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## Dynamic fluorescent cyclic AMP biosensors (cAADis) to study recombinant and endogenous adenylyl cyclase 1 (AC1) signaling in HEK 3/6KO and SH-SY5Y cellular models

S M Sabbir Alam,<sup>1</sup> Emily K. Davidson,<sup>1</sup> Kayla Johnson,<sup>2</sup> Camryn Fulton,<sup>1</sup> Amanda Klein,<sup>3</sup> and Val Watts<sup>1</sup>

<sup>1</sup>Borch Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, IN, USA; <sup>2</sup>Department of Pharmacy Practice and Pharmaceutical Sciences, University of Minnesota, MN, USA; and <sup>3</sup>Department of Pharmacology and Toxicology, University at Buffalo, NY, USA

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Adenylyl cyclases mediate the production of cyclic AMP (cAMP) and plays a pivotal role in regulating important physiological processes. Adenylyl cyclase 1 (AC1) is robustly activated by Ca<sup>2+</sup>/calmodulin (CaM), highly expressed in the central nervous system, and has been implicated in chronic pain modulation, alcohol behaviors, and memory and learning processes. However, the absence of a robust neuronal cell model along with technological limits for measuring cAMP have posed challenges in studying AC1, and potential therapeutic interventions. Specifically, most cell-based methods lack essential neuronal properties necessary for mimicking native cellular conditions, leading to limitations in assessing endogenous AC activity and inhibitor responses in a neuronal model. To address this gap, we aimed to establish a neuronal model utilizing SH-SY5Y neuroblastoma cells expressing genetically encoded cAMP biosensors to study endogenous adenyl cyclase activity and evaluate potential inhibitors of AC1 signaling. The initial studies used our novel HEK AC3/6 KO cells stably expressing AC1 (ACΔ3/6KO-AC1) to assess cAMP signaling with several dynamic cAMP biosensors (cAADis). Progress to date includes significant advancements in successful expression of all cAADis sensors in HEK AC∆3/6KO-AC1 cells and subsequent stimulation of AC activity by forskolin, calcium ionophore (A23187), isoproterenol, and capacitive calcium entry. Both a Neo2 plate reader and Cytation 3-based imaging were utilized for fluorescent measures across different plate configurations, including 96-well and 384-well formats. Studies with constitutively active Gs and Gi-linked receptors (u-opioid and D2 dopamine receptors) were also conducted. Additionally, cAMP overshoot or heterologous sensitization experiments using cAADis cAMP biosensors were successfully executed in the AC∆3/6KO-AC1 cells. A second series of experiments used the best performing cAADis cAMP biosensors in SH-SY5Y neuroblastoma cells to investigate endogenous AC1 activity and explore potential inhibitors of AC1. SH-SY5Y cells express high levels of AC1 mRNA, however, significant Ca<sup>2+</sup>/CaM-stimulated cAMP accumulation is not readily observed. We hypothesized that cell-selective analysis of cAMP using the targeted cAADis biosensors in SH-SY5Y cells would improve our overall signal to noise window of AC1 activity. Stimulation of SH- SY5Y cells with calcium ionophore, A23187 revealed a significant increase in the endogenous cAMP response of approximately 20% versus the maximal forskolin response. This study presents promising advancements towards establishing a robust neuronal model expressing genetically encoded cAMP biosensors, to study endogenous AC1 activity and explore potential AC inhibitor responses pertinent to chronic pain treatment.

**Keywords:** Adenyl Cyclase; cAMP Signaling; cAMP Biosensors; Chronic Pain; Neuronal Models; HEK 293 Cells; SH-SY5Y Cells; Calcium Ionophore.

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