Cell-Type Specific Analysis of Antipsychotic Drug Action in D_1 and D_2 Neurons of the Striatum

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Introduction

Antipsychotic drugs (APDs) have been the primary treatment for the positive symptoms of Schizophrenia for over fifty years. Although these drugs were found to block dopamine D_2 receptors, the downstream mechanisms of therapeutic action remain unclear. Furthermore, APDs usually produce potentially irreversible side effects (including movement and metabolic disorders), indicating the need for a more targeted approach.

Both D_1 and D_2 dopamine receptors are highly expressed in the striatum. However, D_1 and D_2 receptors generally do not colocalize on the same striatal neurons, and also exert opposing effects on downstream signaling. Cell-type specific effects can be masked in extracts of whole striatum.

Transgenic mice expressing differentially tagged protein in D_1 versus D_2 neurons allow cell-type specific analysis of downstream signaling elements of the dopamine pathway (Bateup et. al. 2008), such as the striatal enriched phosphoprotein DARPP-32 (Dopamine and Adenosine Regulated Phosphoprotein of 32 KDa).

DARPP-32 was shown to be a potent inhibitor of Protein Phosphatase 1 (PP1) when phosphorylated on the threonine 34 (T34) residue. More recently (Stipanovich et. al. 2008), dephosphorylation of the serine 97(S97) residue of DARPP-32 was found to promote DARPP-32 nuclear retention and accumulation.

Mutation of the S97 residue was found to impair dopamine regulated responses and histone phosphorylation states (Stipanovich et. al. 2008**). The aim of this project is to elucidate the effects of antipsychotic drugs on the phosphorylation and intracellular localization of DARPP-32**. We further plan to assess the role of nuclear DARPP-32 on histone modifications and transcriptional regulation.

Methods

Animals: 10-12 week old doubly transgenic (D_1 , DARPP-32-Flag/ D_2 DARPP-32-Myc) were received from the Greengard lab (Rockefeller University) and bred in the Yale Animal Facility. Acute drug groups were given a single intraperitoneal injection of either drug or vehicle (n=10) and sacrificed 15 minutes later by focused microwave irradiation. Chronic drug groups received once daily injections for 21 days, and sacrificed 24 hours after the final dose. Care and use of animals was in compliance with IACUC (Institutional Animal Care and Use Committee) regulations for ethical treatment of animals.

Drugs: Haloperidol, Clozapine, and Risperidone (Sigma-Aldrich) were dissolved in saline (Braun 0.9% NaCl). Glacial acetic acid was added for solubility, and the pH was adjusted back up to 6 with sodium hydroxide. Vehicle contained saline and a corresponding volume of glacial acetic acid, with an adjusted pH of 6.

Tissue Preparation and Immunoprecipitation: Mice were sacrifice by focused microwave irradiation and tissue preparation was carried out according to the methods from Bateup et.al. 2008. Striatum were dissected, sonicated in 500µL homogenization buffer, and centrifuged for 20 minutes at 13,000RPM. An aliquot of total striatal homogenate was separated from each sample and the remaining homogenate was added to 50µL flag agarose beads (Sigma EZView red) and rotated overnight at 4˚C. Flag beads were washed three times and eluted in 2X sample buffer. Flow through was transferred to 50µL slurry Myc-conjugated sepharose beads (Cell Signaling), and rotated overnight at 4˚C. Myc beads were washed three times and eluted in 3X sample buffer with β-mercaptoethanol. Flow through was collected and all samples were frozen at -80˚C until processing.

Immunoblotting : Samples from total, flag, and myc extracts were loaded on 4-20% tris-glycine midi gels (Life Technologies) and transferred overnight to PVDF membrane. Membranes were blocked for one hour with 5% non-fat dry milk and incubated overnight with phospho-S97 DARPP-32 Ab (1:1000, Cell Signaling). HRP-conjugated anti-rabbit secondary (1:2000, Pierce) was added for one hour at room temperature. Blots were developed with Pico ECL solution (Pierce) and imaged using the Bio-Rad Chemi-doc XRS+ imaging system. Blots were immediately stripped for three hours in stripping buffer (25mM Glycine 2% SDS, pH 2) at 50˚C. Blocker was added for one hour , followed by total DARPP-32 Ab (1:2000, Greengard Lab) overnight at 4˚C. Membranes were incubated with HRP-conjugated anti-mouse secondary Ab (1:2000, Pierce) for one hour at room temperature, and developed with Pico ECL solution using the BioRad Imager. (Thr-34 blots were processed according to this protocol using primary pT34 DARPP-32 Ab(1:1000 , Greengard Lab), and secondary HRP-conjugated anti-Rabbit (1:1000, Pierce).)

Analysis: All bands were quantified , and pSer-97 values were normalized to corresponding total DARPP-32 values by sample. Normalized values were adjusted to the average of normalized control values (this was carried out by condition: total extracts were normalized to total control , flag to flag control, myc to myc control) and plotted using GraphPad/Prism. Values were excluded as outliers if they varied by greater or less than two standard deviations from the mean. Significance was evaluated by t-test, and marked as significant with p-value less than 0.05.

FIGURE 1

Figure 1:

A) Transgenic mice are doubly homozygous for DARPP-32-Flag and DARPP-32-Myc expressed under the control of the D_1 or D_2 promoter, respectively.

B) Phosphorylation states of DARPP-32: T34 Phosphorylation allows DARPP-32 to become a potent inhibitor of PP1, while S97 phosphorylation regulates nuclear localization of DARPP-32.

C) Immunohistochemical analysis shows effective labeling of D_1-expressed flag-tagged (green), and D_2-expressed myc-tagged (red) DARPP-32. The merged image confirms that these two receptors are not colocalized in single neurons.

D) Hypothesized effect of APDs on downstream dopamine signaling: APDs oppose D_2 signaling and increasing cAMP production and activation of PKA. PKA phosphorylates the T34 residue and activates phosphatase PP2A. T34 phosphorylation allows inhibition of PP1, while PP2A dephosphorylates the S97 site and induces DARPP-32 nuclear retention. Inhibition of nuclear PP1 contributes to regulation of epigenetic and transcriptional changes.

RESULTS

FIGURE 2

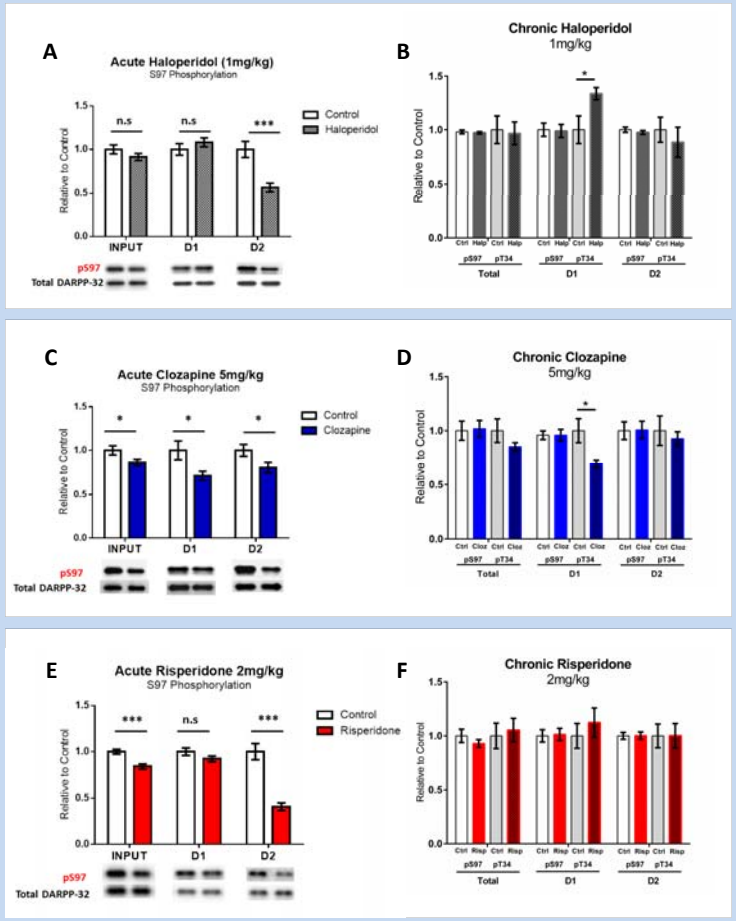

Figure 2:

(A,C, E) *Acute Haloperidol, Clozapine, and Risperidone Decreased DARPP-32 Serine 97 Phosphorylation in Striatal Medium Spiny Neurons:* Serine 97 (S97) phosphorylation was assessed in total striatum (input), D1, or D2 receptor containing neurons from DARPP-32 tagged animals. Mice were injected with vehicle or drug and sacrificed after 15 minutes. All three drugs significantly decreased S97 phosphorylation in D2 receptor neurons. Sample western blot bands from pS97 and total DARPP-32 are shown beneath each acute graph.

A) *Acute haloperidol* (1mg/kg) reduces pS97 phosphorylation in D_2 dopaminoceptive neurons. This change was not detected in total striatal extracts, and no significant effect was observed in D_1 fraction. (n = 10 animals/group, *p-value = 0.0007*)

C) *Acute clozapine* (5mg/kg) treatment decreases pS97 in total extracts, D_1, and D_2 fractions. This is consistent with the broader receptor profile of clozapine. (n = 9/10 animals/group *p-value = 0.04, 0.02, 0.04*)

E) *Acute Risperidone* (2mg/kg) decreased pS97 in the D_2 fraction, but not in the D_1 fraction. This change was detected in total striatal extracts as a significant decrease in overall pS97 levels. (n = 9/10 animals/group, *p-value = 0.0005, 0.00002*)

(B, D, F) *Chronic Haloperidol, Clozapine, and Risperidone did not produce any observable change in Serine 97 DARPP-32 Phosphorylation*: Mice were given once daily I.P. injections with vehicle or drug for 21 days and sacrificed 24 hours after the final injection. Chronic drug exposure did not produce any significant changes in pS97 phosphorylation. However, chronic haloperidol **(B)** increased threonine 34 phosphorylation in D_1 neurons (n = 9/10 animals/group, *p-value = 0.038*), while chronic clozapine **(D)** significantly decreased pT34 in D_1 neurons (n = 9/10 animals/group, *p-value = 0.021*).

Conclusions

- Acute haloperidol decreased pS97 phosphorylation only in D2 neurons.
- Acute Clozapine decreased pS97 in total extracts, D1, and D2 fractions.
- Acute Risperidone decreased pS97 in total extracts and D2 fractions.
- Chronic haloperidol, clozapine, and risperidone treatment has no significant effect on pS97 and varied effects on pT34.
- Immunohistochemical analysis to correlate phosphorylation state with nuclear localization of DARPP-32 is in progress. Data is not available at this time.

References

Stipanovich, A., *et al.* A phosphatase cascade by which rewarding stimuli control nucleosomal response. *Nature* **453**, 879-884 (2008).

Bateup, H.S., *et al.* Cell type-specific regulation of DARPP-32 phosphorylation by psychostimulant and antipsychotic drugs. *Nature neuroscience* **11**, 932-939 (2008).

Supported by NIH grant MH090963