptor and the adenosine A_{2b}-receptor on astrocytes and by microglia in the presence of LPS.

Acknowlegdements:

I would like to thank Marrit Agema and Carlyn Mamber for proof reading this thesis and Knut Biber for supervising and grading it. Also I would like to thank Uli Eisel being the second supervisor on this thesis.

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