Abnormal Cyclic AMP Production in Human Fragile X Neural Tissue: A Proof of Principle Study

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Introduction:

Fragile X syndrome, a neurodevelopmental disorder that is the most common inheritable cause of mental retardation, is due to a CGG trinucleotide amplification on the X chromosome (Xq27.3) in the 5' untranslated region of the fragile X mental retardation-1 gene (FMR1) that suppresses production of the fragile X mental retardation protein (FMRP). FMRP is an mRNA binding protein thought to shuttle mRNA between the nucleus and cytoplasm. The mRNA sequences for adenylate cyclases (AC) III, V, and VI are known to associate with FMRP. With reduced levels of FMRP in fragile X, mistranslocation of these mRNAs would reduce the functional availability of AC proteins and impair cAMP production from ATP. Support for the functional consequence of the link between FMRP and cAMP comes from previous studies in human platelets and lymphoblasts in which cAMP was pharmacologically manipulated (Berry-Kravis and Sklena, 1993; Berry-Kravis et. al., 1995). These studies indicated that the fragile X cAMP cascade is dysfunctional at the level of adenylate cyclase, or its regulation (see Figure 1), after observing diminished cyclic AMP elevation in fragile X cells compared to non-fragile X controls. To clarify the role of FMRP and the cAMP cascade in brain, we tested the hypothesis that human fragile X neural tissue would produce less cyclic AMP upon stimulation compared to non-fragile X control tissue.

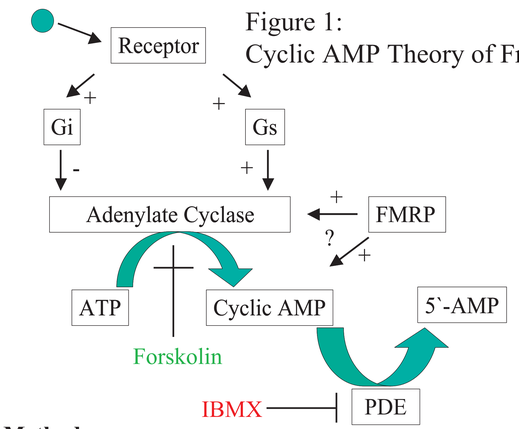


Figure 1: Cyclic AMP Theory of Fragile X

Methods:

In this study, we used neurospheres and differentiated cells from one human fragile X cortical stem cell line and three non-fragile X human cortical stem cell lines as controls. Neurospheres consist of neural precursors and rare stem cells. Differentiated cultures (9 weeks in culture) consist of about 10% GABAergic and glutamatergic neurons and 90% astrocytes. Forskolin stimulated cells were bathed in 5 mM forskolin solution for 30 minutes. All cells were bathed in a 1X KRB-IBMX buffer to inhibit phosphodiesterase reductions in cAMP levels. A fluorescence assay was used to measure cyclic AMP levels (see Figure 2). Fluorescence intensity was read at the Keck-UWCCC (University of Wisconsin Comprehensive Cancer Center) Small Molecule Screening Facility using a Safire II plate reader (Tecan, Inc.). The standard curve was fit using a four parameter dose response fit in Matlab 6.5 (Mathworks) to convert raw fluorescence measures to estimates of cAMP levels.

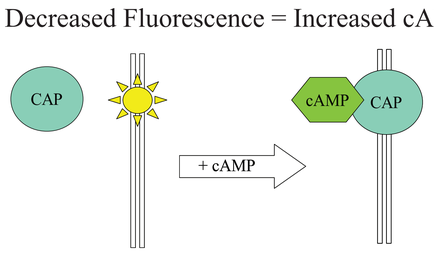


Figure 2: Fluorescence Assay: The Bridge-It cAMP designer system (Mediomics, LLC) has a 5nM cAMP detection level and uses two half-site DNA duplexes that are each fluorescently labelled at the binding site for CAP (cAMP Receptor Protein), a bacterial transcription factor whose DNA binding affinity is high in the presence of cAMP and low in cAMP's absence. Quenching of fluorescence signal by CAP specifically binding to the fluorescently tagged DNA is a quantitative index of cAMP levels since CAP binds selectively to both cAMP and the CAP binding site (Heyduk et al. 2003).

Results:

All cell lines showed increased cAMP production in the presence of forskolin (Table 1). In differentiated cells, fragile X cAMP production was lower than controls; and, in neurospheres, fragile X cAMP production was comparable to controls (Table 2; Figure 3). When differentiated cells are compared to neurospheres, all three control cell lines showed a marked increase in the levels of cAMP production in differentiated cells compared to undifferentiated neurospheres (Mean FDRF Difference [DC-NS] = 0.52); however, stimulated cAMP levels in differentiated cells and neurospheres are comparable in the fragile X line (FDRF Difference [DC-NS] = 0.05). Using a standard curve (Figure 4) to convert fluorescence intensity to cyclic AMP levels, we show stimulated cAMP production is impaired in fragile X cells (Figure 5).

			No Forskolin		Forskolin	
	FDRF	SD	Fluorescence	SEM	Fluorescence	SEM
Controls_D	0.70	0.09	32617	1035	9643	2033
FRAX_D	0.32		30358	---	20685	---
Controls_N	0.19	0.11	28755	213	23318	1759
FRAX_N	0.27		29327	---	21468	---

Table 2. Average fluorescence values for cAMP levels in control (n = 3) and fragile X neurospheres (N) and differentiated (D) cells in the presence or absence of forskolin. Fractional Decrease in Raw Fluorescence (FDRF) = (No Forskolin - Forskolin) / Forskolin

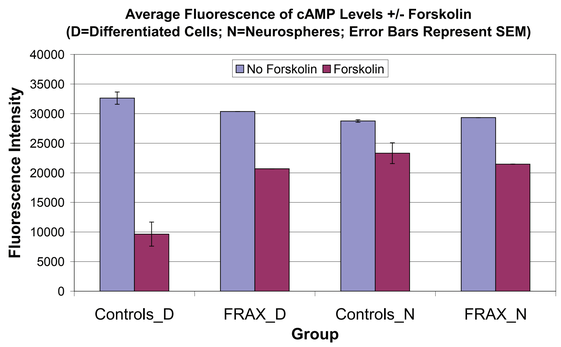


Figure 3: Raw Fluorescence of basal (No Forskolin) and stimulated (Forskolin) cAMP production in differentiated cells (D) and neurospheres (N). The control data represent the average value for 3 non-fragile X control neural stem cell lines. The FRAX data are from a single fragile X neural stem cell line. Unlike controls, the fragile X differentiated cells are unable to respond to forskolin stimulation and continue to behave like undifferentiated neurospheres.

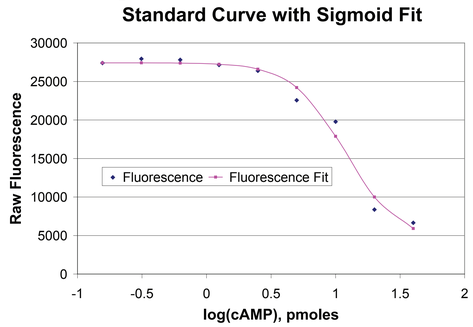
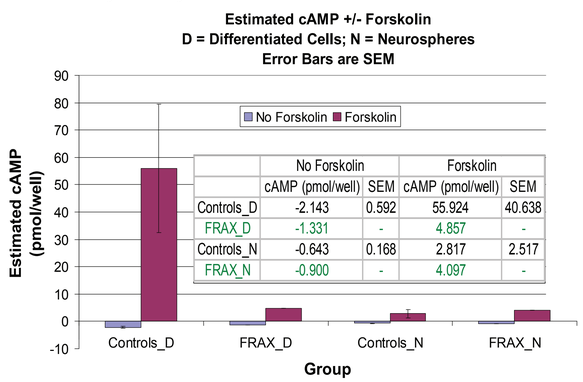


Figure 4: Standard curve to convert Raw Fluorescence Intensity into cyclic AMP levels.



	No Forskolin		Forskolin	
	cAMP (pmol/well)	SEM	cAMP (pmol/well)	SEM
Controls_D	-2.143	0.592	55.924	40.638
FRAX_D	-1.331	-	4.857	-
Controls_N	-0.643	0.168	2.817	2.517
FRAX_N	-0.900	-	4.097	-

Figure 5: Estimated cAMP levels for basal (no forskolin) and stimulated (forskolin) cAMP production in differentiated cells (D) and neurospheres (N). Fluorescence values (Figure 3) were converted to cAMP levels using the standard curve (Figure 4). Data values are shown inset in Table 3.

Conclusion:

As hypothesized, we found that human fragile X neural cells show a stimulated cAMP production that is reduced relative to unaffected neural cells.

These results are the first demonstration of an altered cAMP cascade in human fragile X neural tissue and extend Berry-Kravis' corpus of work which demonstrated an altered fragile X cAMP cascade in non-neuronal human tissue.

Given that cAMP is known to mediate anxiety and memory related processes known to be affected in fragile X, the cyclic AMP cascade may prove to be a potentially useful pharmacotherapeutic target that deserves further investigation.

Future Directions:

We are now concentrating our efforts to clarify if the group difference in cAMP mediated signal transduction is conserved across multiple organs and animal models of fragile X (Figure 6) and to determine which adenylate cyclase isoform cargoes are most affected by reduced levels of FMRP.

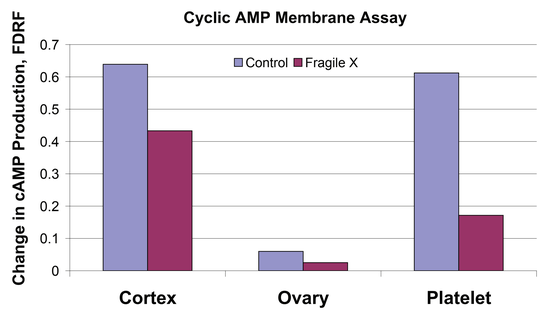


Figure 6: Fractional Decrease in Raw Fluorescence (FDRF) due to forskolin administration in various organs of one wild type and one fragile X KO mouse (C57/B6 FMR1 +/-). Protein content was matched for each group within each organ type (cortex = 42.5 ug; ovary = 30 ug; platelet = 125 ug). FDRF = (No Forskolin - Forskolin) / Forskolin.

Selected References

Berry-Kravis, E. and R. Ciurlionis. Overexpression of fragile X gene (FMR-1) transcripts increases cAMP production in neural cells. J Neurosci Res 51(1): 41-8 (1998).

Berry-Kravis, E., M. Hicar, et al. Reduced cyclic AMP production in fragile X syndrome: cytogenetic and molecular correlations. Pediatr Res 38(5): 638-43 (1995).

Heyduk, E., Fei, Y. & Heyduk, T. Homogeneous fluorescence assay for cyclic AMP. Comb Chem High Throughput Screen 6, 347-54 (2003).

Rius RA, Mollner S, Pfeuffer T, Loh YP. Developmental changes in Gs and G(olf) proteins and adenylyl cyclases in mouse brain membranes. Brain Res.18; 643(1-2):50-8 (1994).

Rius RA, Streaty RA, Peng Loh Y, Klee WA. Developmental expression of G proteins that differentially modulate adenylyl cyclase activity in mouse brain. FEBS Lett. 288(1-2):51-4 (1991).