Published in final edited form as:

Gene Expr Patterns. 2009 March ; 9(3): 144–151. doi:10.1016/j.gep.2008.11.006.

Identification of Zebrafish A2 Adenosine Receptors and Expression in Developing Embryos

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Abstract

The A2A adenosine receptor (AdR) subtype has emerged as an attractive target in the pursuit of improved therapy for Parkinson's disease (PD). This report focuses on characterization of zebrafish a2 AdRs. By mining the zebrafish EST and genomic sequence databases, we identified two zebrafish a2a (*adora2a.1* and *adora2a.2*) genes and one a2b (*adora2b*) AdR gene. Sequence comparisons indicate that the predicted zebrafish A2 AdR polypeptides share 62–74% amino acid identity to mammalian A2 AdRs. We mapped the *adora2a.1* gene to chromosome 8, the *adora2a.2* gene to chromosome 21, and the *adora2b* gene to chromosome 5. Whole mount *in situ* hybridization analysis indicates zebrafish a2 AdR genes are expressed primarily within the central nervous system (CNS). Zebrafish are known to be sensitive to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that causes selective loss of dopaminergic neurons and PD-like symptoms in humans as well as in animal models. Here we show that caffeine, an A2A AdR antagonist, is neuroprotective against the adverse effects of MPTP in zebrafish embryos. These results suggest that zebrafish AdRs may serve as useful targets for testing novel therapeutic strategies for the treatment of PD.

Keywords

A2 adenosine receptors; central nervous system; zebrafish; Parkinson's disease; MPTP; caffeine

Adenosine is an endogenous purine nucleoside that modulates a variety of physiological processes. Adenosine is also a potent anti-inflammatory agent that plays an important role in tissue protection and repair (Jacobson and Gao, 2006). In the central nervous system (CNS), adenosine is involved in regulating neurotransmitter release as well as postsynaptic neuronal responses (Cunha 2001; Fredholm et al. 2005). The effects of adenosine are mediated via the actions of four adenosine receptor (AdR) subtypes (A1, A2A, A2B, and A3). AdRs are members of the superfamily of G-protein coupled receptors (GPCRs) and can be distinguished in part by their pharmacological profiles. The A1 and A3 AdRs are coupled to G_i proteins and inhibit adenylyl cyclase activity, whereas A2A and A2B AdRs are coupled to G_s proteins and stimulate adenylyl cyclase activity (Fredholm et al. 2001). While all four AdR subtypes are expressed in brain, the A2A receptor appears to play the most important role in the control of

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motor behavior and in the modulation of dopamine-mediated responses (reviewed in Pinna et al. 2005).

The A2A AdR has emerged as a potentially attractive therapeutic target for the treatment of Parkinson's disease (PD), based in part on its unique distribution in the CNS. In mammalian CNS, A2A receptors are highly enriched in striatopallidal neurons (Jarvis and Williams 1989; Svenningsson et al. 1997) where they colocalize and form functional heterodimers with D2 dopamine receptors (D2Rs; reviewed in Schwarzschild et al. 2006). Agents that block activation of the A2A receptor in striatopallidal neurons appear capable of reducing the postsynaptic effects of dopamine depletion (Bhidayasiri and Truong, 2008) and in turn lessen the motor deficits that occur in PD (Reviewed in Pinna et al. 2005; Schapira et al. 2006; Schwarzschild et al. 2006).

In humans, the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been shown to cause a selective loss of dopaminergic neurons in the substantia nigra and a corresponding syndrome that resembles PD (Langston and Ballard 1983). MPTP-induced Parkinsonism has become a well-accepted model for PD in a variety of mammalian species including rodents and primates (Betarbet et al. 2002). Recent studies have provided evidence that in zebrafish, dopaminergic neurons are sensitive to MPTP (Anichtchik et al. 2004; Bretaud et al. 2004; Lam et al. 2005; McKinley et al. 2005). The loss of dopaminergic neurons causes concomitant defects in swimming responses (Bretaud et al. 2004; Lam et al. 2005). These effects of MPTP suggest that zebrafish may represent a tractable model system to study the pathogenesis of PD.

Here we have identified three distinct zebrafish a2 (*adora2a.1*, *adora2a.2*, *adora2b*) AdR genes and describe each of their expression patterns during embryogenesis. We also demonstrate that caffeine, an antagonist of the mammalian A2A receptor, can protect zebrafish embryos against MPTP-induced neurotoxicity, a feature that may make zebrafish an attractive model for screening and testing novel drugs for PD therapy.

1. RESULTS and DISCUSSION

1.1. Identification and Characterization of Zebrafish a2 Adenosine Receptor Genes

Zebrafish homologs of mammalian adenosine receptors (AdRs) were identified by blast searches of the zebrafish genome sequence database (http://trace.ensembl.org/perl/ssahaview?server=danio_rerio_translated and http://www.ensembl.org/Danio_rerio/bblastview). RT-PCR was used to amplify full-length cDNAs corresponding to three A2-like AdR genes. Sequence comparisons indicate that the zebrafish A2 AdRs share a high degree (62–74%) of amino acid sequence identity. Two zebrafish clones (*adora2a.1* and *adora2a.2*) show highest identity (71–74%) to the human A2A AdR, while the remaining zebrafish clone (*adora2b*) shares highest identity (65%) with the human A2B AdR. The *adora2a.1* AdR cDNA (GenBank accession no. AY945800) contains a complete 1328-bp open reading frame (ORF). The *adora2a.2* AdR cDNA (GenBank accession no. AY945801) contains a complete ORF that is 1343-bp in length, while the *adora2b* AdR cDNA (GenBank accession no. AY945802) contains a complete ORF 1055-bp in length. Continued mining of the zebrafish genomic and expressed sequence tag (EST) databases failed to uncover any additional a2 AdR genes. Together, these results are consistent with the idea that zebrafish are likely to possess two a2a AdR genes and one a2b AdR gene.

Sequence alignments of the human and predicted zebrafish A2a and A2b AdR polypeptides are shown in Figures 1 and 2, respectively. By aligning the zebrafish and human A2 AdRs, we identified seven putative transmembrane (TM) domains in A2a.1 and A2a.2 highly conserved with the transmembrane segments of the human A2 AdR. The intron-exon organization of the

zebrafish *adora2a* and *adora2b* AdR genes is also identical to their mammalian counterparts (Figs. 1 and 2), strongly suggesting that the zebrafish and mammalian genes arose from a common ancestral gene.

1.2. Phylogenetic Analysis of Zebrafish A2a and A2b Receptors

We examined the evolutionary relationships between zebrafish a2 AdR genes by conducting a phylogenetic analysis using maximum parsimony (MP; Felsenstein 1981) and distance matrix (DM; Fitch and Margoliash 1967) methods (Figure 3). A total of 228 positions at which alignments were unambiguous were used for phylogenetic analysis, while positions at which alignments were ambiguous due to amino acid insertions or deletions were excluded. The sequences retained for analysis aligned to amino acids 9–140, 170–210, 222–260, and 275–290 of the human A2A receptor polypeptide (NP_000666). Clustering of zebrafish A2a.1 and A2a.2 with other vertebrate A2A sequences was strongly supported (MP, 95%; DM, 97%) by trees generated. Clustering of zebrafish A2b with other fish A2B sequences is supported by 91% (MP) and 100% (DM). The results of this phylogenetic analysis therefore confirm the evolutionary relationships amongst zebrafish a2 AdR genes.

1.3. Chromosomal Mapping of Zebrafish a2a and a2b AdR Genes

The zebrafish *adora2a.1* AdR gene was identified on a genomic contig (GenBank accession no. NW_001879350.1) that was mapped to chromosome 8. We then determined the chromosomal positions of the zebrafish *adora2a.2* and *adora2b* AdR genes by using the T51 radiation hybrid panel (Kwok et al. 1998). Gene map positions were calculated with the ZonRH mapper resource (<http://zfrhmaps.tch.harvard.edu/ZonRHmapper>). A summary of the map positions of the individual a2 receptor genes is presented in Table 3. The zebrafish *adora2a.2* gene mapped to chromosome 21 at a distance of 4cR from marker chunp306, while the zebrafish *adora2b* gene was localized to chromosome 5 at a position 9cR from marker zc199f23.za.

1.4. Expression of Zebrafish a2a and a2b Receptor Genes

We used whole-mount *in situ* hybridization to examine the spatio-temporal expression of each of the a2 AdR genes during zebrafish embryogenesis. The expression pattern of the *adora2a.1* gene is shown in Fig. 4. Expression of *adora2a.1* begins at gastrulation with transcripts detected primarily in the enveloping layer (EVL, Fig. 4a). Expression of the *adora2a.1* gene persists in the EVL through early somitogenesis (11 hpf, Fig. 4B) at which time transcripts were also present in the ventral hematopoietic mesoderm (VHM). At mid-somitogenesis (15 hpf), the *adora2a.1* gene was expressed in the VHM and macrophages (Fig. 4C,D). In 24 hpf embryos, *adora2a.1* transcripts were detected in the intermediate cell mass (ICM) of the mesoderm and also in the vasculature (Fig. 4E), while at 36 hpf, *adora2a.1* mRNA expression was detected in blood and in distinct nuclei of the tegmentum and hindbrain (Fig. 4F–G). A similar expression pattern for *adora2a.1* was visualized at 48 hpf (Fig. 4H). Overall, *adora2a.1* expression appeared less robust at 48 hpf compared with *adora2a.1* mRNA levels at 36 hpf. No expression of the *adora2a.1* gene could be detected at 5 dpf (data not shown).

Expression of the *adora2a.2* gene is shown in Fig. 5. mRNA transcripts were first detected at gastrulation in the yolk syncytial layer (YSL) and ventral margin (Fig. 5A–B). Expression of the *adora2a.2* gene persisted in the YSL through early somitogenesis and was also detected in the tail bud (Fig. 5C). At mid-somitogenesis, *adora2a.2* transcripts continued to be detected in the YSL and tail bud, as well as in neurons of the trunk region (Fig. 5D–E), while at 24 hpf *adora2a.2* mRNA was detected in the YSL, interrenal tissue and in a few neurons in the anterior portion of the spinal cord (Fig. 5F–G). The pattern of *adora2a.2* mRNA expression was very similar in 24 hpf and 36 hpf embryos, with the exception that at 36 hpf, *adora2a.2* transcripts were detectable in the telencephalon (data not shown). By 48 hpf, transcripts of the *adora2a.*

2 gene were only present in the telencephalon (Fig. 5H). No *adora2a.2* transcripts were detected in 5 dpf embryos (data not shown).

The *adora2b* expression pattern is shown in Fig. 6. Transcripts of the *adora2b* gene were first detected in the YSL at early somitogenesis (Fig. 6A–B). The expression of *adora2b* persisted in the YSL at mid-somitogenesis (Fig. 6C) and was also detected in somites. At 24 hpf (Fig. 6D–E) and 36 hpf (data not shown), *adora2b* transcripts were expressed in the YSL, vasculature, and spinal cord neurons. At 48 hpf, the *adora2b* gene was expressed at basal levels in brain with more robust labeling in the anterior lateral hindbrain and in the neurons of the spinal cord (Fig. 6F–G). In 5 dpf embryos, basal expression of the *adora2b* gene was found throughout the brain with distinct staining visible in the ventricular zone (Fig. 6H).

One of the most striking features of A2 AdR gene expression is the low abundance of A2 AdR mRNA sequences that are present in early zebrafish embryos. However, our *in situ* hybridization data indicates that *adora2a.1*, *adora2a.2*, and *adora2b* AdR expression in zebrafish for the most part parallels A2-like receptor expression in mammals. For example, mRNA sequences encoding A2A receptors are expressed in lymphocytes and platelets of mammalian species, while *adora2a.1* transcripts are detectable in blood of 24 hpf and 36 hpf zebrafish embryos. The function of these hematopoietic AdRs is largely unknown. Mammalian A2A AdR genes are expressed in the kidney and the activation of these receptors has been reported to reduce renal injury in models of ischemic renal failure (Okusa 2002). At 24 hpf, zebrafish *adora2a.2* transcripts are present in interrenal tissue, an expression site that suggests the possibility that the A2a.2 AdR subtype may also be associated with protection against renal injury in zebrafish. In mammals, A2A AdR transcripts are expressed in several brain regions including striatum (Kull et al. 2000). In zebrafish, basal expression of *adora2a.1* transcripts is found in the diencephalon, tegmentum, and hindbrain of developing embryos, while *adora2a.2* transcripts are present in telencephalon of 48 hpf zebrafish embryos. The overall similarity in A2 AdR expression patterns between zebrafish and mammals suggest that zebrafish A2a and A2b AdRs are likely to share common functions with their mammalian counterparts.

1.5. Caffeine Attenuates MPTP-induced Dopaminergic Toxicity in Larval Zebrafish

MPTP-induced Parkinsonism is now a generally accepted model for Parkinson's disease in a variety of mammalian species (reviewed in Schwarzschild et al., 2006; Kalda et al., 2006). A number of studies have established that MPTP significantly decreases the number of dopaminergic neurons in larval zebrafish (Bretaud et al. 2004; McKinley et al. 2005). It has also been demonstrated in animal model systems that administration of caffeine, an A1 and A2A AdR antagonist, can protect dopaminergic neurons from MPTP-induced toxicity (Chen et al. 2001; Schwarzschild et al. 2003; Kalda et al. 2006). Based on these findings, we examined whether caffeine was also neuroprotective against MPTP in larval zebrafish.

We analyzed the effect of MPTP and caffeine on zebrafish embryos treated with either one or both of these drugs from 24 hpf to 5 dpf (Fig. 7). Thirty embryos were treated per group. The neurotoxicity produced by MPTP treatment was assessed at 5 dpf by *in situ* hybridization using an anti-sense probe that detects expression of the zebrafish dopamine transporter (*dat*) gene, a specific marker for dopaminergic neurons (Holzschuh et al. 2001). At 5 dpf, virtually no *dat* staining was observed in diencephalic or pretectal neurons of larval zebrafish treated with 40 μ M MPTP (Fig. 7B), indicating that MPTP was neurotoxic to dopamine producing neurons. However, when embryos were co-incubated with MPTP and 10 μ M caffeine, the level of *dat* staining in diencephalic dopaminergic was very similar to the level of *dat* staining observed in untreated zebrafish larvae (Fig. 7A, C), suggesting that caffeine was neuroprotective. Interestingly, caffeine did not protect pretectal dopaminergic neurons against MPTP neurotoxicity in this assay (Fig. 7C). Treatment of embryos with 10 μ M caffeine alone appeared to have no effect on *dat* expression in dopaminergic neurons (Fig. 7D). Together, these results

indicate that in zebrafish, caffeine protects diencephalic dopaminergic neurons against MPTP-induced neurotoxicity.

It is of interest to note that ventral diencephalic neurons were protected against MPTP-induced damage by co-incubation with caffeine, whereas the pretectal cluster of dopaminergic neurons was not. In adult zebrafish, axons of pretectal neurons project to the optic tectum (Ma 2003), however, little is known about their function. Our data contrasts with previous studies that showed that pretectal dopaminergic neurons in zebrafish were protected against MPTP-induced damage by co-incubation of embryos with either the monoamine oxidase-B inhibitor L-deprenyl or the dopamine transporter inhibitor nomifensine (McKinley et al. 2005). The reason for this discrepancy is not clear, although it is worth noting that our *in situ* hybridization analysis failed to identify *adora2a.1* or *adora2a.2* transcripts in the pretectal cluster. It is therefore tempting to speculate that the lack of $\alpha 2$ AdR expression may enhance the sensitivity of the pretectal neurons to the damaging effects of MPTP, and that caffeine treatment is not sufficient to protect these cells against MPTP-induced toxicity.

Neuroprotection offered by caffeine against MPTP-induced damage is likely to involve antagonism of A2a receptors. The best evidence for this idea comes from mouse knockout studies that showed that A2a AdR null mice exhibit significantly reduced susceptibility to MPTP-induced neurotoxicity (Chen et al. 2001). It will clearly be of interest to determine whether the neuroprotective effect of caffeine on dopaminergic neurons in zebrafish is mediated through either the *adora2a.1* or *adora2a.2* AdR gene (or both). This issue may be best addressed by using a morpholino-based approach to knock down expression of each of the $\alpha 2$ receptor subtypes in zebrafish embryos.

The current study presents the first detailed description of zebrafish AdR genes and their expression in the developing embryo. Our studies further substantiate zebrafish as a tractable model organism for studying PD, and suggest that zebrafish adenosine receptors may serve as useful targets for testing novel therapeutic strategies for the treatment of this neurodegenerative disease.

2. EXPERIMENTAL PROCEDURES

2.1. Cloning of Zebrafish $\alpha 2$ AdR Genes

Segments of zebrafish $\alpha 2$ AdR genes were identified by using mammalian polypeptide sequences as probes in low stringency Sequence Search and Alignment by Hashing Algorithm (SSAHA; Ning et al. 2001) searches of the zebrafish genomic reads generated by the Sanger Center (http://trace.ensembl.org/perl/ssahaview?server=danio_rerio_translated). Polymerase chain reaction (PCR) primers that overlapped conserved initiation and termination codons were designed based on genomic sequence reads. The corresponding full-length cDNAs were generated via reverse transcriptase (RT)-PCR. First-strand cDNA synthesis was performed by using SuperScript reverse transcriptase (Life Technologies) according to the manufacturer's protocols. Random hexamers were used to prime cDNA synthesis and total RNA from adult zebrafish was used as template. REDTaq DNA Polymerase (Sigma) was used for subsequent amplification. Primers and annealing temperatures are listed in Table 1. PCR was carried out using a TD-7500 Thermal Cycler (Hybaid). An initial 4-min denaturation step at 94°C was followed by 34 cycles at 94°C for 30 sec, 52–54°C for 30 sec, and 72°C for 90 sec. A final elongation step was carried out at 72°C for 10 min.

All PCR-generated cDNAs were sequenced using an ABI 377 automated sequencer. Sequences were compiled by sequencing at least three independent clones and by comparison to genomic sequences. Using this approach, we identified three zebrafish $\alpha 2$ AdR genes (*adora2a.1*, *adora2a.2*, and *adora2b*).

2.2. Phylogenetic Analysis

Amino acid sequences were aligned using the PILEUP program (Devereux et al. 1984). A total of 228 positions at which alignments were unambiguous were used for phylogenetic analysis, while positions at which alignments were ambiguous due to amino acid insertions or deletions were excluded. The sequences retained for analysis aligned to amino acids 9–140, 170–210, 222–260, and 275–290 of the human A2A receptor polypeptide (NP_000666). Phylogenetic analysis was performed using the PHYLIP suite of programs (version 3.573c) described by Felsenstein (1981). Maximum parsimony trees were calculated using PROTPARS. Evolutionary distance trees were constructed using the algorithm of Fitch and Margoliash (1967). For each method, tree reliability was estimated by analysis of 100 half jackknife sub-replicates. Trees were rooted using the human β_2 adrenergic receptor (NP_000015) and histamine H2 receptor (NP_071640).

2.3. Chromosomal Mapping of Zebrafish α_2 AdR Genes

Zebrafish α_2 AdR genes were mapped by using the Goodfellow T51 radiation hybrid (RH) panel (Research Genetics) (Kwok et al. 1998). Primers were designed to unique sequences for the adenosine receptor genes to amplify specific PCR products. A list of the primers used and chromosomal assignments for each of the zebrafish α_2 AdR genes can be found in Table 2. PCR panels for each gene were done in duplicate with PCR conditions optimized for each primer pair. PCR products were run on 2% agarose gels and scored for the presence or absence of the zebrafish-specific amplicon. Chromosomal assignments were computed using the RH Zon mapper resource (<http://zfrhmaps.tch.harvard.edu/ZonRHmapper/>).

2.4. α_2 AdR mRNA Expression

Zebrafish were obtained from Aquatica Tropicals (Plant City, FL). Whole mount *in situ* hybridization was performed as described (Thisse and Thisse, 2008; <http://zfin.org/cgi-bin/webdriver?Mivalaa-pubview2.apg&OIDZDB-PUB-010810-1>). The following probes were used to analyze AdR expression: *adora2a.1* (accession no. AY945800), nucleotides 1–1373; *adora2a.2* (accession no. AY945801), nucleotides 1–1455; *adora2b* (accession no. AY945802), nucleotides 1–1094. A probe derived from *slc6a3* (accession no. NM131755) nucleotides 391–1055 (from W. Driever), was used to analyze expression of the dopamine transporter.

2.5. Drug Treatment

Single cell zebrafish embryos were incubated for 24 hrs at 28.5°C in charcoal filtered water. At 24 hours post fertilization (hpf), embryos were dechorionated, transferred to a 6-well tissue culture dish (30 embryos/well) and grown in charcoal filtered water (untreated), or charcoal filtered water containing either 40 μ M MPTP, 10 μ M caffeine, or 40 μ M MPTP + 10 μ M caffeine. Each well contained 0.003% 1-phenyl-2-thiourea (PTU) to prevent pigment biosynthesis, and water (with or without drugs) was changed every 24 hrs. At 120 hpf (5 dpf), larvae were harvested and dopamine transporter expression analyzed by whole mount *in situ* hybridization.

Acknowledgements

We thank Joe Bednarczyk of the Molecular Genetics Core Facility, Penn State College of Medicine, for DNA sequencing. This work was supported by an NIH Conte Center Grant (MH 068789) and the Pennsylvania Department of Health (Tobacco Settlement Funds).

References

- Anichtchik OV, Kaslin J, Peitsaro N, Scheinin M, Panula P. Neurochemical and behavioural changes in zebrafish *Danio rerio* after systemic administration of 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J Neurochem* 2004;88:443–453. [PubMed: 14690532]
- Betarbet R, Sherer TB, Greenamyre JT. Animal models of Parkinson's disease. *Bioessays* 2002;24:308–318. [PubMed: 11948617]
- Bhidayasiri R, Truong DD. Motor complications in Parkinson disease: clinical manifestations and management. *J Neurol Sci* 2008;266:204–215. [PubMed: 17897677]
- Bretaud S, Lee S, Guo S. Sensitivity of zebrafish to environmental toxins implicated in Parkinson's disease. *Neurotoxicol Teratol* 2004;26:857–864. [PubMed: 15451049]
- Chen JF, Xu K, Petzer JP, Staal R, Xu YH, Beilstein M, Sonsalla PK, Castagnoli K, Castagnoli N Jr, Schwarzschild MA. Neuroprotection by caffeine and A(2A) adenosine receptor inactivation in a model of Parkinson's disease. *J Neurosci* 2001;21:RC143. [PubMed: 11319241]
- Cunha RA. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem Int* 2001;38:107–125. [PubMed: 11137880]
- Devereux J, Haeblerli P, Smithies O. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* 1984;12:387–395. [PubMed: 6546423]
- Felsenstein J. Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* 1981;17:368–376. [PubMed: 7288891]
- Fitch WM, Margoliash E. Construction of phylogenetic trees. *Science* 1967;155:279–284. [PubMed: 5334057]
- Fredholm BB, Chen JF, Masino SA, Vaugeois JM. Actions of adenosine at its receptors in the CNS: insights from knockouts and drugs. *Annu Rev Pharmacol Toxicol* 2005;45:385–412. [PubMed: 15822182]
- Fredholm BB, Irenius E, Kull B, Schulte G. Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. *Biochem Pharmacol* 2001;61:443–448. [PubMed: 11226378]
- Holzschuh J, Ryu S, Aberger F, Driever W. Dopamine transporter expression distinguishes dopaminergic neurons from other catecholaminergic neurons in the developing zebrafish embryo. *Mech Dev* 2001;101:237–243. [PubMed: 11231083]
- Jacobson KA, Gao ZG. Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov* 2006;5:247–264. [PubMed: 16518376]
- Jarvis MF, Williams M. Direct autoradiographic localization of adenosine A2 receptors in the rat brain using the A2-selective agonist, [3H]CGS 21680. *Eur J Pharmacol* 1989;168:243–246. [PubMed: 2558026]
- Kalda A, Yu L, Oztas E, Chen JF. Novel neuroprotection by caffeine and adenosine A(2A) receptor antagonists in animal models of Parkinson's disease. *J Neurol Sci* 2006;248:9–15. [PubMed: 16806272]
- Kull B, Svenningsson P, Fredholm BB. Adenosine A(2A) receptors are colocalized with and activate g (olf) in rat striatum. *Mol Pharmacol* 2000;58:771–777. [PubMed: 10999947]
- Kwok C, Korn RM, Davis ME, Burt DW, Critcher R, McCarthy L, Paw BH, Zon LI, Goodfellow PN, Schmitt K. Characterization of whole genome radiation hybrid mapping resources for non-mammalian vertebrates. *Nucleic Acids Res* 1998;26:3562–3566. [PubMed: 9671819]
- Lam CS, Korzh V, Strahle U. Zebrafish embryos are susceptible to the dopaminergic neurotoxin MPTP. *Eur J Neurosci* 2005;21:1758–1762. [PubMed: 15845104]
- Langston JW, Ballard PA Jr. Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *N Engl J Med* 1983;309:310. [PubMed: 6602944]
- Ma PM. Catecholaminergic systems in the zebrafish. IV. Organization and projection pattern of dopaminergic neurons in the diencephalon. *J Comp Neurol* 2003;460:13–37. [PubMed: 12687694]
- McKinley ET, Baranowski TC, Blavo DO, Cato C, Doan TN, Rubinstein AL. Neuroprotection of MPTP-induced toxicity in zebrafish dopaminergic neurons. *Brain Res Mol Brain Res* 2005;141:128–137. [PubMed: 16209898]

- Ning Z, Cox AJ, Mullikin JC. SSAHA: a fast search method for large DNA databases. *Genome Res* 2001;11:1725–1729. [PubMed: 11591649]
- Okusa MD. A(2A) adenosine receptor: a novel therapeutic target in renal disease. *Am J Physiol Renal Physiol* 2002;282:F10–18. [PubMed: 11739107]
- Pinna A, Wardas J, Simola N, Morelli M. New therapies for the treatment of Parkinson's disease: adenosine A2A receptor antagonists. *Life Sci* 2005;77:3259–3267. [PubMed: 15979104]
- Schapira AH, Bezard E, Brotchie J, Calon F, Collingridge GL, Ferger B, Hengerer B, Hirsch E, Jenner P, Le Novere N, Obeso JA, Schwarzschild MA, Spampinato U, Davidai G. Novel pharmacological targets for the treatment of Parkinson's disease. *Nat Rev Drug Discov* 2006;5:845–854. [PubMed: 17016425]
- Schwarzschild MA, Agnati L, Fuxe K, Chen JF, Morelli M. Targeting adenosine A2A receptors in Parkinson's disease. *Trends Neurosci* 2006;29:647–654. [PubMed: 17030429]
- Schwarzschild MA, Xu K, Oztas E, Petzer JP, Castagnoli K, Castagnoli N Jr, Chen JF. Neuroprotection by caffeine and more specific A2A receptor antagonists in animal models of Parkinson's disease. *Neurology* 2003;61:S55–61. [PubMed: 14663012]
- Svenningsson P, Le Moine C, Kull B, Sunahara R, Bloch B, Fredholm BB. Cellular expression of adenosine A2A receptor messenger RNA in the rat central nervous system with special reference to dopamine innervated areas. *Neuroscience* 1997;80:1171–1185. [PubMed: 9284069]
- Thisse C, Thisse B. High resolution in situ hybridization to whole-mount zebrafish embryos. *Nature Protocols* 2008;3:59–69.

Figure 1. Comparison of zebrafish and mammalian A2a adenosine receptors

Human, mouse, and zebrafish (ZF; A2a.1, A2a.2) A2a receptors were aligned using CLUSTALW. Ellipses in sequences allow optimal alignment for amino acid insertions/deletions. Identical amino acids are highlighted in black, and conserved amino acids are highlighted in gray. Amino acids are numbered to the left of each line. The transmembrane (TM) domains are indicated by solid lines above the sequence. Arrow indicates the location of the intron and is flanked by the corresponding exon numbers.

Figure 2. Comparison of zebrafish and mammalian A2b adenosine receptors

Human, rat, and zebrafish (ZF; A2b) A2b receptors were aligned using CLUSTALW. Ellipses in sequences allow optimal alignment for amino acid insertions/deletions. Identical amino acids are highlighted in black, and conserved amino acids are highlighted in gray. Amino acids are numbered to the left of each line. The transmembrane (TM) domains are indicated by solid lines above the sequence. Arrow indicates the location of the intron and is flanked by the corresponding exon numbers.

Figure 3. Phylogenetic analysis of vertebrate adenosine receptors

Tree was rooted using the human $\beta 2$ adrenergic receptor (NP_000015) and the human histamine receptor (NP_071640) peptide sequences. Branch lengths were estimated by the method of Fitch and Margoliash (1967) using all aligned positions. Evolutionary distance scale is below tree. The maximum parsimony and Fitch/Margoliash consensus trees obtained by bootstrap analysis showed identical topologies. Numbers to the left of each node indicate percent support from bootstrap analysis (Fitch/Margoliash below, maximum parsimony above). Both methods strongly support clustering of all A2a and A2b sequences (100%). Sources of sequences (accession number or SwissProt identifier): zebrafish A2a.1, A2a.2, and A2b (this paper); chicken A1: P_989647, chicken A2a: XP_425280, chicken A2b: P_990418,

chicken A3: NP_989482, mouse A1: NP_001008533, mouse A2a: NP_033760, mouse A2b: NP_031439, mouse A3: NP_033761, *Xenopus laevis* A2a: AAH84390, human A1: NP_000665, human A2a: NP_000666, human A2b: NP_000667, human A3: NP_000668. The following sources of sequences are DNA accession numbers of species-specific genomes: medaka A2b: BAAF02026954, fugu A2b: CAAB01003799, fugu A2a: CAAB01000409.

Figure 4. Expression of the *adora2a.1* gene

Lateral view of embryos at **A**: gastrula stage, **B**: early somitogenesis (11 hpf), **C**: mid-somitogenesis (18 hpf), **D**: dorsal view of a flat mounted embryo at 18 hpf in the caudal region. Lateral view of embryos at **E**: 24 hpf, **F**: 36 hpf, **G**: dorsal view of an embryo at 36 hpf after dissecting off the yolk. **H**: lateral view of embryo at 48 hpf. B, blood; EVL: enveloping layer; HB: hindbrain; HT: hypothalamus; ICM: inner cell mass; VHM: ventral hematopoietic mesoderm; VTG: ventral tegmentum.

Figure 5. Expression of the *adora2a.2* gene

Lateral view of embryos at **A**: gastrulation, **B**: gastrulation, focussing on the lateral part of the YSL and showing accumulation of transcripts in perinuclear area of the YSL, **C**: early somitogenesis (11 hpf), **D–E**: mid-somitogenesis (15 hpf) with **E** focussing on the trunk region of the embryo, **F–G**: 24 hpf, with **G** focussing on the trunk region, **H**: 48 hpf. IT, interrenal tissue; MG, margin; SCN, spinal cord neurons; T, telencephalon; TB, tail bud; YSL, yolk syncytial layer.

Figure 6. Expression of the *adora2b* gene

A: Lateral view of embryo at early somitogenesis (11 hpf), **B:** Dorsal view of embryo at early somitogenesis (11.5 hpf) showing labelling in YSL nuclei. Lateral view of embryos at **C:** mid-somitogenesis (15 hpf), **D:** 24 hpf, **E:** 24 hpf in the trunk region, **F:** 48 hpf. Dorsal view of the head region at **G:** 48 hpf, **H:** 5 dpf. ASC, anterior spinal cord; AV, axial vasculature; CV, caudal vein; H, hindbrain; SCN, spinal cord neurons; R, retina; T, telencephalon; VZ, ventricular zone; YSL, yolk syncytial layer.

Figure 7. Caffeine protects dopaminergic neurons from MPTP-induced neurotoxicity
Dorsal views of 5 dpf embryos hybridized with *dat* probe. Embryos were treated with drug from 24 hpf through 5 dpf. n=30 embryos/treatment group. **A:** Untreated control embryo. **B:** Embryo treated with 40 μ M MPTP. **C:** Embryo treated with 10 μ M caffeine and 40 μ M MPTP. **D:** Embryo treated with 10 μ M caffeine. Neurons of the ventral diencephalon are circled. Black arrows point to the bilateral pretectal cluster of neurons.

Table 1

Map position of Zebrafish A2 Adenosine Receptor Genes

Gene	Accession No.	Forward primer	Reverse primer	Zebrafish Chromosome
adora2a.1	AY945800	tacattgaggcgaggatgggcc	gatggcaatgtatcgggtcaatggc	8
adora2a.2	AY945801	cacactgggtgatagcaccatg	ctgccctcaaccaatacacgg	21
adora2b	AY945802	catggcatcttaagtgaagtgagc	tcatgcgcggaatgctgtgca	5

Table 2

PCR Primers for the Amplification of Zebrafish A2 Adenosine Receptors

Clone	Forward primer	Reverse primer	Ta (°C)
adora2a.1	⁻⁴⁴ tacattgagcgagggcatgtcc ⁻²²	¹³⁷³ tcaggaaacctccgtgagttc ¹³⁵³	51
adora2a.2	⁻¹⁹ cacctgggtgatagcaccatg ⁺³	¹⁴⁵⁵ gcgcacaccactgattacttc ¹⁴³⁵	51
adora2b	⁻²⁸ catggcatcttaagtgaagtgagc ⁻⁵	¹⁰⁹⁴ acaccggcgtctatagcagag ¹⁰⁷⁴	51

Nucleotide +1 is the A of the ATG of the initiating methionine