Dopamine response gene pathways in dorsal striatum MSNs from a gene expression viewpoint: cAMP-mediated gene networks

Vladimir Babenko, Institute of Cytology and Genetics SB RAS, Novosibirsk, Russia 630090, Novosibirsk State University, Novosibirsk, Russia 630090; Anna Galyamina, Institute of Cytology and Genetics SB RAS, Novosibirsk, Russia 630090; Igor Rogozin, National Institutes of Health, Rockville Pike, Bethesda, Maryland, USA 20892; Dmitry Smagin, Institute of Cytology and Genetics SB RAS, Novosibirsk, Russia 630090; Natalia Kudryavtseva, Institute of Cytology and Genetics SB RAS, Novosibirsk, Russia 630090

*Corresponding author e-mail address: bob@bionet.nsc.ru (Vladimir Babenko)

Abstract

A mouse model of chronic social conflicts was used to analyze dorsal striatum neurons implicated in cAMP-mediated phosphorylation activation pathways specific for Medium Spiny Neurons (MSNs). Based on expression correlation analysis, we succeeded in dissecting Drd1- and Drd2-dopaminoceptive neurons (D1 and D2, correspondingly) gene pathways. We also found that D1 neurons feature previously reported two states, passive and active ones, represented in our analysis by distinct, negatively correlated gene clusters.

The correlation based gene pathways strongly corroborate the phosphorylation cascades highlighted in the previous studies, implying that the expression-based viewpoint corresponds to phosphorylation/ dephosphorylation interplay in each type of neurons. Notably, D2 neurons showed the largest *Ppp1r1b* (encoding DARPP-32*)* expression modulation impact, implying that *Ppp1r1b* expression dynamics is mostly associated with neuroendocrine response mediated by *Penk/Pdyn* genes expression in D2 neurons.

We observed that under defeat stress in chronic social conflicts mice exhibited reduced motor activity as well as overall depression of dopamine-mediated MSNs activity, while aggressive mice exhibited motor hyperactivity and an increase in both D1-active phase and D2 MSNs genes expression.

24   Based on alternative transcript isoforms expression analysis, it was assumed that many genes (*Drd1,*

25   *Adora1, Pde10, Ppp1r1b, Gnal*), specifically those in D1 neurons, apparently remain transcriptionally

26   repressed via the reversible mechanism of promoter CpG island silencing, resulting in alternative promoter

27   usage following profound reduction in their expression rate.

Significance statement

29   Medium Spiny Neurons (MSNs) comprise the main body of dorsal striatum neurons and represent

30   dopaminoceptive GABAergic neurons. The cAMP- mediated cascade of excitation and inhibition responses

31   involved in dopaminergic neurotransmission is crucial for neuroscience research due to its involvement in the

32   motor and behavioral functions. In particular, all types of addictions are related to MSNs. Shedding the light

33   on the mechanics of the above-mentioned cascade is of primary importance for this research domain. In this

34   paper MSNs steady states will be elucidated based on pooled tissue RNA-Seq data not explicitly outlined

35   before and connected with dynamic dopamine neurotransmission cycles.

Introduction

37   The dorsal striatum is responsible for the regulation of motor activity and stereotypical behaviors and is

38   also potentially involved in a variety of cognitive, reward and social hierarchy processes (Gerfen and

39   Surmeier 2011; Lobo and Nestler 2011; Lee et al. 2018; Stagkourakis et al. 2018). Through afferent and

40   efferent projections to associative, motor and sensorimotor cortical areas and other brain structures, dorsal

41   striatum participates in the initiation and execution of movements, as well as in the regulation of muscle tone

42   (Devan et al. 2011; Liljeholm and O'Doherty 2012).

43   Medium Spiny Neurons (MSNs) represent 95% of neuron population within the dorsal striatum in mice

44   (Kemp and Powel 1971). Notably, MSNs are GABAergic neurons thatalso have dopamine/glutamate

45   receptors in postsynaptic dendrites. The phosphorylation cascades throughout the dorsal striatum neurons

46   play a central role in motion and emotion signals transduction (Surmeier et al. 2007). In particular,

47   striatonigral "direct" and striatopallidal "indirect" pathways represent opposite excitatory and inhibitory

48 signal transmissions, respectively, depending on MSN dopamine receptor types: D1-dopaminoceptive, or D2-

49 dopaminoceptive (Gerfen et al. 1990; Ouimet et al. 1998).

50   Reciprocal protein phosphorylation/dephosphorylation cascades constitute a major regulatory

51 mechanism of intracellular signal transduction. Its functioning in MSNs, where the corresponding gene

52 pathways are highly expressed and coordinated, provides a vivid illustration. The networks comprise the

53 pathways mediated by PKA and Cdk5 kinases for cAMP activated kinases, Mapk2-4 for mitogen-activated

54 kinases, serine/threonine phosphatases PP1, PP2A, PP2B and two tyrosine phosphatases Ptpn5, Ptpn7

55 (Surmeier et al. 2007).

56   Due to the specifics of dephosphorylation machinery regulation, multiple inhibitor subunits are

57 recruited in the phosphatase complex formation. In particular, protein phosphatase 1 (PP1) represented by

58 three catalytic subunits alpha, beta, gamma, encoded by *Ppp1ca, Ppp1cb, Ppp1cc* genes, can associate with

59 more than 100 inhibitor subunits (Eto 2009), while PP2A (2 catalytic subunits) inhibitors are represented by

60 15+distinct subunits, and PP2B (3 catalytic subunits) is regulated by four inhibitor subunit genes. However, it

61 was found that only one inhibitor can bind the catalytic core at a time via the same catalytic site (Aggen et al.

62 2000). Thus, the repressor subunits play a pivotal role in regulating phosphatases in a tissue and stage specific

63 manner.

64   Since protein phosphatases act in a wide range of cell types and are commonly associated with

65 deactivation and ubiquitylation of proteins (Nguyen et al. 2013), *Ppp1r1b* encoding PP1 inhibitor subunit

66 DARPP-32 was underlined as one of the rare neurospecific genes expressed at very high rates specifically in

67 striatum medium spiny neurons and playing a crucial role in the 'orchestration' of neurotransmission

68 (Svenningsson et al. 2004). *Ppp1r1b* is expressed in the upper bound of the expression range for dorsal

69 striatum-related protein-encoding genes and is implicated as an ultimate factor of MSNs phosphorylation

70 kinetics regulation (Scheggi et al. 2018). It has been proven to be involved in many pathophysiological

71 processes (Albert et al. 2002; Svenningsson et al. 2005; Santini et al. 2007). Notably, this gene from the

72 family of protein phosphatase inhibitors solely is directly associated with aggressive behavior as previous

73 studies have revealed (Reuter et al. 2009).

74   In D1 MSNs cAMPs activate PKA which phosphorylates DARPP-32 on Thr34, transforming it into a

75   of PP1 inhibitor (Stoof and Kebabian 1981; Nishi et al. 1997; Missale et al. 1998). Calcineurin (PP2B, its

76   catalytic subunit expressed at the highest rates is encoded by *Ppp3ca*), is also expressed in D1 neurons,

77   dephosphorylates DARPP-32 at Thr34, mediating DARPP-32 phosphorylated homeostasis state, in particular

78   turning it off upon signal abrogation. Conversely, Cdk5, which is activated in D2-dopaminoceptive MSNs by

79   Ca2+ provided by AMPA/NMDA receptors, phosphorylates DARPP-32 at Thr75, turning DARPP-32 into a

80   PKA inhibitor (Nishi et al. 2002).The pathway is being regularly updated, and published elsewhere (Scheggi

81   et al. 2018). Besides, MSNs express dorsal striatum specific Tyrosine phosphatase STEP (encoded by *Ptpn5*),

82   which functions alternatively to serine/threonine phosphatases (Fitzpatrick and Lombroso 2011).

83   In the current study we investigated a mouse chronic social conflicts model with antagonizing

84   emotional states and motor activity (hyperactivity and total immobility) (Smagin et al. 2018; Smagin et

85   al.2019) to analyze the involvement of dorsal striatum neurons in cAMP-mediated phosphorylation activation

86   pathways specific for Medium Spiny Neurons (MSNs). We aimed at assessing the overall profile of the

87   D1/D2 neurons based on RNA-Seq expression profiling by considering the reported genes involved in

88   phosphorylation kinetics in MSNs.

89   **Results**

90   **Compilation of gene sets**

91   To gain fuller insight into the D1/D2 MSNs we retrieved the annotated genes implicated in PP1-

92   regulated posphorylation pathways in MSNs based on available literature (Nishi et al. 2002; Svenningsson et

93   al. 2004; Nishi et al. 2011; Nishi et al. 2017; Yapo et al. 2017). The compiled core gene set comprising

94   cAMP-mediated dopamine response genes expanded with NMDA glutamate receptor subunits *Grina*, *Grin1-*

95   *Grin2* and MAP kinases is presented in Table 1. Their expression profiles are presented in Supplemental

96   Table S1. We used *Cdk5r1,* neuron-specific activator of cyclin dependent kinase 5 (CDK5) p35 subunit, as a

97   CDK5 activation marker. Three major serine-/threonine-specific phosphatases involved in MSN cascades are

98   PP1, PP2A and PP2B. The PP1 catalytic core comprises 3 subunits (encoded by *Ppp1ca, Ppp1cb, Ppp1cc*),

4

99   PP2A is represented by 2 catalytic subunits (*Ppp2ca* and *Ppp2cb*), and PP2B includes 3 subunits (*Ppp3ca,*

100   *Ppp3cb, Ppp3cc*). For all three phosphatases the 'alpha' subunit exhibits the highest levels of expression

101   (Supplemental Table S2), so these subunits were used as the major phosphatase gene markers, except for PP1

102   (all subunits were considered).

103   **Basic confirmation of 4 clusters based on dorsal striatum data using the core genes set (Table 1).**

104   We clustered the core dopamine response cAMP-mediated genes (Table 1) by Agglomerative

105   Hierarchical Cluster (AHC) analysis (Fig. 1) and observed 4 distinct clusters. Strikingly, each of the clusters

106   corresponds to a specific signal transduction cascade observed in cAMP-mediated response to dopamine,

107   which can be ascribed to the key genes. In particular, there is a cluster comprising *Drd2* receptor distinct from

108   one comprising *Drd1* receptor. Another cluster marks PKA-phosphorylation cascade *(Prkaca)*, and the fourth

109   clusterrepresented by a single gene (*Tac2*) indicates the absence of dopamine signaling.
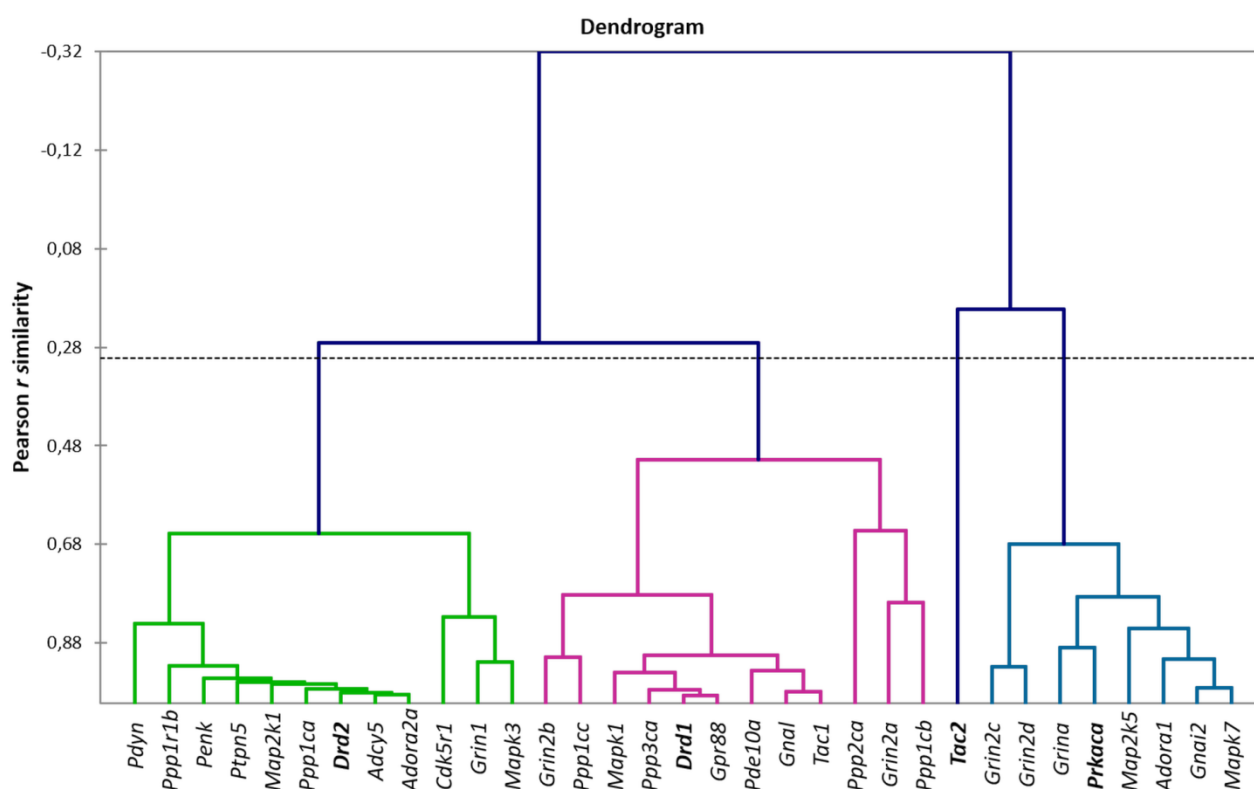

110

111   Fig. 1. AHC analysis reveals 4 consequent clusters of a) D2-associated genes (green); b) D1-associated

112   passive state genes (red); c) D1-associated active state genes (blue). Single gene corresponding to DA

113   depletion is represented by *Tac2* expression.

5

114   To gain an expanded view on the clusters elucidated by AHC we performed the Principal

115   Components Analysis (PCA) on the same core genes set (Fig. 2). It has been confirmed that the gene clusters

116   are highly synchronized (high correlation rate), each corresponding to a particular neuron type (D1/D2)

117   according to their annotation in publications. Also, as follows from Fig. 2, we can observe antagonistic
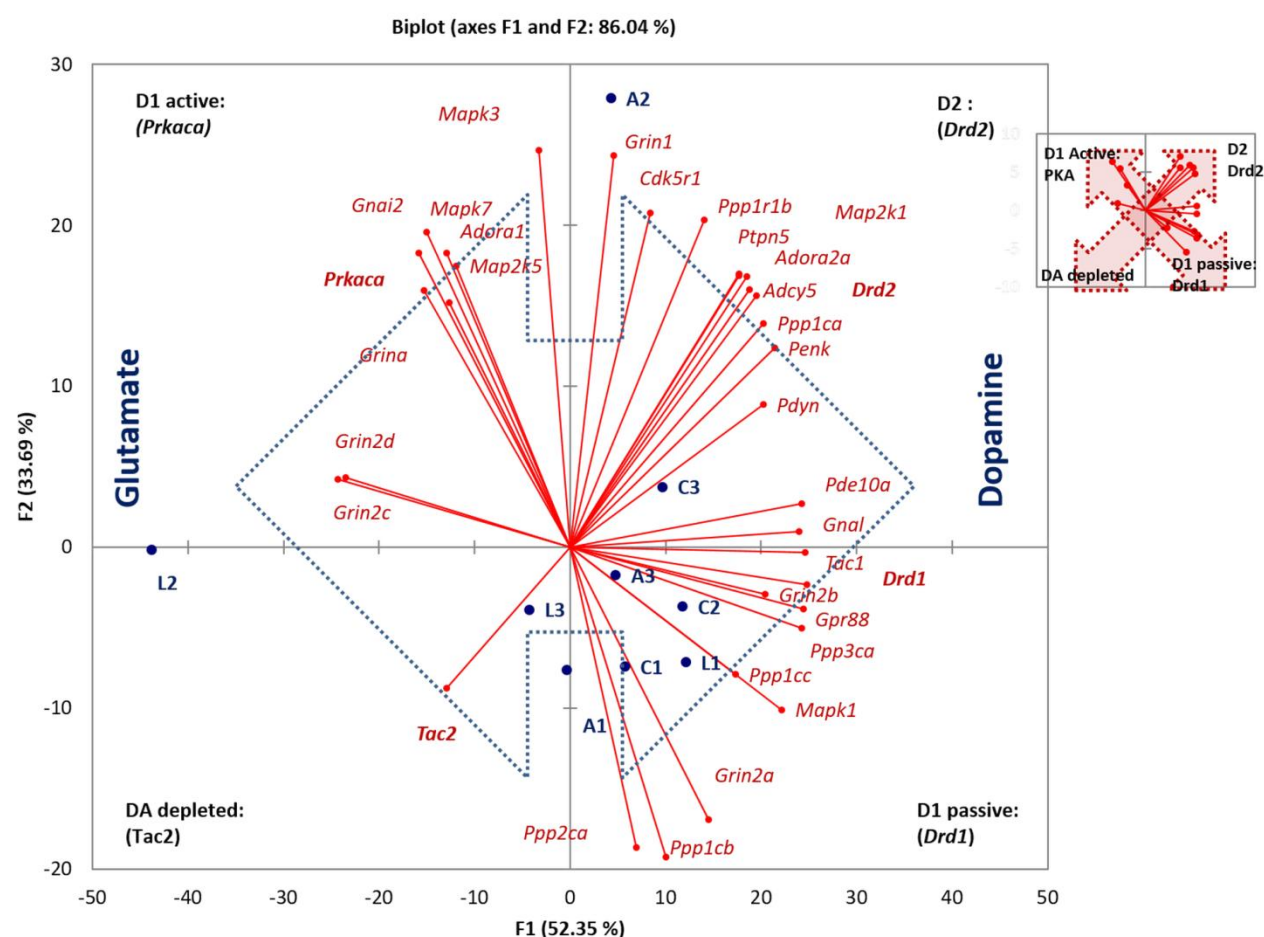

Biplot (axes F1 and F2: 86.04 %)

118

119   Figure 2.Major clusters detected by AHC and PCA: C1, C2, C3 – control; A1, A2, A3 – aggressive mice; L1,

120   L2, L3 – losers, defeated mice. 4 quadrants are indicated with corresponding (D1/D2/DA depleted) states and

121   marker genes. Inserted is a scheme of antagonistic clusters: the arrows depict alternative states of D1 MSN,

122   and D2/DA-depleted states. Blue arrow indicates antagonistic gradient of dopamine and glutamate according

123   to the corresponding receptors distribution. Inserted (small figure) is a scheme of antagonistic clusters: the

124   arrows depict alternative states of D1 MSN, and D2/DA-depleted states.

125

126   clusters of the D1 geneindicating its stable rather than passive state followed by a short firing time span upon

127   activation represented by D1 active state as shown in (Yapo et al. 2017). The details of the PCA analysis are

128   shown in Fig. 2. According to the genes in these clusters they were designated as D2 cluster, D1 passive, D1

129   active and DA depletion. Two major components are responsible for 86% of the variability in the expression

130   of the genes evaluated, underscoring consistency and exhaustive representation of genes variation.

131

132   *Regulation of Ppp1r1b* **expression**

133   It is known that in phosphorylated state DARPP-32 effectively inhibits both PP1 alpha (encoded by

134   *Ppp1ca;* Hemmings et al. 1984) and PP1 gamma (encoded by *Ppp1cc;* Hemmings et al. 1989). As we can see

135   from the plot in Fig.2 *Ppp1r1b* is located in the D2 cluster along with *Ppp1ca* encoding PP1 alpha subunit,

136   which indicates that this cluster is a major expression site. However, another catalytic subunit of PP1, *Ppp1cc,*

137   is located in D1 passive cluster (Fig. 2). Given *Ppp1cc* is committed specifically to D1 passive state (Fig. 2)

138   one may expect its coexpression with *Ppp1r1b* in D1 neurons.

139   Similar to *Grin1* (major subunit of NMDA) committed to D2c luster, *Grin2a-d* subunits are distributed

140   across distinct quadrants in Fig. 2: *Grin2a,b* are associated with D1 neurons passive state, while *Grina,*

141   *Grin2d,c* are located in D1 neurons active state cluster.

142   **Expression rates of dorsal striatum-specific genes as compared to four other brain regions**

143   Average expression rate of the genes has been analyzed in distinct clusters (described above) across 5

144   brain regions: hippocampus (HPC), hypothalamus (HPT), dorsal striatum (STR) ventral tegmental area

145   (VTA), midbrain raphe nuclei (MRN). Several genes have been identified as the genes preferentially

146   expressed in dorsal striatum. Expression profiles of the selected genes are shown in Fig.3a, b. They happen to

147   be expressed specifically in D2 and D1 passive state clusters. As for D1 active cluster, we identified *Prkaca*

148   (encoding one of the PKA subunits) and *Gnai2* as the genes with the lowest expression levels in dorsal

149   striatum across 5 brain regions (Fig. 3c). Fig.3 illustrates that the most actively expressed genes are

150   associated with D2 cluster (Fig. 3a). *Ppp1r1b* and *Penk* genes with more than 1000 FPKM units may be

151 considered as the signature, driver genes within D2 cluster. The selected genes in D1 passive cluster also

152 maintain a dorsal striatum-specific expression pattern (Fig. 3b), but their average expression rate was lower

153 than that of D2 cluster genes (Fig. 3a), implying the major role of D2 cluster, and DARPP-32 in particular as

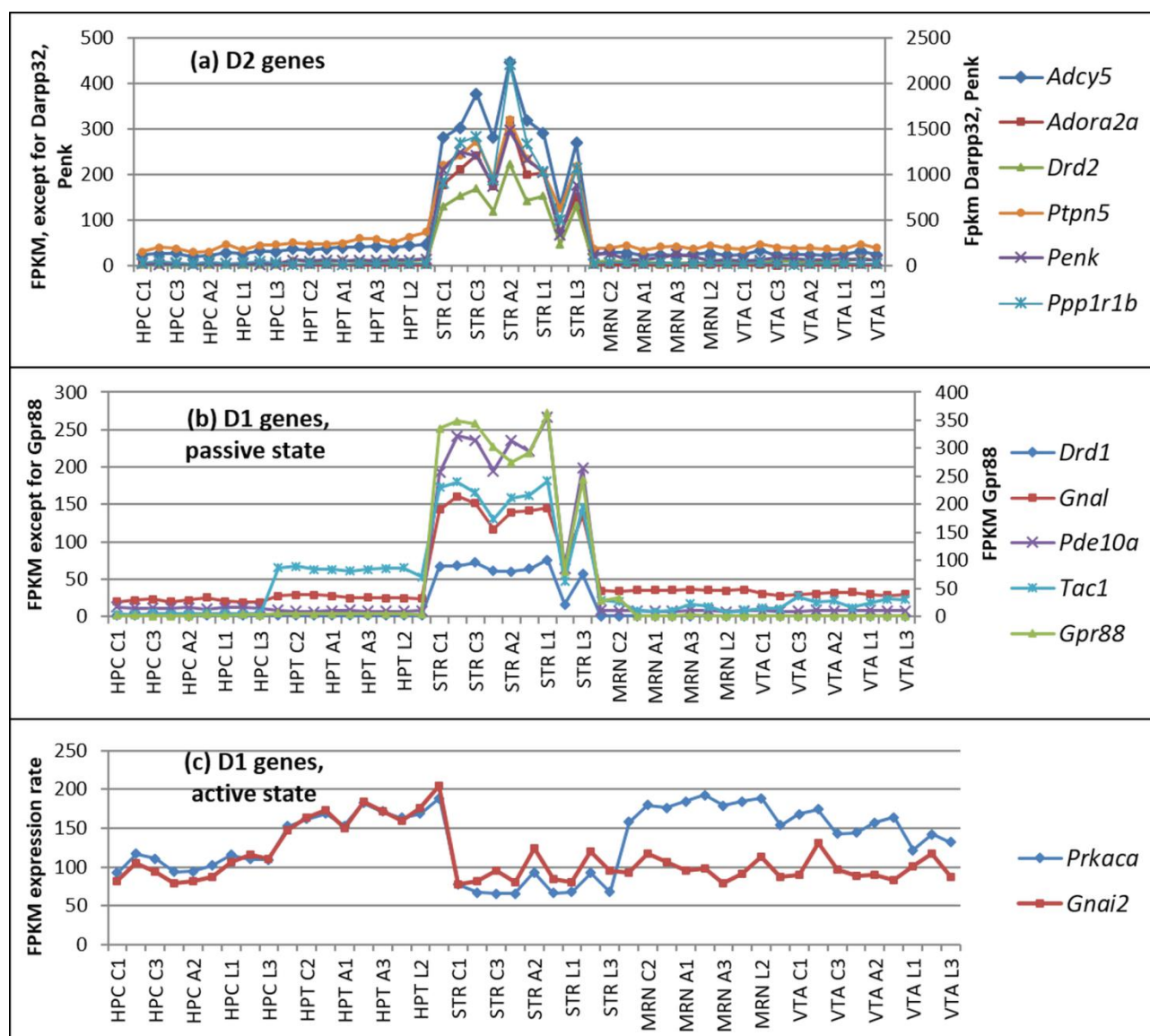154 the basic maintenance unit of dorsal striatum functioning.

155

156 Figure 3. Profiles of D1/D2 striatum specific genes expression (a,b), and non (anti) -specific ones in D1-

157 active pathway (c) across 5 brain regions.C1, C2, C3 – control; A1, A2, A3 – aggressive mice; L1, L2, L3 –

158 losers, defeated mice. HPC-Hippocampus, HPT – hypothalamus, STR-dorsal striatum, MRN – midbrain

159 raphe nuclei, VTA – ventral tegmental area.

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160

161   Notably, in the above-mentioned two clusters (D2 and D1 passive), the expression rate of selected

162   genes was significantly higher in dorsal striatum compared to other four brain regions. On the contrary,

163   assessment of the signature genes (*Prkaca, Gnai2*) of D1-active cluster demonstrated patently decreased

164   expression rates of *Prkaca* and *Gnai2* genes as compared to other brain regions (Fig. 3c), implying a short

165   expression time span upon firing as one of the reasons.

166   **Alternative splicing analysis**

167   We retrieved 13 splice isoforms for 12 genes from our set of 33 core genes (Supplemental Table S3).

168   After that we performed Principal Components Analysis (PCA) for the Pearson pairwise correlation matrix.

169   A circular plot for 46 resulting transcripts is shown in Fig.4. Most splicing isoforms, namely for genes

170   *Prkaca, Adora1, Mapk7, Mapk1, Tac1, Drd1, Penk, Ppp3ca* (Fig. 4), exhibit concordant expression patterns

171   in the same clusters or close to them. However, we identified negatively correlated, mutually exclusive

172   splicing isoforms for three pairs of gene transcripts: *Pde10-Pde10_1, Ptpn5-Ptpn5_1, Gnal-Gnal_1* (Fig. 4).

173   Notably, *Pde10a_1, Ptpn5_1* and *Gnal_1* represent minor long isoforms (Supplemental Fig. S1a-c) with

174   negligible expression rates (4-100-fold lower; Supplemental Table S3) as compared to corresponding major

175   transcripts. According to the results shown in Fig.4 one may suggest that the transition from D1 passive to D1

176   active state is accompanied by the switch between transcription patterns of *Pde10 and Gnal* genes. Also, the

177   transition from D2 state to dopamine depleted state is signified by the switch between transcription patterns
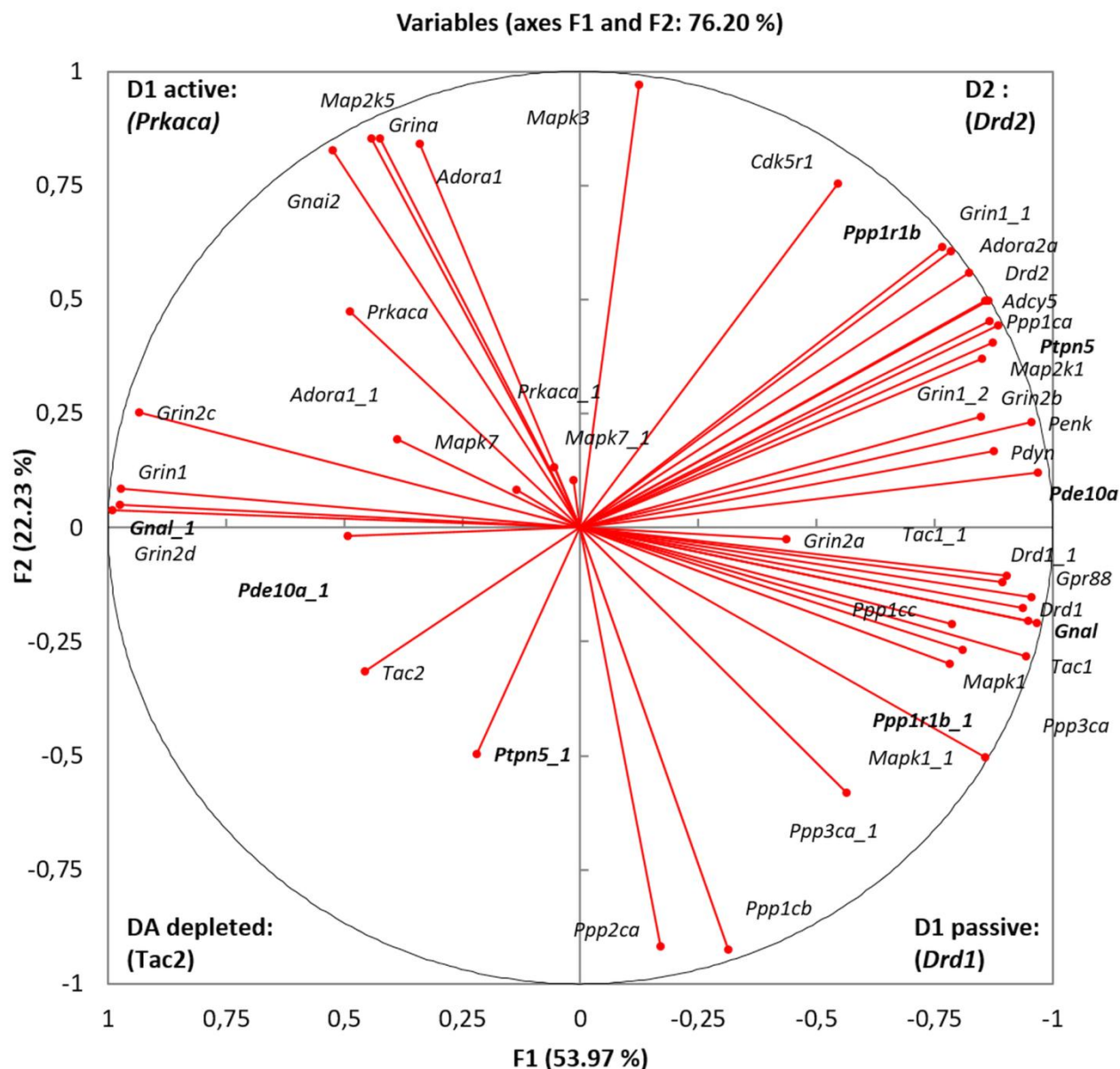
178   of *Ptpn5*.

Figure 4. Distribution of splice isoforms across D1/D2 clusters. In bold are splice isoforms that are negatively correlated, or significantly differed (bold italic) in their location. Transcript denotations are listed in Supplemental Table S3.

The *Ppp1r1b* minor splice variant (*t-DARPP* or *Ppp1r1b*_1 in Supplemental Table S3) was found to be specific to D1 MSNs in a passive mode and to exhibit 5-fold reduction in expression level ascompared to

10

186 that of major D2 cluster-associated transcript (Fig. 4). The truncated isoform of *Ppp1r1b* (*t-DARPP;*

187 NM_001313970; Avanes et al. 2019) (Supplemental Fig. S2a) lacking 37aa at *N*-terminal makes T34

188 inaccessible to phosphorylation by PKA as well as prevents its overall binding to PP1 while retaining the

189 ability to inhibit PKA upon T75 phosphorylation.

190 **Role of CpG islands in alternative transcription**

191 The genes listed above (*Ppp1r1b, Ptpn5, Pde10a* and *Gnal*) are CpG islands associated with promoter

192 genes. Remarkably, upon transition of D1 passive to D1 active state and consequent gene networks

193 rearrangement, some of the alternative long isoforms start being transcribed from its closest distal CpG

194 islands (Supplemental Fig. S1, S2). This alteration is witnessed by negative correlation of the splicing

195 isoforms. This is the case with *Pde10a*, *Gnal* genes (Supplemental Fig. S1), implying that the mechanism of

196 transcription pattern switching is based on the inhibition of proximal promoter CpG island of the major

197 isoform transcript. Two other genes, *Ppp1r1b* and *Ptpn5,* maintain CpG promoter in the major isoform but

198 with non-CpG mediated alternative transcription start site (Supplemental Fig. S1, S2). As the above-

199 mentioned genes are involved in D1 passive/active state dynamics, CpG island inhibition process may

200 support the propensity for rapid reciprocity upon D1 active state abrogation and the restoration of the genes'

201 default isoforms.

Discussion

203 The dynamics of MSN related cAMP-mediated dopamine response, even though intensively studied,

204 needs to be elaborated further (Yapo et al. 2017). After the emergence of the single cell transcriptome

205 protocol, in the study by Gokce et al. 2016 and in a range of subsequent publications dedicated to single cell

206 analysis the taxonomy of D1/D2 neurons and glial cells in dorsal striatum was depicted. Still, the current

207 study showed that the pooled tissue analysis may yield a unique insight based on the consideration of specific

208 genes subsets.

209 We pursued the task of elucidating the phase portrait of dopamine cAMP-mediated response based on

210 specific genes subset expression profiles to assess the asymptotic steady states of MSNs in the dorsal striatum,

11

211   taking into account the fact that the dorsal striatum cell content is highly homogeneous. It was postulated that

212   cell identity is determined by specific gene expression signature as it is in single cell analysis. In our work we

213   found four distinct gene clusters, namely: one corresponding to D2 MSNs, another one - to dopamine

214   depleted state, and two clusters - to opposite D1 passive/active states. The analyzed clusters feature the

215   following properties:

216   1) **D2 cluster.** While it is known that *Ppp1r1b is* expressed both in D1- and D2 MSNs, in the current

217   study the expression rate of *Ppp1r1b* proved to be strongly correlated with those for *Cdk5 (p35)*

218   along with *Drd2, Adora2* and *Penk,* implying that a major fraction of *Ppp1r1b* expression rate in

219   dorsal striatum has to be ascribed to D2 MSNs. Notably, phosphorylation of DARPP-32 and D1R by

220   CDK5 has been reported previously (Jeonget al. 2013. Expression of *Ppp2ca, Ppp3ca* genes

221   encoding the catalytic subunits forspecific serine-/threonine- protein phosphatases PP2A, PP2B was

222   not found in this cluster by the clustering analysis (Fig. 1, 2). Instead, the previously reported tyrosine

223   phosphatase STEP encoded by *Ptpn5* is intensively expressed in the neurons of this type (Gerfen and

224   Surmeier 2011). In D2 MSNs *Adora2A-Drd2-Adcy5* genes constitute a highly interlinked heteromeric

225   transmembrane receptor complex elucidated recently and featuring D2-MSNs (Ferré et al. 2018;

226   Navarro et al. 2018).

227   2) **D1 cluster (passive phase).** *Ppp2ca* (PP2A) and *Ppp3ca*(PP2B*)* were found to be specifically

228   expressed in D1-neurons, apparently exemplifying dephosphorylation of DARPP-32 upon activation

229   signal abrogation, thereby maintaining homeostasis of phosphorylated DARPP-32 proteins at a

230   certain level mediated by AMPA/NMDA Ca2+ input rate. Also, experimental findings (Massart et al.

231   2009) that *Golf*, *Gpr88* are the major G-proteins facilitating preferential maintenance of *Drd1*

232   receptors in MSNs have been confirmed. As mentioned earlier (Sin et al.2008) preprotachykinin A

233   (*Tac1) e*xpression is highly correlated with that of D1 receptor. As follows from correlation analysis,

234   ERK2(*Mapk1)* kinase is also involved in maintaining D1 MSN passive phase and was shown to be

235   associated with D1 neurons (Gerfen et al. 2002). Notably, the truncated form of *Ppp1r1b* (*t-DARPP*)

236   was previously reported for several brain structures (Straccia et al. 2016), but it is the first time that *t-*

237   *DARPP* has beenmappedto MSNs network D1 cluster.

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238   3) **D1 cluster (active phase).** We observed coordinated expression of *Prkaca, Grina* and *Adora1a*

239   genes in active D1 MSNs. Their relation to D1 neurons was reported previously (Svenningssonet al.

240   2004), along with *Grin1*, *Map2k1*/*Mapk3*(*ERK1*) and *Map2k5*/*Mapk7*(*ERK5*) genes (Grissom et al.

241   2018), which were also localizedin this cluster in our study.

242   4) The fourth cluster was represented by single gene, *Tac2* (Fig. 2) which maintains minimal striatal

243   FPKM level of 0.5-1 units observed in the current study. Preprotachykinin B (*Tac2*) is known to be

244   expressed in only 5% of dorsal striatum non-MSN neurons (Zhou et al. 2004). It implies that *Tac2*

245   gene may be chosen as a signature indicator of non-D1/D2 MSN neuron type lacking specific

246   dopamine response, thus indicating the dopamine-depleted state (Fig. 2b).

247   The expression profiling of the genes involved in D1/D2 MSNs phosphorylation cascades has proved

248   informative and concordant with our current knowledge of gene pathways in these neurons. As an illustration

249   in Fig. 5a, b the proteins ascribed in our study to the genes from D1/D2 clusters are marked with color. This

250   technique and the schematics proposed by (Yapo et al. 2017) can be helpful for gaining the expression-based

251   insight into the specifics of MSNs functioning.

252   While the variation among the groups of experimental animals yielded no particular group-specific

253   clustering (Fig.2), the observed samples obtained from defeated mice (L2, L3) exhibit a bias toward the

254   dopamine-depleted quadrant (Fig. 2). Aggressive mice (A2- winner) display an increase in both D1 and D2

255   expression (Fig. 2). Other samples tend to maintain passive D1 phase and moderate D2 MSNs genes

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256   expression (Fig. 2). Thus, it can be stated that a behavior model featuring distinct physiological states is a
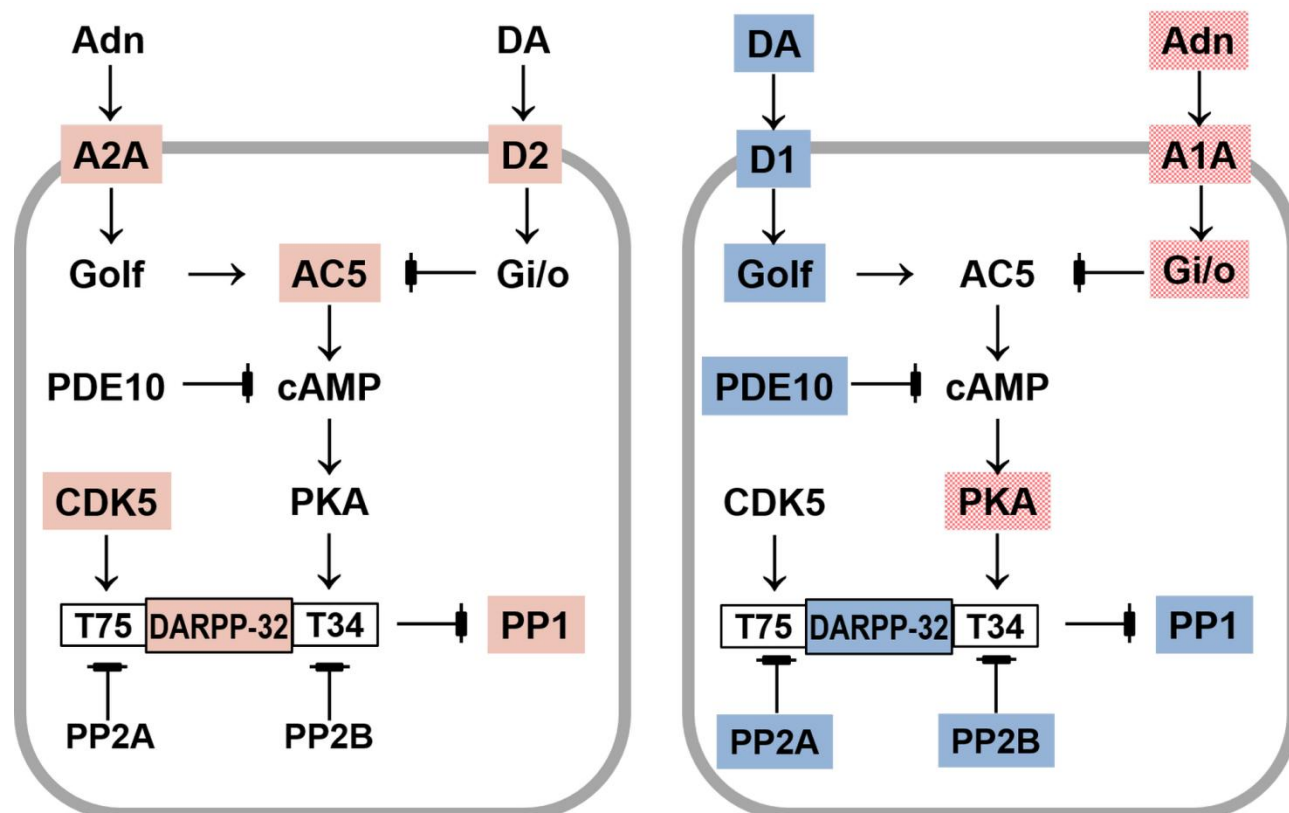

257

258   Fig. 5 (a). Stable D2-A2A-Golf-Gi/o complex features preferential inhibition of AC5 -> cAMP synthesis

259   upon DA (dopamine) signal, though stimulants can invoke it (Ferré et al. 2018; Navarro et al. 2018). Genes

260   encoding proteins shaded red are coordinately regulated in D2 neurons as observed in our RNA-Seq data

261   (Fig.1).Genes encoding uncolored proteins are absent in the D2 cluster (Fig.1), implying they may be present

262   with minor expression ranges and are not involved in coordinated variation.

263
264   Figure 5 (b). Oscillating passive-active cascades in D1 neurons. Genes encoding proteins of the same color

265   manifest correlated clusters in our data presented in Fig. 2. Blue (D1 passive state) vs Red (D1 active state)

266   clusters are antagonistic ones according to AHC and PCA analyses (Fig.1; Fig. 2).Genes encoding uncolored

267   proteins are absent in the D1 cluster (Fig.1), implying they may be present with minor expression ranges and

268   are not involved in coordinated variation.

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271   potent tool to enhance variation in expression profiles of observed genes and to ascertain dopamine-depleted

272   area specific states (D1 active, DA depleted). Graphically it is represented in Fig. 2 by blue arrow underlined

273   dopamine-glutamate gradient.

274   The 'up' and 'down' states of D1 neurons were reported previously (Wickens and Wilson 1998;

275   Surmeier et al. 2007): the 'firing' of D1 neurons upon glutamate input is reported to maintain peak-like

276   induction of voltage increase spanning about 1 sec (Wickens and Wilson 1998) incomparable with the down

277   state span for the major time lapse. Stress model used in the study helped to visualize the D1 active state in

278   detail.

279   The performed correlation analysis was based on the previous experimental evidence that the synaptic

280   genes network expression is highly coordinated and specific (Trinidad et al. 2008). Our attempt to shed a

281   light on such intricate genes networks was based on the presumption that proteins phosphorylation/

282   dephosphorylation cascades provide relevant feedback for genes expression rate, which was found to be well-

283   grounded in our study.

284   As it is known, besides dopamine, there are multiple other neurotransmitters that regulate the

285   excitability of dopaminoceptive neurons: glutamate, GABA, opiates, and adenosine, which are involved in

286   signaling through DARPP-32 in medium spiny neurons (Nishi et al. 2017). In particular, in our previous

287   studies the expression rates of catecholaminergic, glutamatergicand GABAergic receptors in the dorsal

288   striatum were shown to be significantly altered as compared to the control group, in reverse directions for the

289   defeated and aggressive mice (Smagin et al. 2018; Smagin et al. 2019).

290   DARPP-32 serving as a PKA inhibitor upon Thr75 phosphorylation by CDK5, as reported elsewhere

291   (Bibb et al. 1999; Nishi et al. 2002; Nishi et al. 2017) proved to be specific to D2 neurons. According to our

292   data, the genes with dorsal striatum expression profiles most correlated with that of *Ppp1r1b Cdk5r1* along

293   with *Ppp1ca* (Fig. 2) in D2 cluster. For D1 passive state *Ppp1cc* exhibits a distinct expression pattern (Fig. 2)

294   implying also that *Ppp1r1b* encodes the PP1 subunit.

295   Notably, phosphorylation of DARPP-32 Thr34 cascade upon PKA activation (Nishi et al. 2017)

296   apparently leads to PP1 phosphatase depletion according to the absence of *Ppp1** genes encoding the PP1

297   subunit in D1 active cluster (Fig. 2).Consequently, theinhibition of PP1 by DARPP-32Thr34 phosphorylation

298   in D1 active state upon PKA activation appears to be a fairly rapid and effective process.

299   Specific combinatorial usage of NMDA receptor subunits (*Grin2a*, *b, c, d*) with content alterations

300   across various clusters (Fig. 2) has been found within the dopamine-dependent cAMP-related genes network.

301   When analyzing alternatively spliced transcripts, we found a wide range of genes employing

302   alternative promoter usage (Supplemental Fig. S1). Most of them are associated in particular with D1 neurons

303   (*Drd1, Gnal, Ppp1r1b, Pde10a;* Supplemental Table S3; Supplemental Fig. S1, S2)*,* implying a possible

304   mechanism for gene/transcripts switching through reversible blocking of CpG island promoters

305   (Supplemental Table S3; Supplemental Figs. S1, S2). It is worth noting that the minor isoform is usually

306   longer (Supplemental Table S3), implying the inhibition of proximal CpG island, as reported earlier (Sarda et

307   al. 2017). As the switching between D1 active – passive states is of oscillatory nature, CpG islands are

308   apparently not modified by DNA methylation as was the case in (Sarda et al. 2017), nor are alternative

309   histone modifications (Pal et al. 2011), since both isoforms are present in D1 neurons. It is also intriguing that

310   the majority of the isoforms encode alternative functional proteins, which are expressed in D1 active state,

311   but usually at significantly lower rates (Supplemental TableS3).

312   Notably, D1 MSN excitation kinetics exerts the major impact on the D1/D2 cascades dynamics as

313   follows from D1-split states observations (featuring PKA cascade activation), while D2 neurons manifest the

314   strongest coordinated background expression of the target genes, including postsynaptic *Drd2- Adora2-*

315   *Adcy5* receptor complex, *Ppp1r1b*, and *Penk* (Fig.2). In particular, *Drd2* expression rate in dorsal striatum is

316   twofold higher than that of *Drd1* (Supplemental Table S1)*.* High *Penk* expression rate observed in D2

317   neurons (Fig. 3a; Supplemental Table S1) pinpoints D2 MSNs entity as a major neuroendocrine response

318   body in dorsal striatum previously reported in (Dunn et al. 2012; Bali et al. 2015; Belujon and Grace 2015).

319   In conclusion, we correspond that using RNA-Seq data as the basis for an asymptotic stationary model

320   of gene pathways we were able to elucidate the cAMP-mediated phosphorylation activation pathways.

321   Dopamine response gene pathways specific to D1- and D2-neurons represent the tight clusters of highly

322   correlated genes based on the expression rate profiles. In Fig.5a the signature genes elucidated for the D2

323   cAMP cascade by cluster analysis are depicted (Fig. 1).Moreover, the clustering was effective in

324   distinguishing the passive and active states of D1-neurons which employ distinct gene non-overlapping

325   pathways instantiation, thereby implementing the oscillatory states of D1 neurons (Fig. 5b).

326   According to our data, *Ppp1r1b* proved to be one of the most highly expressed protein-encoding genes in

327   dorsal striatum MSNs,. Based on the correlation analysis, the major activities of DARPP-32 in dorsal

328   striatum MSNs are assumed to be related specifically to D2 neurons, including, besides the regulation of

329   PKA activity, the involvement in neuroendocrine stress response. It has to be stressed that the pathways

330   reconstruction attempt succeeded due to the unexpectedly high coordination of the sampled genes expression,

331   as well as the homogeneity/dominance of MSNs content in dorsal striatum. In PCA analysis the overall

332   variation for the first two components reached 86% (Fig. 2), which underscores that our model presentation is

333   exhaustive and consistent. We believe that the stationary phase expression profile provided for dopamine

334   cAMP-mediated response in MSNs will help gaining further insight into the MSNs molecular dynamics.

335   **Materials and methods.**

336   **Animals**

337   Adult C57BL/6 male mice were obtained from Animal Breeding Facility, Branch of Institute of

338   Bioorganic Chemistry of the RAS (ABF BIBCh, RAS) (Pushchino, Moscow region). Animals were housed

339   under standard conditions (12:12 hr light/dark regime starting at 8:00 am, at a constant temperature of 22+/-

340   2°C, with food in pellets and water available *ad libitum*). Mice were weaned at three weeks of age and housed

341   in groups of 8-10 in standard plastic cages. Experiments were performed with 10-12 week old animals. All

342   procedures were in compliance with the European Communities Council Directive 210/63/EU of September

343   22, 2010. The protocol for the studies was approved by Scientific Council No 9 of the Institute of Cytology

344   and Genetics SD RAS of March, 24, 2010, N 613 (Novosibirsk, http://spf.bionet.nsc.ru/).

345   **Generation of alternative social behaviors under agonistic interactions in male mice**

346   Prolonged negative and positive social experience, social defeats and wins, in male mice were

347   induced by daily agonistic interactions (Kudryavtseva 1991; Kudryavtseva et al. 2014). Pairs of weight-

348   matched animals were each placed in a steel cage (14 x 28 x 10 cm) bisected by a perforated transparent

349   partition allowing the animals to see, hear and smell each other, but preventing physical contact. The animals

350   were left undisturbed for two or three days to adapt to new housing conditions and sensory contact before

351   they were exposed to encounters. Every afternoon (14:00-17:00 p.m. local time) the cage lid was replaced by

352   a transparent one, and 5 min later (the period necessary for individuals' activation), the partition was removed

353   for 10 minutes to encourage agonistic interactions. The superiority of one of the mice was firmly established

354   within two or three encounters with the same opponent. The superior mouse would be attacking, biting and

355   chasing another, who would be displaying only defensive behavior (sideways postures, upright postures,

356   withdrawal, lying on the back or freezing). As a rule, aggressive interactions between males are discontinued

357   by lowering the partition if the sustained attacks have lasted 3 min (in some cases less), thereby preventing

358   the damage of defeated mice. Each defeated mouse (loser) was exposed to the same winner for three days,

359   while afterwards each loser was placed, once a day after the fight, in an unfamiliar cage with an unfamiliar

360   winner behind the partition. Each winning mouse (winners, aggressive mice) remained in its original cage.

361   This procedure was performed once a day for 20 days and yielded an equal number of the winners and losers.

362   Three groups of animals used were: 1) Controls – mice without a consecutive experience of agonistic

363   interactions; (2) Losers – chronically defeated mice; (3) Winners – chronically aggressive mice. The losers

364   and winners with the most expressed behavioral phenotypes were selected for the transcriptome analysis. The

365   affected mice and the control animals were simultaneously decapitated. The brain regions(hippocampus

366   (HPC), hypothalamus(HPT), dorsal striatum(STR), midbrain raphe nuclei (MRN), ventral tegmental area

367   (VTA) were dissected by one experimenter according to the map presented in the Allen Mouse Brain Atlas

368   [http://mouse.brain-map.org/static/atlas]. All biological samples were placed in RNAlater solution (Life

369   Technologies, USA) and were stored at -70° C until sequencing.

18

370   It has been shown in previous experiments (Smagin et al. 2018; Smagin et al. 2019) that the chronically

371   aggressive mice develop motor hyperactivity, enhanced aggressiveness and stereotypic-like behaviors.

372   Chronically defeated mice manifest low motor activity and depression-like behaviors.

373   **RNA-Seq data collection**

374   The collected brain samples were processed at JSC Genoanalytica (www.genoanalytica.ru, Moscow,

375   Russia). According to the protocol therein, mRNA was extracted using a Dynabeads mRNA Purification Kit

376   (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). cDNA libraries were constructed using the

377   NEBNext mRNA Library PrepReagent Set for Illumina (New England Biolabs, Ipswich, MA USA)

378   following the manufacturer's protocol and were subjected to Illumina sequencing. More than 20 million reads

379   were obtained for each sample. The resulting "fastq" format files were used to align all reads to the

380   GRCm38.p3 reference genome using the STAR aligner (Dobin and Gingeras 2015) followed by *Cuffnorm*

381   software to assess the expression rate of the transcripts (Trapnell et al. 2012). The identification of

382   alternatively spliced events in the RNA-Seq data was performed with Cuffnorm procedure.

383   **Statistical analysis**

384   For the transcriptome data, a Principal Component Analysis (PCA) was conducted using the XLStat

385   software package (www.xlstat.com). PCA was based on Pearson product moment correlation matrix

386   calculated on the FPKM value profiles of analyzed genes. We also used a Pearson correlation as a similarity

387   metric for the Agglomerative Hierarchical Clustering (AHC). The agglomeration method comprised an

388   unweighted pair-group average. We avoided using commonly accepted WGCNA method (Langfelder and

389   Horvath 2008) due to its detrimental impact on clustering density because of limited amount of our genes

390   sample (up to 100 transcripts).

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393

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523

524   TABLES

525   Table 1. Core genes set of cAMP-mediated dopamine response involved in the *Ppp1r1b* mediated

526   phosphorylation cycles expanded with NMDA glutamate receptor set (*Grina, Grin1-Grin2*) and Map kinases

527   genes.

Gene symbol	Protein name	Description	STR–specific*
Adcy5	AC5	adenylate cyclase 5	yes
Adora1	A1A	adenosine A1 receptor	no
Adora2a	A2A	adenosine A2a receptor	yes
Cdk5r1	CDK5	cyclin-dependent kinase 5, regulatory subunit 1 (p35)	no
Drd1	D1R	dopamine receptor D1	yes
Drd2	D2R	dopamine receptor D2	yes
Gnai2	Gi/o	guanine nucleotide binding protein (G protein), alpha inhibiting 2	no
Gnal	Golf	guanine nucleotide binding protein, alpha stimulating, olfactory type	yes
Gpr88	STRG	G-protein coupled receptor 88	yes
Grin1	NMDA1	glutamate receptor, ionotropic, NMDA1 (zeta 1)	no
Grin2a	NMDA2A	glutamate receptor, ionotropic, NMDA2A (epsilon 1)	no
Grin2b	NMDA2B	glutamate receptor, ionotropic, NMDA2B (epsilon 2)	no
Grin2c	NMDA2C	glutamate receptor, ionotropic, NMDA2C (epsilon 3)	no
Grin2d	NMDA2D	glutamate receptor, ionotropic, NMDA2D (epsilon 4)	no
Grina	NMDARA1	glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1 (glutamate binding)	no
Map2k1	ERK1	mitogen-activated protein kinase kinase 1	no
Map2k5	ERK5	mitogen-activated protein kinase kinase 5	no
Mapk1	ERK2	mitogen-activated protein kinase 1	no
Mapk3	ERK1	mitogen-activated protein kinase 3	no
Mapk7	ERK5	mitogen-activated protein kinase 7	no
Pde10a	ADSD2	phosphodiesterase 10A	yes
Pdyn	PPD	prodynorphin	no
Penk	PPE	preproenkephalin	yes
Ppp1ca	PP1	protein phosphatase 1, catalytic subunit, alpha isoform	no
Ppp1cb		protein phosphatase 1, catalytic subunit, beta isoform	no
Ppp1cc		protein phosphatase 1, catalytic subunit, gamma isoform	no
Ppp1r1b	Darpp32	protein phosphatase 1, regulatory (inhibitor) subunit 1B	yes
Ppp2ca	PP2A	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	no
Ppp3ca	PP2B	protein phosphatase 3, catalytic subunit, alpha isoform (Calcineurin).	no
Prkaca	PKA	protein kinase, cAMP dependent, catalytic, alpha	no
Ptpn5	STEP	protein tyrosine phosphatase, non-receptor type 5	yes

Tac1	PPTA	tachykinin 1	yes
Tac2	PPTB	tachykinin 2	no

528   *'Striatum (STR) -specific' column indicates genes maintaining striatum specific expression profile

529   (average expression rate threefold higher in dorsal striatum than in any of the other four brain regions).