

Supplementary Information for

Adenosine A_{2A} receptor blockade prevents cisplatin-induced impairments in neurogenesis and cognitive function

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Extended Methods

Mouse husbandry

All experiments were performed on 3-4 month old female C57BL/6J mice (Jackson Laboratory). A_{2A}R-Cre homozygous mice were originally provided by Dr. Jiang-Fan Chen at Wenzhou Medical University, and were crossed to Ai9-tdTomato mice (Jackson laboratory, stock # 007909) to generate A_{2A}R-Cre^{+/+};Ai9^{+/+} mice. Female mice were utilized to visualize cell type-specific A_{2A}R expression in the hippocampus. All mice were housed in standard ventilated cages under a 12-hour light/dark cycle with water and food *ad libitum*. Mouse experiments were conducted in accordance with National Institutes of Health guidance on the care and use of laboratory animals. All procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC protocol # A00005043 and A00004190).

Drugs

KW-6002 (Istradefylline, 3 mg/kg *i.p.*, Tocris Bioscience, Bristol, UK), was prepared in 15 % DMSO (MilliporeSigma, St. Louis MO, USA), 15 % Cremophor (MilliporeSigma) and 0.9% saline (MilliporeSigma). Cisplatin (Fresenius Kabi, Lake Zurich IL, USA) was dissolved in 0.9% saline and all compounds were delivered at a 0.1 ml/10 g body weight.

Cisplatin and KW-6002 administration

Although chemotherapy-induced cognitive impairment (CICI) is observed in both males and females, CICI is commonly reported among female cancer patients as afflicted by breast and ovarian cancer (1, 2). Therefore, we focused on females for this study. To induce cognitive dysfunction in our mouse models that approximate clinical

cognitive impairments (3, 4), we used the platinum based compound cisplatin, which has been shown to penetrate the blood-brain barrier and accumulate in brain in clinical and preclinical studies (5, 6). In addition, the two primary domains that are commonly reported in clinical neuropsychiatry arising from cisplatin chemotherapy is psychological distress, such as anxiety and depression, as observed in testicular cancer survivors (7, 8). The other complication is the long-term cognitive functional decline of learning and memory, attentional processing speed and executive function that is observed in patients with testicular, lung, and/or head-and-neck cancers receiving cisplatin (9-13). Although our mouse model has some natural limitations in modelling all clinical cognitive domains of CICI, it does recapitulate a similar pattern of neuropsychological and neurocognitive function in several domains such as increased anxiety, delayed learning ability and memory impairment (6). From a mechanistic point of view, our approach is justifiable, because isolating cisplatin induced cognitive effects from cancer pathology has the advantage to study mechanisms independent of possible confounding factors from cancer pathology. Our implementation of one treatment cycle is operationally defined as 5 consecutive daily injections of cisplatin (2.3 mg/kg *i.p.*) or 0.9 % saline vehicle followed by 5 days of rest from injections. The investigations in this report utilized either 4 treatment cycles of cisplatin or vehicle (Fig. 1A, B, E) or 3 treatment cycles of cisplatin (Fig. 1C, D, G, H and Fig. 2), cisplatin in combination with KW-6002 (CIS + KW), as well as KW-6002 or vehicle alone (Fig. 2A). KW-6002 administration occurred 2 h prior to cisplatin administration. The dose of cisplatin was chosen based on our previous study demonstrating memory impairment by cisplatin while effective in its antitumor efficacy (6). This cisplatin regimen is known to be comparable to clinical treatment regimens for the patients with various cancers (3, 4, 14, 15). In addition, the dose of KW-6002 used in our study was chosen based on the dosing of a previous study published in PNAS demonstrating that reductions in weight gain, synaptic alterations and

memory dysfunction triggered by chronic stress were attenuated by A_{2A}R inhibition via KW-6002 (16). Body weight measurements (Fig. 2B) were recorded daily during treatment cycles, during behavior testing, and during the recovery period following behavior testing.

Brain tissue preparation

To extract fresh brain tissue from mice administered 3 or 4 cycles of cisplatin or KW-6002 in combination with cisplatin, mice were anesthetized via a ketamine (K; 100 mg/kg), xylazine (X; 10 mg/kg), and acepromazine (A; 10 mg/kg) cocktail (KXA, 0.1ml/10g body weight. *i.p.*) as previously described (6). Whole brains were then washed in ice cold 1x phosphate buffered saline (PBS; BioRad, Hercules CA, USA) and subsequently the whole hippocampus was dissected on an ice-cold aluminium block with the aid of a surgical microscope. The dissected brain tissue was then snap-frozen on dry ice and stored at -80°C until processing for RNA sequencing, cAMP measurement or western blot analysis.

RNA-sequencing analysis

For RNA-sequencing analysis presented in Fig. 1A, 3-4 month old female C57BL/6J mice were injected with cisplatin (2.3 mg/kg *i.p.*) or vehicle for 4 treatment cycles. Tissues were freshly collected (~10-25 mg/tissue sample), snap frozen in dry ice and stored at -80°C until processed. RNA libraries were prepared according to the manufacturer's instructions from the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA) using 100 ng of total and incorporating a poly-A mRNA enrichment using oligo dT magnetic beads. The final adapter-modified cDNA fragments were enriched by 12 cycles of PCR using Illumina TruSeq PCR primers. The concentration and size

distribution of the completed libraries were determined using a Fragment Analyzer (AATI, Ankeny, IA) and Qubit fluorometry (Invitrogen, Carlsbad, CA). Libraries were sequenced at 6 samples per lane following Illumina's standard protocol using the Illumina cBot and HiSeq 3000/4000 PE Cluster Kit. The flow cells were sequenced as 100 X 2 paired end reads on an Illumina HiSeq 4000 using HiSeq 3000/4000 sequencing kit and HD 3.4.0.38 collection software. Base-calling was performed using Illumina's RTA version 2.7.7.

MAP RSeq computational workflow pipeline

Following our sequencing procedure, raw *Fastq* files were processed through Mayo Clinic's internal MAP-RSeq pipeline (17) (Version 3.0). MAP-RSeq uses a variety of publicly available bioinformatic tools tailored by in-house developed methods. Briefly, the alignment and mapping of sequence reads are performed using Star aligner against mm10 reference genome (18). The gene and exon counts are generated by *FeatureCounts* using the gene definition files from *Ensembl v79* (19). Quality control was carried out using *RSeqQC* (20) and some additional metrics (21) to ensure the results from each sample are reliable and can be collectively used for differential expression analysis. Differential expression analysis was carried out using version 3.20.1 of *EdgeR* (22). Differentially expressed genes (DEGs) were identified with an average expression larger than 2 counts per million (CPM), and an absolute Log_2 fold change larger than -2.0 (significantly downregulated) or +2.0 (significantly upregulated), and a multiple comparison adjusted *P* value ≤ 0.05 . Statistical analysis was carried out utilizing *R* under *R version 3.4.2*.

Ingenuity pathway analysis (IPA)

As presented in Fig. 1E, network analysis from our RNAseq gene set enrichment analysis (GSEA) dataset was performed with Ingenuity Pathway Analysis (IPA; QIAGEN Inc., Redwood City CA, USA) as previously described (23). Our complete dataset of identified differentially expressed genes was uploaded into IPA for stratification and categorization of direct and indirect network interactions using an IPA functional CORE analysis with default settings and excluding cancer cell lines. Expression value threshold filters were set to an absolute Log_2 fold change larger than -2.0 (significantly downregulated) or +2.0 (significantly upregulated) and a confidence value of $P \leq 0.05$ to distinguish non-significant reference genes from significant *focus genes* for generation of statistically significant associations with canonical pathways and biological process gene networks. The IPA bioinformatic algorithm takes into consideration the total number of reference genes from our data set participating in biological processes (derived from the IPA Ingenuity Knowledge Base) in combination with focus gene signatures to generate biological process gene networks that connect with significantly upregulated (red color intensities) or downregulated (blue color intensities) focus genes.

Quantitative RT-PCR and primer sequencing information

For quantitative RT-PCR analysis presented in Fig. 1B, 3-4 month old female C57BL/6J mice were injected with cisplatin (2.3 mg/kg *i.p.*) or vehicle for 5 consecutive days followed by 5 days of rest from injections for 4 treatment cycles. Tissues were freshly collected (~10-25 mg/tissue sample), snap frozen in dry ice and stored at -80°C until processed. Total RNAs were extracted using Trizol reagent (Invitrogen, 221706) according to the manufacturer's instructions. RNase-free DNase I (Thermo Fisher, EN0525)-treated total RNAs were used in cDNA synthesis using a SuperScript III First-strand Synthesis System (Invitrogen, 18080-051). Reverse transcription was carried out

according to the manufacturer's instructions in 20 μ L reaction mixtures containing oligo(dT) primer. Quantitative real-time PCR was performed using Fast SYBR Green Master Mix (Applied biosystems, 4385612) and the QuantStudio 3 Real-Time PCR sequence detection system (Thermo Fisher, A28136). The reaction was carried out in a total volume of 12 μ L, which contained 6 μ L of 2 \times SYBR premix, 0.5 μ L of each oligonucleotide primer and 1 μ L of cDNA. The amplification conditions were an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The sequences of the sense and antisense primers for adenosine receptor families were as follows: A₁R sense: 5'-GATCGGTACCTCCGAGTCAAGA-3'; A₁R antisense: 5'-CACTCAGGTTGTTCCAGCCAAAC-3'; A_{2A}R sense: 5'-CACGCAGAGTTCCATCTTCAGC-3'; A_{2A}R antisense: 5'-CCCAGCAAATCGCAATGATGCC-3'; A_{2B}R sense: 5'-TTCGTGCTGGTGCTCACACAGA-3'; A_{2B}R antisense: 5'-AAGGACCCAGAGGACAGCAATG-3'; A₃R sense: 5'-GCCATTGCTGTAGACCGATACC-3'; A₃R antisense: 5'-CCCACCAGAAAGGAAACTAGCC-3'; Actin sense: 5'-TTCTACAATGAGCTGCGTG-3'; Actin antisense: 5'-GGGGTGTGAAGGTCTCAA-3'. After completion of the cycling process, the data were analyzed using QuantStudio 3 Real-Time PCR sequence detection system software. The expression of mRNA was normalized using Actin.

Western blot analysis

For western blot analysis presented in Fig. 1C and Fig. 1H, 3-4 month old female C57BL/6J mice were injected with cisplatin (2.3 mg/kg *i.p.*) or vehicle for 3 treatment cycles and brains were harvested 24 h following the last injection. Following whole

hippocampus dissection from fresh brain, tissues were magnetically homogenized (Storm 24 Bullet Blender, Next Advance, Troy NY, USA) with 0.5 mm ZrO₂ beads at a setting of 4 for 2 min in Neuronal Protein Extraction Reagent (N-PER, Thermo Scientific, Cat# 87792) containing protease inhibitor cocktail (Cell Signaling) followed by centrifugation at 14,000 x g for 15 min at 4°C as previously described (23). The supernatant protein concentrations were measured by the BCA assay kit (Pierce, Cat# 23227). Equal amounts of protein per gel lane were separated by 4-15% SDS-PAGE and transferred to nitrocellulose membranes, which were blocked in 5% non-fat dry milk in TBST. Membranes were incubated overnight at 4°C with anti-A_{2A}R (mouse, 1:500, EMD Millipore, Cat# 05-717), anti-pCREB (rabbit, 1:1000, Cell Signaling, Cat# 9198S), anti-CREB (rabbit, 1:1000, Cell Signaling, Cat# 9197S), and anti-GAPDH (rabbit, 1:1000, Cell Signaling, Cat# 2118) antibodies followed by HRP-linked secondary antibody. Membranes were then washed and visualized with enhanced chemiluminescence (GE Healthcare Life Science, RPN2232). Expression was normalized to that of GAPDH for loading controls.

Perfusion process and immunostaining information

To visualize cell type-specific A_{2A}R expression presented in Fig. 1D, 3-4 month old female A_{2A}R-Cre mice with Ai9 mice expressing tdTomato (A_{2A}-Cre;Ai9-mice) were injected with cisplatin (2.3 mg/kg *i.p.*) or vehicle for 3 treatment cycles. Mice were then anesthetized with KXA and perfused brains were harvested 24 h following the last injection. Brains were processed for histological analysis as previously described (6). Briefly, brains were coronally sectioned (40 µm in thickness) from anterior to posterior through the entire brain in serial order and processed for histological analysis. At least 3-5 sections per brain in serial order were mounted and immunostained in at least 2-

independent immunostaining experiments. Immunostaining was performed with primary antibody specific to neuronal nuclei (NeuN; mouse, 1:500, EMD Millipore, Cat# MAB377) used in this study followed by appropriate secondary antibody. Because the enhanced Tomato signal corresponds to enhanced recombination, we have also performed immunohistochemistry with the specific A_{2A}R antibody (Cat# AAR-002, Alamone) to directly test the expression pattern of endogenous A_{2A}R following cisplatin administration. For neurogenesis analysis presented in Fig. 2H, immunostaining was performed with primary antibody specific to doublecortin (DCX; rabbit, 1:500, Cell Signaling, Cat# 4604S) used in this study followed by appropriate secondary antibody. Images were acquired on a Zeiss LSM 780 single-photon confocal system using a multi-track configuration.

Analysis of cAMP in hippocampal tissue

For assessment of cAMP levels from adult mouse hippocampus presented in Fig. 1G, tissues were magnetically homogenized (Storm 24 Bullet Blender, Next Advance, Troy NY, USA) with 0.5 mm ZrO₂ beads at a setting of 4 for 2 min in Neuronal Protein Extraction Reagent (N-PER, Thermo Scientific, Cat# 87792) containing Halt™ Protease Inhibitor Cocktail (Thermo Scientific, Cat# 78440) followed by centrifugation at 14,000 x g for 15 min at 4°C as previously described (23). The supernatant protein concentrations were measured by the BCA assay kit (Pierce, Cat# 23227). Equal amounts of protein concentration from hippocampal tissue were used for cAMP assay. cAMP levels from hippocampal tissue were determined using cyclicAMP XP® assay kit (Cell Signaling, Cat #4339) according to the manufacturer's protocol. Briefly, brain tissues were lysed using a lysis buffer including protease inhibitor cocktail and the lysate was added to the cyclicAMP XP® assay kit to displace HRP-linked cAMP bound to an anti-cAMP XP®

Rabbit mAb immobilized onto a 96-well plate. After removing displaced HRP-linked cAMP, HRP substrate TMB was added and cAMP concentration was measured colorimetrically at 450 nm.

Behavioral analysis

Mice were transferred from colony housing to the behavior testing suite and allowed acclimation to this suite for 1 hr before onset of testing to minimize stress during behavior testing. All tests were performed during the light phase (6 am-to-6 pm) of the 12-hr light/dark cycle. All behavior experimental data was video recorded with a monochrome camera mounted to the ceiling of the testing suite and analyzed with EthoVision-XT 14 video tracking (Noldus Information Technology, Leesburg, VA, USA). Where appropriate, testing chambers were sanitized with 70% ethanol between trials. At the end daily experimental testing, mice were returned to their home cage and transferred back to colony housing.

Elevated plus maze: The elevated plus maze (EPM; ENV-560A, Med-Associates St. Albans VT, USA) presented in Fig. 2C, takes advantage of the proclivity for rodents to avoid open, unprotected areas, making this simple and efficient test appropriate to measure anxiety-like behavior in the rodent. The EPM is elevated 50 cm above the floor and consists of two open arms and two closed arms of similar dimensions (L 34.9 cm x W 6 cm). The closed arms are lined with a 26 cm high opaque black wall. Mice are released at a center location (L 6 cm x W 6 cm) between the open and closed arms and allowed to freely explore every section of the maze for 5 min. Time spent in the open and closed arms of the maze was video tracked and recorded (Noldus).

Novel object recognition: The novel object recognition (NOR) task presented in Fig. 2D is a well-established recognition memory behavioral assay which takes advantage of the rodent's inherent ability to familiarize/habituate to a context/object and subsequently use memory recall to distinguish between a previously habituated/familiar object and exposure to a novel object (24). NOR testing was conducted in the same cohorts of experimental mice across the daily 10 min habituation, training and testing sessions as used in our previous report (6). On Day 1, mice were allowed to freely explore and habituate to the empty arena (L 42 cm x W 42 cm x H 24 cm). On Day 2 (training phase), two identical (Familiar) objects were placed at the bottom of the arena diagonally opposed to each other (i.e. in the NW and SE corners) and mice were allowed to explore the objects. We did not detect a location preference between the two familiar objects among the treatment groups (Day-2). On Day 3 (testing phase), mice were presented with and allowed to explore a new (novel) object which replaced one of the familiar objects. The Familiar object shape was a blue rectangular wood cube shaped to a point (H 14 cm x W 3.7 cm), while the Novel object was a wooden bishop chess piece (H 11 cm x W 4 cm) that had black circles and squares randomly drawn at its sides. Nose-point exploration time of familiar in relation to novel object and object bound zone (1.5 cm surrounding the object) was video tracked, quantified and analyzed (Noldus), thus providing an index of recognition memory.

Morris Water Maze: The Morris water maze (MWM) presented in Fig. 2E-G is a behavioral procedure that is widely used in rodents to study spatial learning and memory which are crucial processes that rely on adequate hippocampal-dependent cognitive function (25). The MWM task relies on rodents learning to utilize spatial cues placed around a circular tank (120 cm diameter and 90 cm deep; ENV594M-B, Med-Associates)

to locate and escape onto a hidden, submerged platform. The submerged platform (1.25 cm below the water surface) measured 10.2 cm in diameter and was concealed by white opaque non-fat dry milk mixed water. Visible spatial cues of equal size were placed at North (N; star), South (S; circle), East (E; square), and West (W; triangle) coordinate locations within the tank which divided the tank into NE, SE, SW, NW quadrants. To measure learning during MWM training, mice were released from NE, SE, SW, NW points where swim latencies and trajectories to escape onto the hidden platform were video tracked, traced and analyzed (Noldus). The test was performed across 6 consecutive days consisting of visible platform days (days 1 and 6), training days (days 2-4), and a memory probe test day (day 5). During the visible platform days, the platform was in the SE quadrant and signaled by an orange flagpole protruding 8 cm above water's surface. To begin, mice were released from the NE release point and allowed 2 min to see the flag and escape onto the platform. If mice found the platform in less than 2 min, they were allowed to remain on the platform for an additional 30 seconds, thus habituating mice to remain on the platform. If mice were unable to locate and escape onto the platform within the 2 min trial, they were manually guided to the platform and allowed to remain there for an additional 30 seconds. This process was repeated in subsequent SE, SW, and NW release trials for visible platform day 1 and day 6. On test days 2-4, the flag was removed, and mice had to rely on spatial cues to learn to navigate and escape onto the submerged platform following release from NE, SE, SW and NW release points. Each release point was an individual trial where mice had 2 min to find the escape platform. If mice found the platform in less than 2 min, they were removed from the platform and placed in a drying cage until the next release trial. If mice were unable to locate and escape onto the platform within the 2 min trial, they were manually guided to the platform and allowed to remain there for an additional 30 seconds. For

data analysis of MWM performance during training days and visible platform days, the escape latencies from each release point were averaged for each animal per day.

To specifically probe spatial memory retention (day 5 Probe test), the hidden platform was removed, and release trials were conducted for 2-min in the same fashion as during MWM training (Days 2-4). To determine whether mice memorized the location of the platform by relying on spatial cues to find its location, the frequency of platform zone crosses and trajectories were recorded during the probe test. Across each test day, following completion of every release trial, mice were placed in a drying cage lined with paper towels and under a warming lamp, to await onset of their next trial. Water temperature was maintained between $25 - 27 \pm 1$ °C across testing days.

Retrovirus production, stereotaxic surgery, and dendrite analysis

Engineered self-inactivating murine onco-retroviruses were used to express GFP selectively in proliferating neural progenitors as previously described (6). Briefly, high titers of engineered retroviruses were produced by co-transfection of retroviral vectors and Vesicular Stomatitis virus G (VSVG) into HEK293 gp cells followed by ultra-centrifugation of viral supernatant. Dendrite morphology of adult-born neurons was visualized by stereotaxic injection (26) of retroviruses into the dentate gyrus at 4 sites (0.5 μ l per site at 0.25 μ l/min) with the following coordinates (in mm): posterior = - 2 from Bregma, lateral = \pm 1.6, ventral = 2.5; posterior = - 3 from Bregma, lateral = \pm 2.6, ventral = - 3.2. Three days after performing the retroviral injections, the following treatments were administered on mice for 3-cycles: vehicle, cisplatin, or cisplatin in conjunction with KW-6002 (Fig. 2/). One day following the completion of cisplatin and KW-6002 administrations, brains were extracted, sectioned and total dendrite length of GFP⁺ adult-born neurons were analyzed. Utilizing Z-series stacks of confocal images (Zeiss

LSM780), three-projection images were semi-automatically traced with the Neuron J plugin (NIH Image J) and the total dendritic length of individual GFP⁺ neurons were analyzed. The total dendritic length of each individual neuron for each treatment group is shown in dot plot distribution.

Xenograft tumor models

As shown in Fig. 2L, the effect of KW-6002 and cisplatin on tumor growth was studied *in vivo* by orthotopically injecting MCF-7-ER α ⁺ cells (estrogen receptor-alpha) into 4-5 months-old CB17/Icr-Prkdc^{scid} SCID female mice (Charles River, Strain Code, 356231). As used in our previous study (6), MCF-7-ER α ⁺ cells with a concentration of $\sim 1 \times 10^6$ cells in 100 μ L + 100 μ L of Matrigel (Corning, 356231) were injected into the mammary fat pads of 17 β -estradiol (0.54 mg/90-day release; Innovative Research of America, Cat# NE-121) pellets were implanted into the nape of the neck using a trochar to induce tumor growth. After the tumor volume reached approximately 50 mm³, mice were treated with 3-cycles of the following treatments: vehicle, cisplatin, KW-6002 or cisplatin in combination with KW-6002. One day after completion of drug administration, tumors were isolated, and their weights were measured.

Statistical analysis

All statistical analyses were executed using GraphPad Prism 8.02 (GraphPad Software, La Jolla, CA, USA). For molecular and behavioral results, a two-tailed unpaired student's t-test, standard or repeated-measures one-or two-way ANOVA followed by Dunnett's, Tukey's or Bonferroni's *post hoc* multiple comparisons were used, as appropriate for each experiment. For pathway analysis, IPA incorporates a right-tailed Fisher's exact test as part of its CORE analysis algorithm. Statistical significance was

defined as $P < 0.05$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). $P > 0.05$ results were defined as statistically not significant (n.s.). Statistical outliers were detected in Fig. 2F with GraphPad Prism QuickCalcs Grubb's outlier test (<https://www.graphpad.com/quickcalcs/grubbs1/>) and removed equally in an unbiased manner between all groups tested. All experiments and data analyses were performed in a blinded fashion.

Datasets S1. Differential gene expression in the hippocampus derived from 4-cycles of cisplatin or vehicle administration.

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