Therapeutic efficacy of trigonelline-based standardized extract of fenugreek seeds on Levodopa induced dyskinesia in 6-OHDA lesioned rat model of Parkinson's disease

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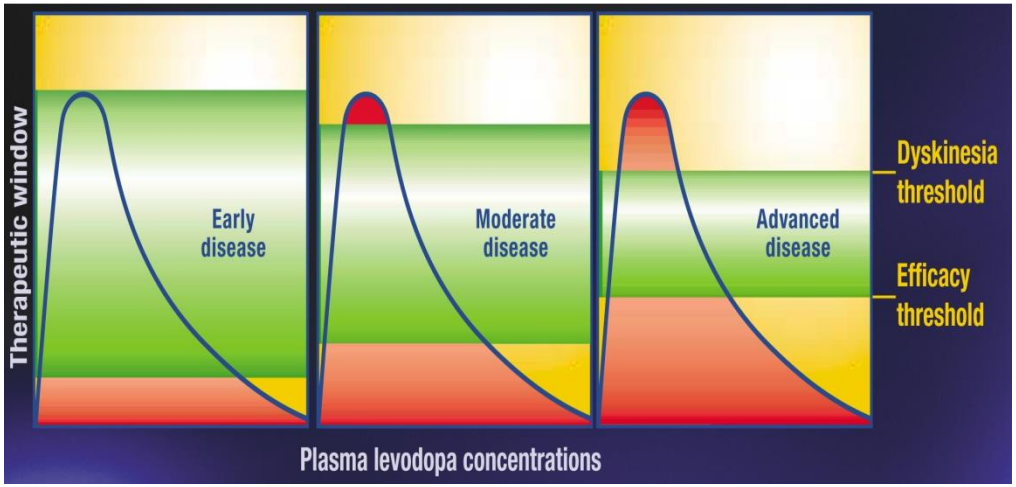
Introduction

- Parkinson's disease (PD): second most common neurodegenerative disorder
- Approximately 4 million people worldwide and expected to doubled by 2030 (Arsene et al., 2009, Buck and Ferger, 2010).
- L-DOPA remains the standard pharmacotherapy for Parkinson's disease (PD).
- L-Dopa induced dyskinesia (LID): a major side effect
  - characteristics idiosyncratic mixture of choreic and dystonic movements called as abnormal involuntary movements (AIMs)



Arsene et al (2009) Farmacia, 4:3-9
Buck & Ferger (2010) Drug discovery today, 15:867-75

L-dopa induced dyskinesia - Off label treatment options

L-dopa dose to reduce when PD is in advanced stage and dose needs to be increased

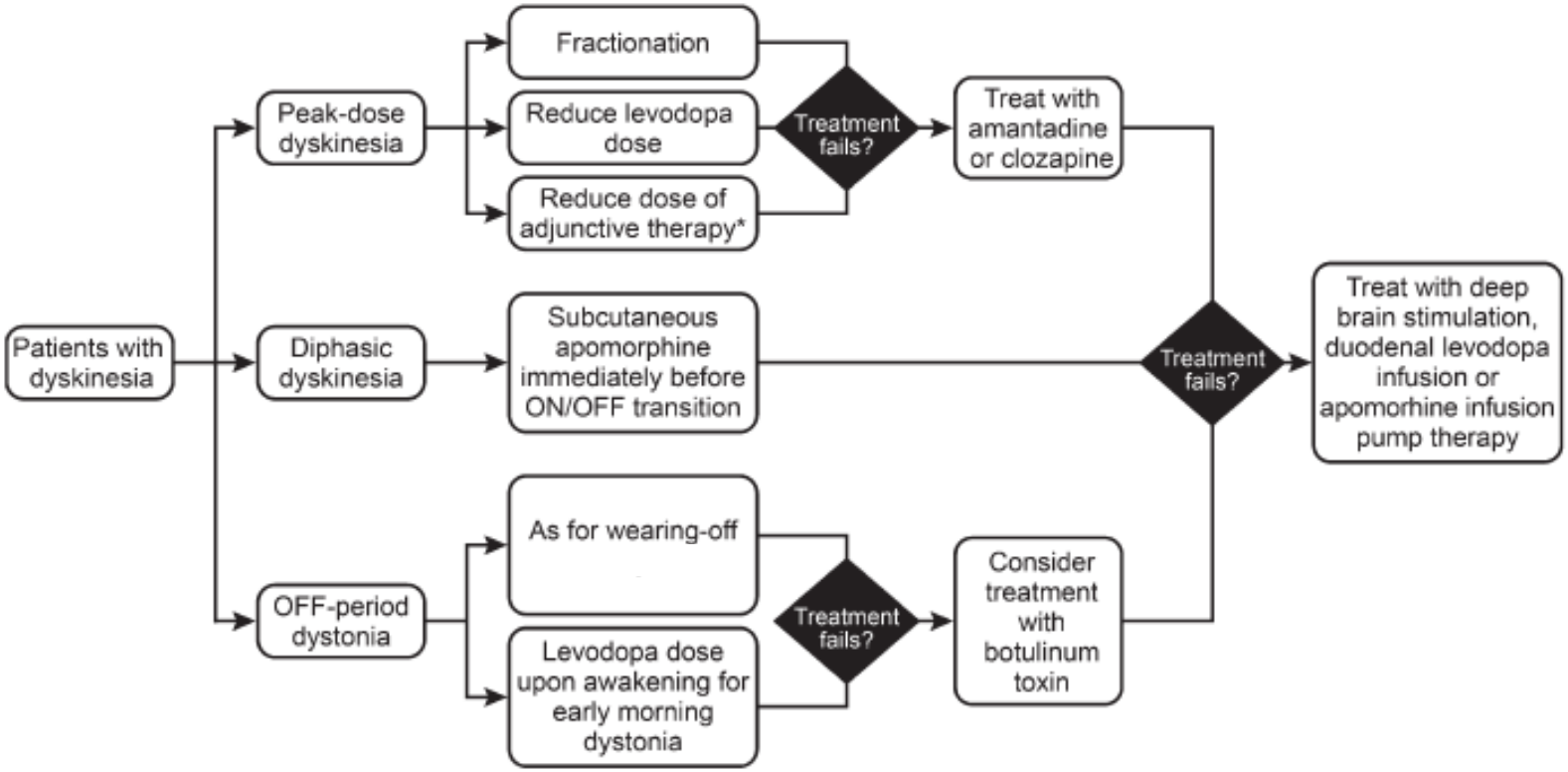


Fig. 2. Schematic diagram for treatment optimization in patients with dyskinesias. *Includes adjunctive catechol-*O*-methyl transferase inhibitors, monoamine oxidase B inhibitors, dopamine agonists.

Salat and Tolosa (2013). J Parkinsons Dis 3(3): 255-69.

LID: Need of New agents

- LID: dose limiting side effect
  - Around 90% patients suffers after 9-15 years of initiation of L-DOPA treatment (Bishop et al., 2006)
  - reduces therapeutic window with time

- No approved agent for LID
  - Amantadine (a non-competitive antagonist of NMDA receptor) showed promise and in Phase III clinical studies

- Need to address BOTH: symptoms of LID and progression of PD
  - L-DOPA → free radical mediated damage → mitochondrial dysfunction → accelerates nigral neuronal cell dysfunction.
  - An energy imbalance between increased demands and a reduced synthesis is also observed in LID.

Bishop et al (2006) Eur J of Neurosci., 23:2669-76.

The test compound, IBHB - Introduction

- Fenugreek ((*Trigonella foenum-graecum* L., Family: Fabaceae) seeds
  - Potent antioxidant effects (Devasena & Menon, 2002)

- Trigonelline (TGN) is a major alkaloid in fenugreek seeds
  - Potent antioxidant properties (Yen et al., 2005).

- TGN : Protect oxidative stress to isolated goat mitochondrial systems *in vitro* (Dutta et al, 2014)

- IBHB : TGN based standardized fenugreek seed extract (Gaur et al, 2013) with 72% w/w TGN as marker compound

- Yen et al (2005). Journal of Agricultural and Food Chemistry 53(7): 2658-2663.
- Devasena and Menon (2002) J Biochem Mol Biol Biophys 6(4): 289-292
- Dutta et al (2014) Journal of Pharmacy Research 8(6): 1694-1718.
- Gaur et al (2013).Pharm Biol 51(5): 550-7.

IBHB : Promise against LID

- Gaur et al, 2013: strong neuroprotective activity in animal models of PD

- Nathan et al, 2014: Proven efficacy and safety as an adjuvant to L-Dopa therapy in early PD patients
  - Significant Clinical important difference (CID) in total UPDRS and motor symptoms score V/s. Placebo
  - Excellent safety profile
  - Found beneficial to reduce symptoms of LID in some advanced PD patients (Investigator's observation)

- Therefore, the present work was undertaken to explore therapeutic (curative) efficacy of IBHB against LID using suitable animal model

- Gaur et al (2013).Pharm Biol 51(5): 550-7.
- Nathan et al (2014). Phytotherapy Research 28: 172-178.

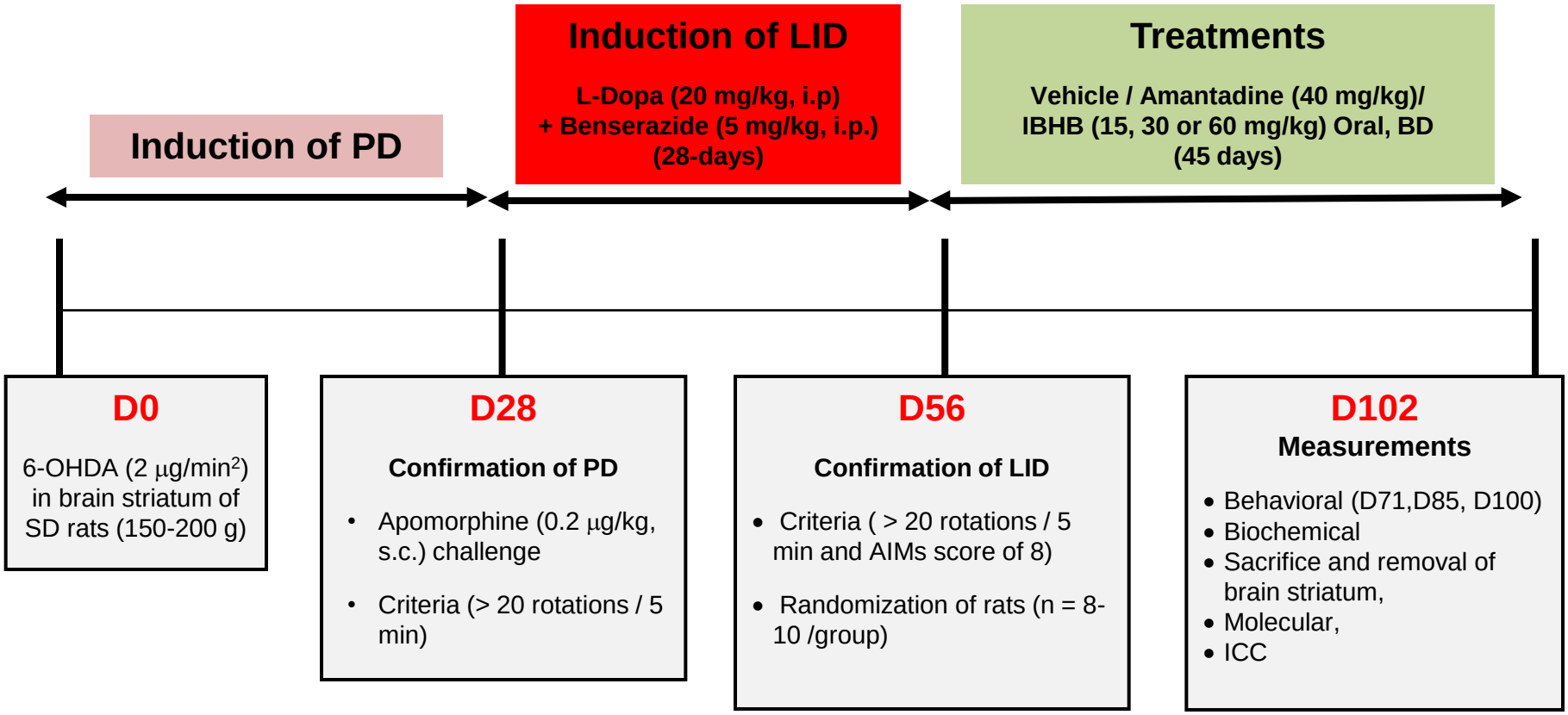
Objectives of work

Objective and Parameters

- To explore the efficacy and possible mechanism of subacute treatment of IBHB on L-dopa induced dyskinesia (LID) using 6-hydroxydopamine (6-OHDA) lesioned rat model of PD

- Parameters:
  - **Behavioral**
    - Abnormal involuntary movements (AIMs)
    - Forelimb adjusting steps (FAS)
  - **Biochemical**
    - Brain and Plasma: 3-O-methyldopa (3-OMD)
    - Brain DA and 5-HT metabolism (5-HT, DA, DOPAC, 5-HIAA and their turnover)
    - Brain: Mitochondrial respiratory chain complexes I, II, III and IV activity
  - **Molecular**: mRNA expression of C-Fos, Arc, Nurr77, Gad67, Homer, PDyn, Jun D, Penk, PINK1, Parkin, DJ-1 and CO-1 in striatum area of brain by RT-PCR analysis
  - **Immunocytochemistry (ICC)** : FosB, Tyrosine hydroxylase (TH), 5-HT and 5-HT2c in stratum area of brain

Study Flow chart



Animal ethics committee approval: IAEC of Poona college of Pharmacy, Bharati Vidyapeeth Deemed University, Pune (CPCSEA/PCL/38/2014-15)

IBHB on LID (Grouping and treatments)

Treatment Groups	No. of animals	Treatment schedules (days)		
		0	29th to 56th day	57th to 102th day
Sham	10	L-ascorbate-saline(12 µg)	Saline (1 ml/kg, i.p.)	Vehicle (10 mg/kg, p.o.)
Sham-L-Dopa	10	L-ascorbate-saline(12 µg)	L-DOPA + Benserazide ---	L-DOPA + Benserazide + Vehicle (10 mg/kg, p.o.)
Hemiparkinsonian	8	6-OHDA (12 µg)	Saline (20 mg/kg, i.p.)	Vehicle (10 mg/kg, p.o.)
LID control	9	6-OHDA (12 µg)	L-DOPA + Benserazide --	L-DOPA + Benserazide + Vehicle (10 mg/kg, p.o.)
Amantadine (40)	8	6-OHDA (12 µg)	L-DOPA + Benserazide ---	L-DOPA + Benserazide + Amantadine (40 mg/kg, i.p. once daily)
IBHB (15)	9	6-OHDA (12 µg)	L-DOPA + Benserazide ---	L-DOPA + Benserazide + IBHB (15 mg/kg, p.o.)
IBHB (30)	9	6-OHDA (12 µg)	L-DOPA + Benserazide ---	L-DOPA + Benserazide + IBHB (30 mg/kg, p.o.)
IBHB (60)	10	6-OHDA (12 µg)	L-DOPA + Benserazide ---	L-DOPA + Benserazide + IBHB (60 mg/kg, p.o.)

L-DOPA + Benserazide: L-DOPA (20 mg/kg, i.p., once daily) + Benserazide (5 mg/kg, i.p., once daily)

Behavioral Tests

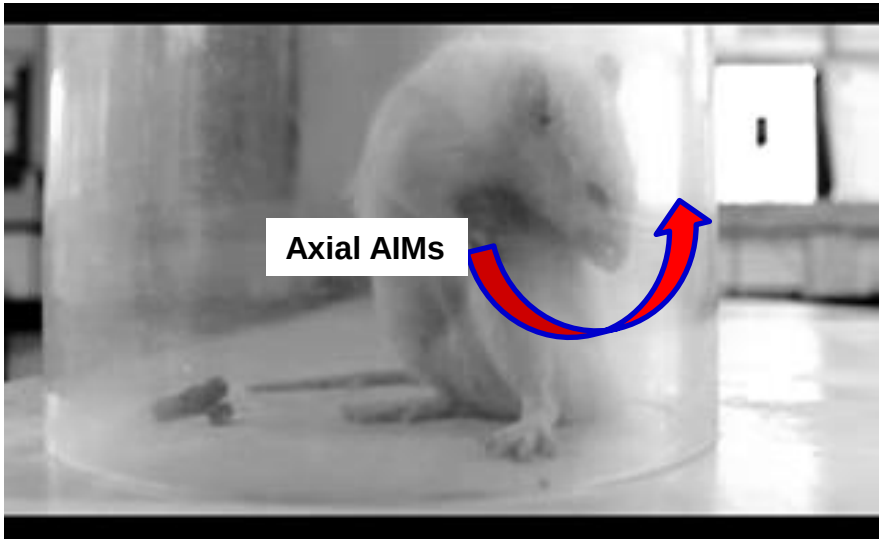
Abnormal Involuntary movements (AIMs) - Method

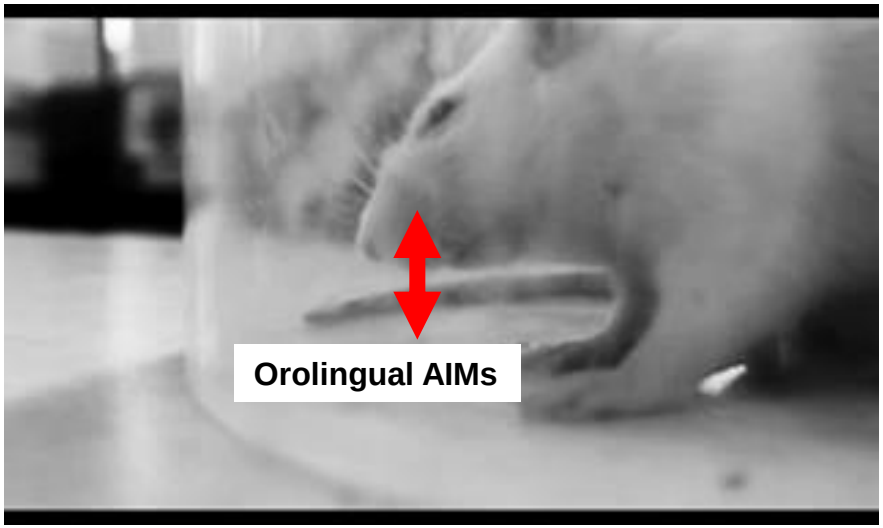
- **Abnormal involuntary movements (AIMs test)** (Bishop et al., 2006)
  - **Axial AIMs**: Dystonic posturing of the neck and torso, involving positioning of the neck and torso in a twisted manner directed toward the side of the body contralateral to the lesion
  - **Forelimb AIMs:** Rapid, purposeless movements of the forelimb located on the side of the body contralateral to the lesion.
  - **Orolingual AIMs:** Repetitive openings and closings of the jaw and tongue protrusions. The movements are considered abnormal since they occur at times when the rats are not chewing or gnawing on food or other objects.
  - **Rotational AIMs:** Rats ambulated in a contralateral circular direction.

- The rats were observed every after 20 min for 2 min duration till 2 h.

- Rats were rated for AIMs during the 1st min and rotational behavior for next 1 min.
- AUC of total AIMs score of treated group was compared with LID control group

AIMs scoring system - Method



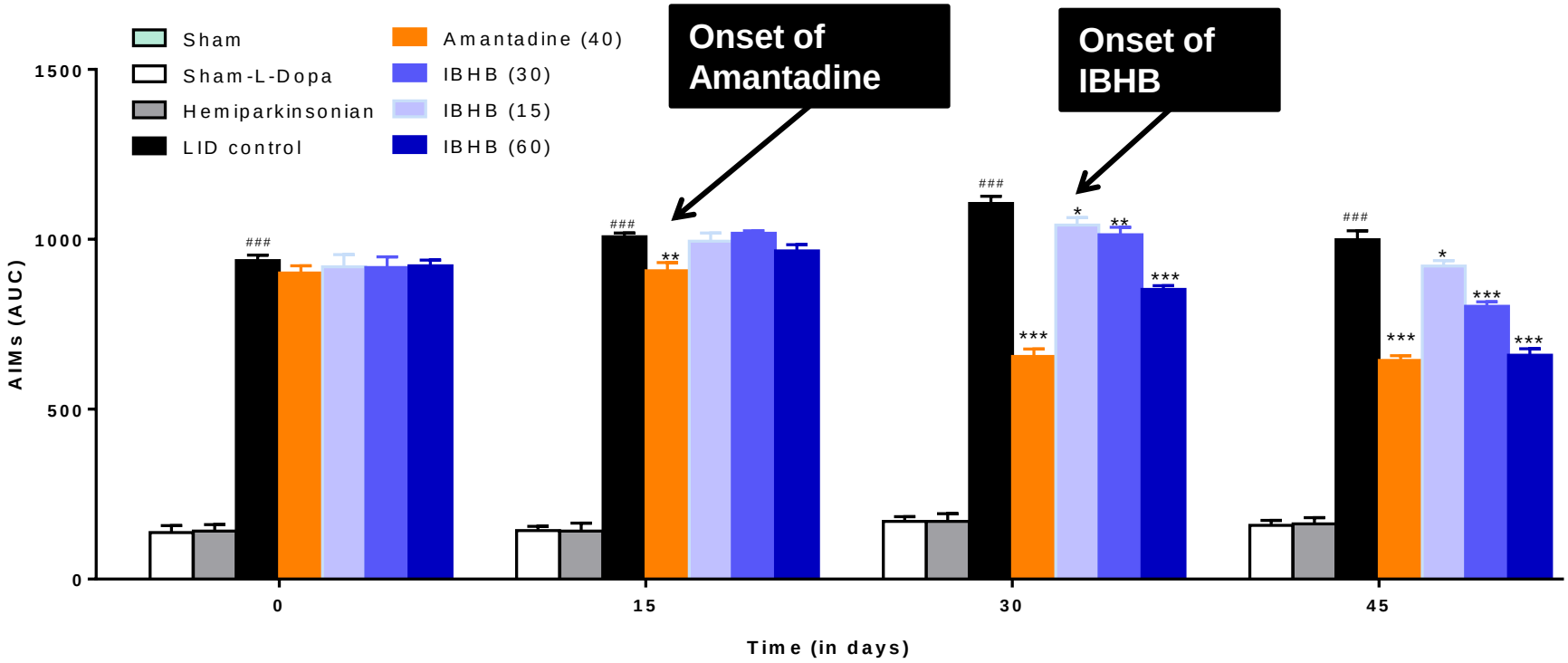


AIMs scoring

0 = not present
1 = present for < 50% of the observation period (i.e. **1–29 s).**
2 = present for >= 50% of the observation period (i.e. **30–59 s**).
3 = present for 100% of observation period (i.e. **60 s**) but interrupted by a loud stimulus (e.g. tap)
4 = present for the 100% observation period and not interrupted by a loud stimulus.

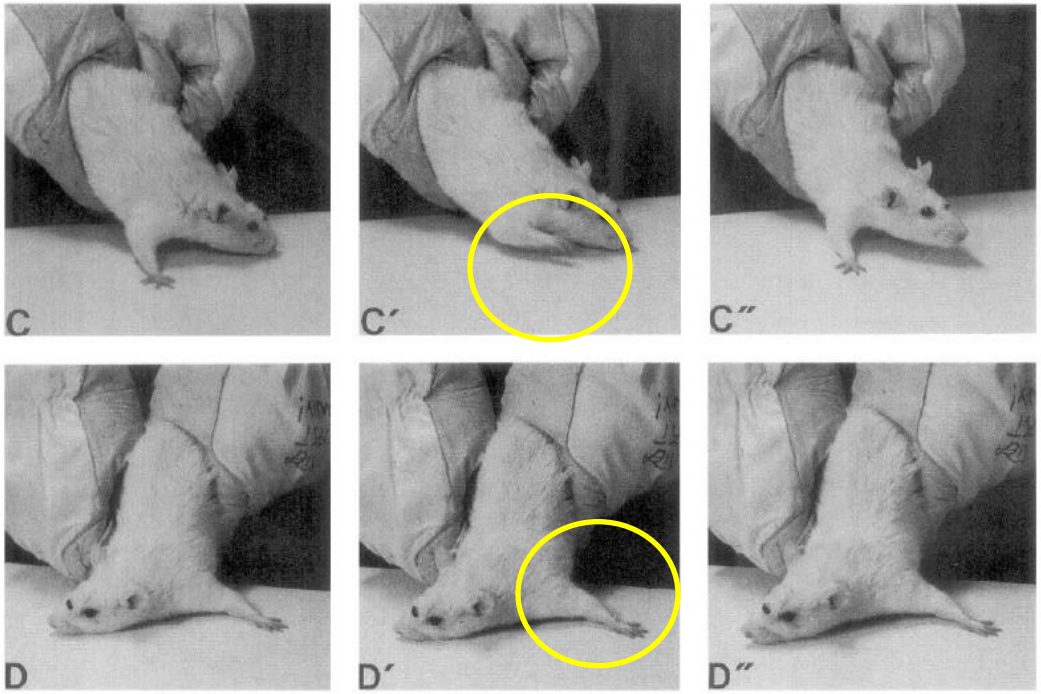
*** Photos of rats from present experiments

Results: AUC of Total AIMs



Data was analyzed by Non-parametric ANOVA and Mann-Whitney post tests. $^{###}P < 0.001$ as compared with sham group, and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with LID control on respective days.

Forelimb adjusting steps (FAS) - Method

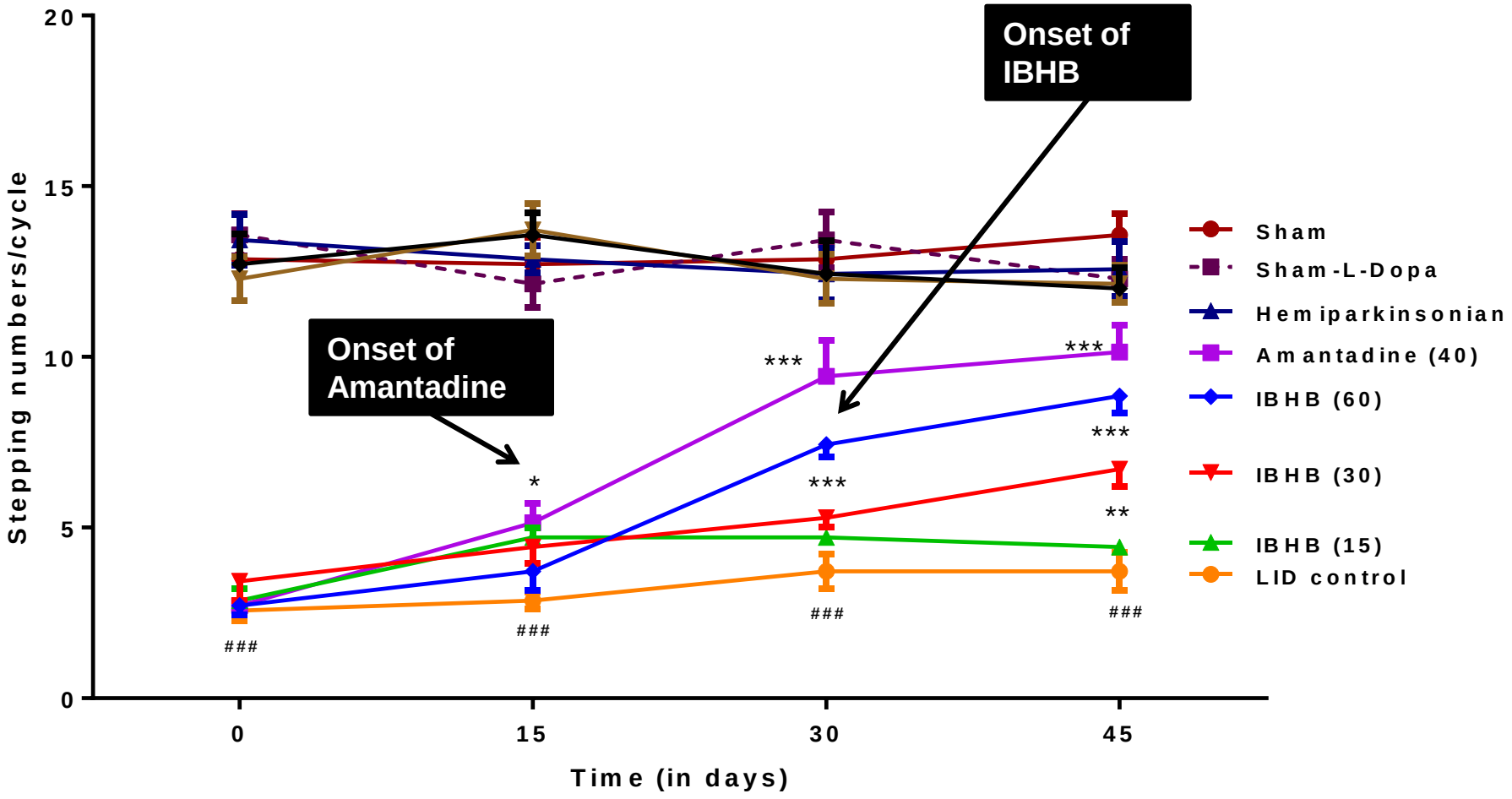


Sequence of FAS in LID control group
Ipsilateral paw– Lesioned side (C-C”)
contralateral paw - Other side ((D-D”)

Source : Olsson et al (1995). Journal of Neuroscience **15(5 Pt 2): 3863-75.**

- Rats was moved laterally across a table at a steady rate of 90 cm/10 s.
- The rear part of the torso and the hind limbs was lifted from the table and one forepaw was held by the experimenter so as to bear weight on the other forepaw.
- Each stepping test consisted of 6 trials for each forepaw (Olsson et al., 1995)

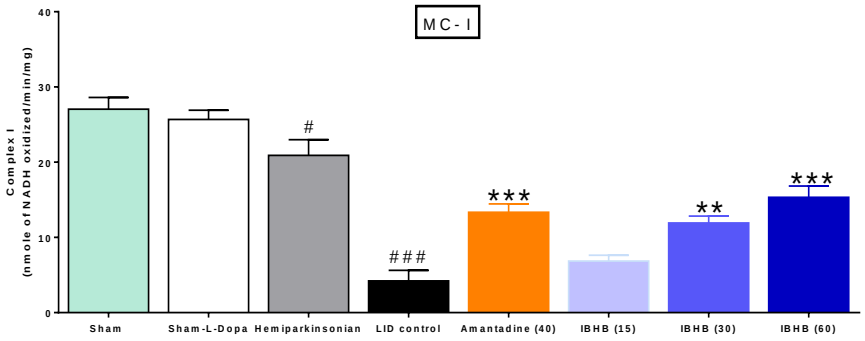
Results: Numbers of steps in FAS

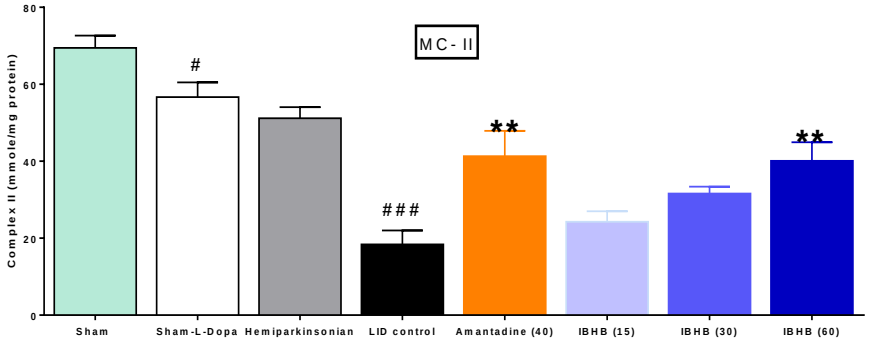


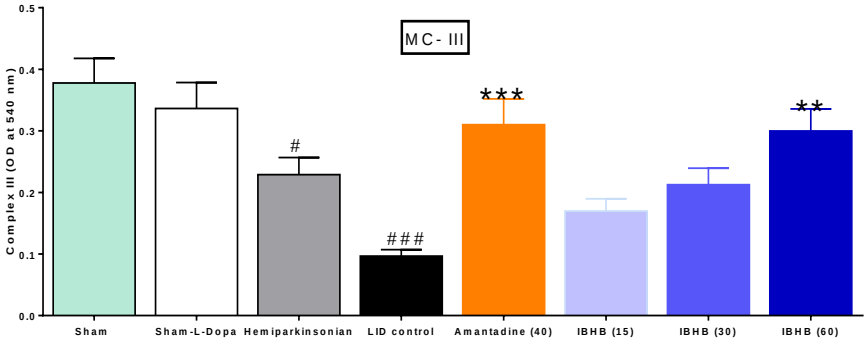
Data was analyzed by Non-parametric Mann-Whitney post tests. ###P < 0.001 as compared with sham group, and **P < 0.01, ***P < 0.001 as compared with LID control group on respective days.

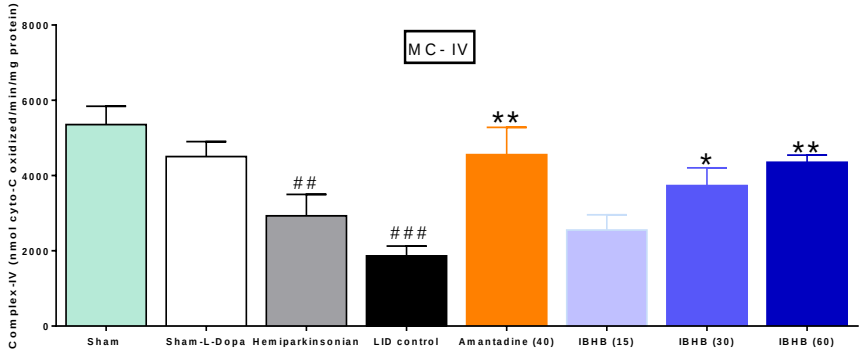
Biochemical Parameters

Results: Striatal Mitochondrial Complex



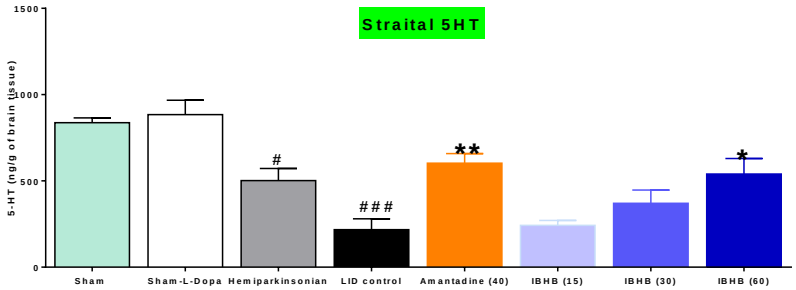


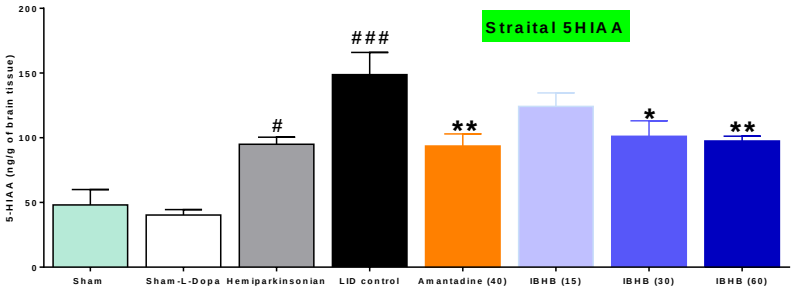


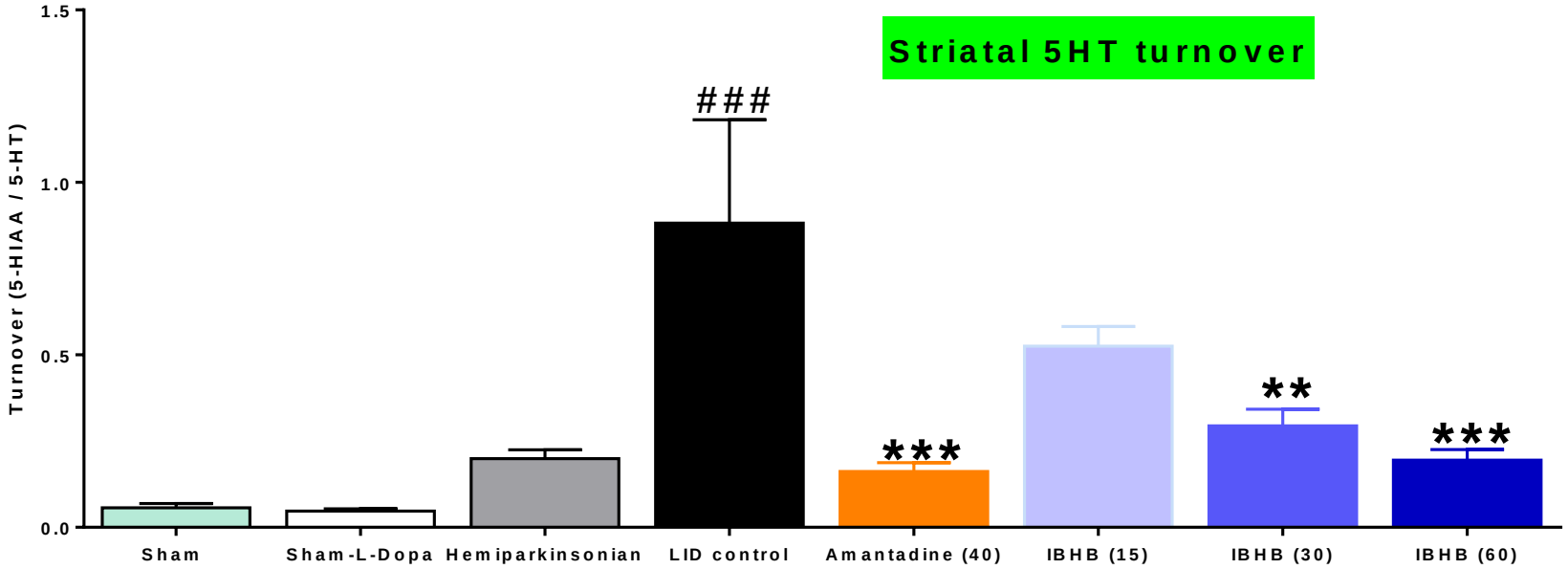


Each set of data was analyzed by One-Way ANOVA followed by Dunnett's tests $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (v/s. sham group), * P < 0.05, **P < 0.01, ***P < 0.001 (v/s. LID control)

Results: Striatal 5-HT metabolism

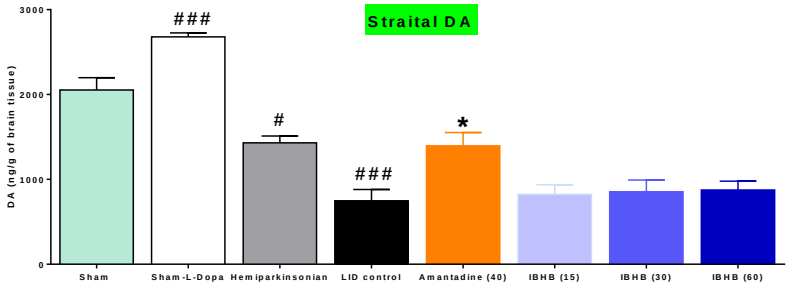


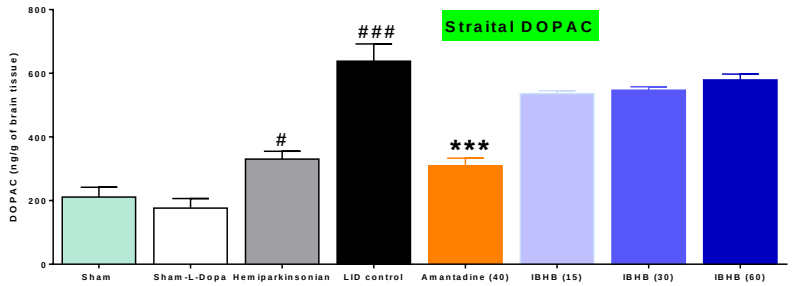


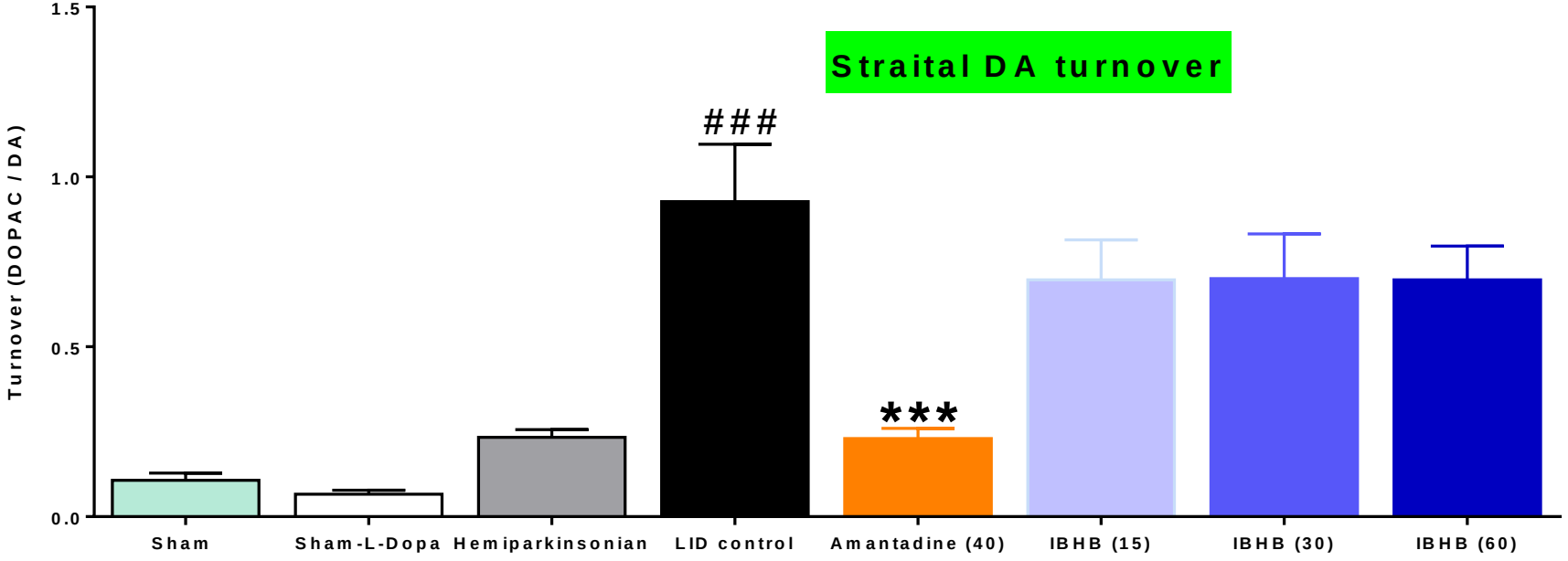


Data of each parameter was analyzed by One-Way ANOVA followed by Dunnett's post tests $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (v/s. sham group), * $P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (v/s. LID control)

Results: Striatal DA Metabolism

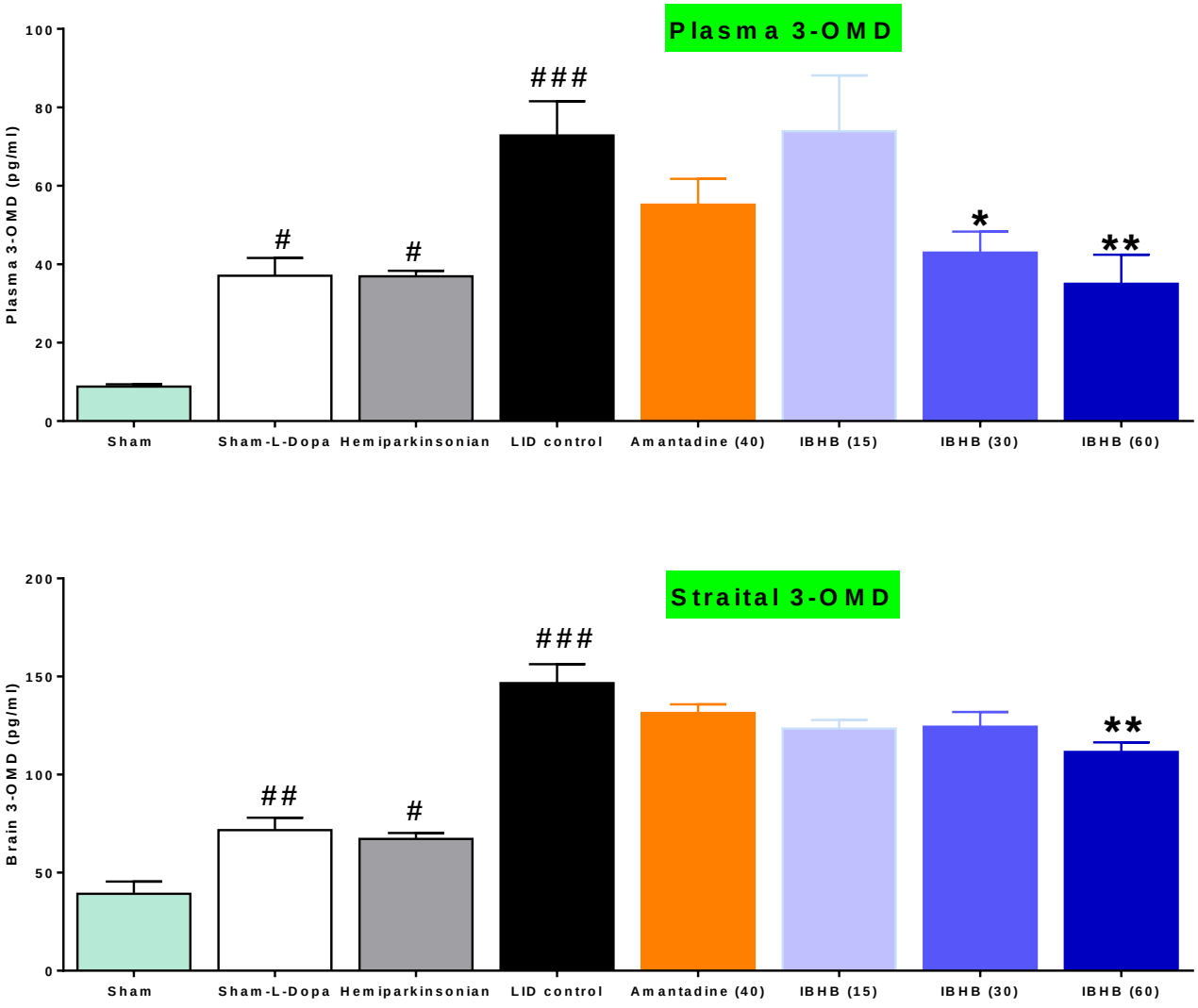






Data of each parameter was analyzed by One-Way ANOVA followed by Dunnett's post tests $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (v/s. sham group), * P < 0.05, **P < 0.01, ***P < 0.001 (v/s. LID control)

Results: Plasma and striatal 3-OMD levels



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Molecular parameters

Gene expression study by RT-PCR - Method

- RT-PCR analysis was performed in striatum of brain by using 10 µg/µl of RNA from about 30 mg of tissue.
- ß-actin served as a control for sample loading and integrity.
- The amplicons were visualized, and image was captured using gel documentation system.
- The expression of all the genes : densitometry data by analyzing the gel images (Image J program, Version 1.33, NIH), USA) semiquantitatively.

PD Progression		LID symptoms		LID Mechanisms	
Name	**Relevance**	**Name**	**Relevance**	**Name**	**Relevance**
Arc	DA overexpression	cFos	AIMs and LID marker	CO-1	Mitochondrial Dysfunction
Homer	Dopamine → Glutamate interaction	Gad67	LID induction v/s non-LID status	DJ-1	Apopotosis and neuronal antioxidant
Nur71	Arc → DA pathway	JunD	cFos Related, NMDA related	Perkin	Mitochondrial dysfunction
Pink-1	Progression and onset of PD (early PD)	PDyn	Progression of dyskinesia		
		Penk	cFos Related, JunD->Pyn pathway		

Results: Gene expression - PD Progression

Arc/ß-actin	ns	ns	*↑	***↑	*↓	ns[2]	ns[2]	ns[2]
Homer/ß-actin	ns	ns	*↑	***↑	*↓	ns[2]	*↓	**↓
Nurr-77/ß-actin	ns	ns	*↑	***↑	*↓	ns[2]	ns[2]	ns[2]
PINK1/ß-actin	ns	ns	*↑	***↑	*↓	ns[2]	*↓	***↓

Each parameter was analyzed by One-Way ANOVA followed by Dunnett's tests $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (v/s. sham group), ns2- not significance, * P < 0.05, **P < 0.01, ***P < 0.001 (v/s. LID control), ↑ **or** ↓**: significantly increased or decreased (v/s. sham group),** ↑ **or** ↓**: significantly increased or decreased (v/s. LID control)**

Arc: Activity-Regulated Cytoskeleton-Associated Protein
Nurr77: transcription factor, an orphan nuclear receptor,
PINK1: PTEN-induced putative kinase 1

Results: Gene expression - LID symptoms

cFos/ß-actin	ns	ns	*↑	***↑	**↓	ns[2]	ns[2]	**↓
Gad-1/ß-actin	ns	ns	*↑	***↑	*↓	ns[2]	ns[2]	ns[2]
junD/ß-actin	ns	ns	*↓	***↓	*↑	ns[2]	ns[2]	**↑
Pdyn/ß-actin	ns	ns	ns	***↑	***↓	ns[2]	ns[2]	*↓
Penk/ß-actin	ns	ns	ns	***↑	**↓	ns[2]	*↓	*↓

Each parameter was analyzed by One-Way ANOVA followed by Dunnett's tests $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (v/s. sham group), ns2- not significance, * P < 0.05, **P < 0.01, ***P < 0.001 (v/s. LID control), ↑ **or** ↓**: significantly increased or decreased (v/s. sham group),** ↑ **or** ↓**: significantly increased or decreased (v/s. LID control)**

cFos: Indirect marker of increased neuronal activity (LID), accumulated in dopamine D1-type medium spiny neurons.
Gad1- Glutamate decarboxylase 1
JunD : a Proto-Oncogene
Pdyn: Prodynorphin, basic building-block of endorphins
PENK: Proenkephalin, endogenous opioid hormone

Results: Gene expression - Mitochondrial Dysfunction

CO-1/ß-actin	ns	ns	*↑	***↑	***↓	ns[2]	ns[2]	**↓
DJ-1/ß-actin	ns	ns	ns	ns	ns[2]	ns[2]	ns[2]	ns[2]
Parkin /ß-actin	ns	ns	*↑	***↑	**↓	ns[2]	ns[2]	*↓

Each parameter was analyzed by One-Way ANOVA followed by Dunnett's tests $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (v/s. sham group), ns2- not significance, * P < 0.05, **P < 0.01, ***P < 0.001 (v/s. LID control), ↑ **or** ↓**: significantly increased or decreased (v/s. sham group),** ↑ **or** ↓**: significantly increased or decreased (v/s. LID control)**

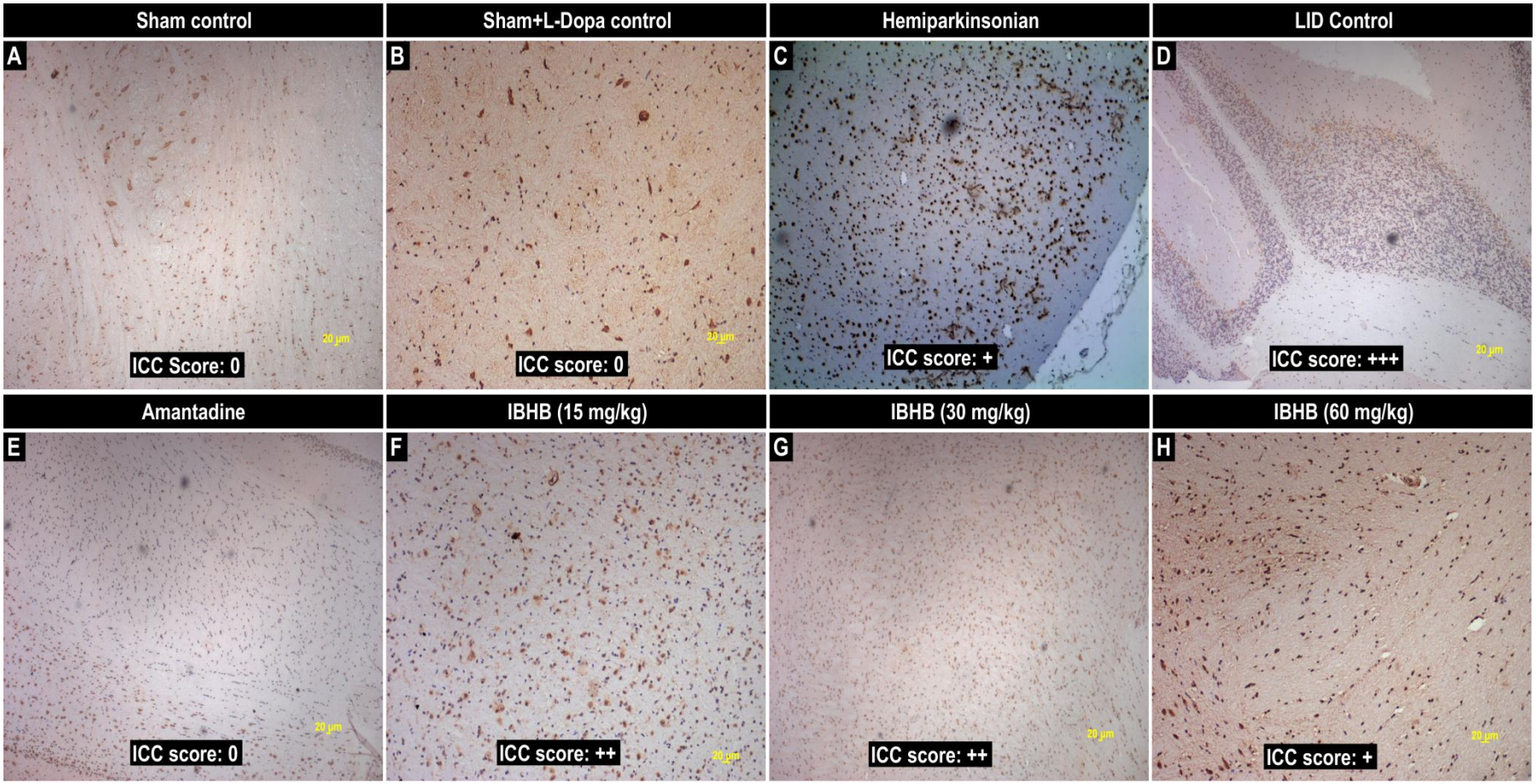
CO-1:Cytochrome c oxidase, Mitochondrial oxidative enzyme
DJ-1- A stress sensor, mitochondrial regulator
Parkin: an important ATP dependent protein degradation machinery

Immunocytochemical studies

Immunocytochemistry of striatum of brain - Method

- Peroxidase-based method (Zhang et al., 2007)
  - Sections were incubated with different antibody (1:200) overnight at 4°C
    → Biotinylated secondary antibody and HRP conjugated streptavidin.
    → developed using diaminobenzidine (DAB) as the chromogen.
- Antibodies
  - **Fos B**: Linked with L-DOPA-induced AIMs
  - **Tyrosine hydroxylase (TH):** Enzyme responsible for catalyzing the conversion of the amino acid L-tyrosine to DOPA
  - **5-HT and 5-HT2c**: Release of 5-HT and 5-HT turnover

Results: FosB Immunoreactivity in Striatum



ICC scoring

-- No immunoreactivity,
+ Mild increase in immunoreactivity,
++ Moderate increase in immunoreactivity
+++ Strong increase in immunoreactivity

Results: Summary - Immunocytochemistry study

- Striatal immunoreactivity scores of different groups

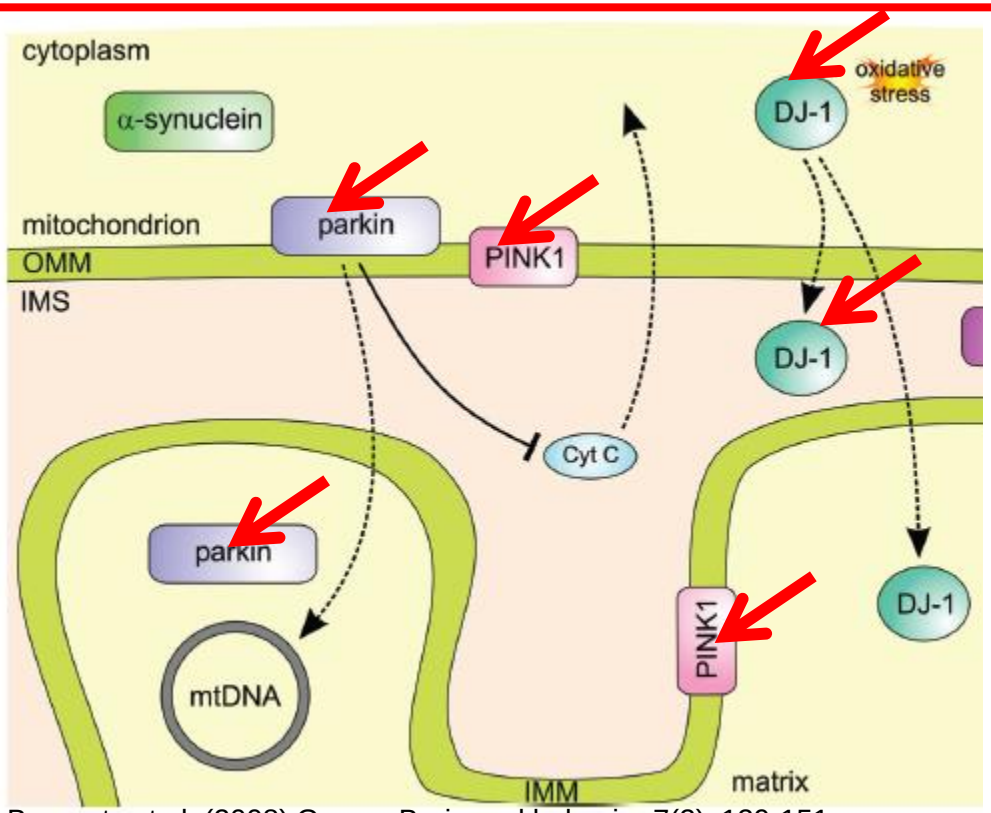
Antibody used	Sham	Sham + L-Dopa	Hemi PD	LID control	Aman-tadine (40)	IBHB (15)	IBHB (30)	IBHB (60)
TH	-	-	+	+++	-	++++	++	++
FosB	-	-	+	+++	-	++	++	+
5-HT	-	-	+	+++	-	+++	++	+
5-HT2c	-	-	+	+++	-	+++	++	+

FosB- FBJ Murine Osteosarcoma Viral Oncogene Homolog B)

ICC scoring
-- No immunoreactivity,
+ Mild increase in immunoreactivity,
++ Moderate increase in immunoreactivity
+++ Strong increase in immunoreactivity

Discussion

IBHB : restores mitochondrial dysfunction



Bogaerts et al. (2008) Genes, Brain and behavior 7(2): 129-151.

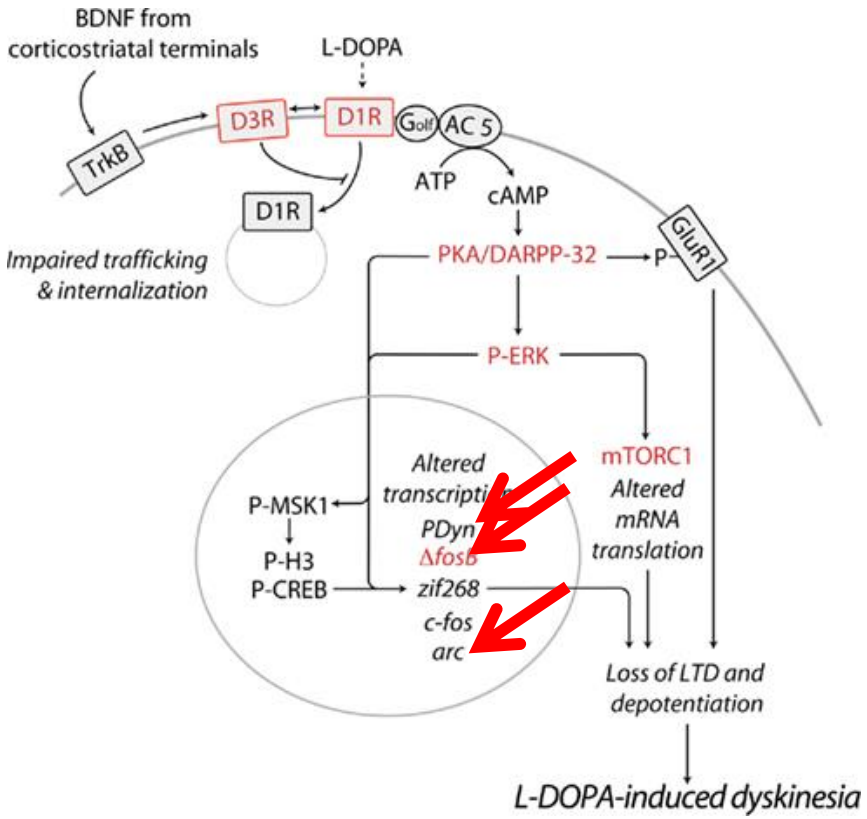
- Aggregation of α -syncluein might be an upstream actor of mitochondrial alterations.
- **Parkin-** role in mitochondrial biogenesis
- **PINK1** - localized to mitochondrial membranes
- **DJ-1** - oxidation of a key Cys-residue in DJ-1 leads to relocalization of PINK1 to mitochondria.

OMM – Outer mitochondrial membrane
IMS - Intermembrane space
IMM – Inner mitochondrial membrane

- Reduced LID-induced mitochondrial energy demand in mitochondria (FosB) immunoreactivity, striatal gene expression of cFos, Pdyn, JunD).
- Reversal of LID induced gene expression (PINK1, Perkin, JunD)
  → reduction to oxidative stress
  → prevents mitochondrial complex-I inhibition

Targets for IBHB

IBHB : reduces LID symptoms



Feyder et al (2001) Front Behav Neurosci **5: 71.**

In PD, the loss of striatal DA
→ sensitization of D1 receptors (D1R) on the striatonigral region
→ appearance of dyskinesia

Chronic administration of L-DOPA
→ up-regulation of FosB, Pdyn and Arc
→ development of dyskinesia

ΔFosB overexpression in the striatum
→ correlated with LID (Cao et al 2010)

In present study, IBHB treated rats showed
→ reduction in LID induced upregulated gene expressions and immunoreactivity in striatum area
→ reduces LID symptoms

Targets for IBHB

Cao et al (2010 J Neurosci 30(21): 7335-43.

IBHB : Normalizes 3-OMD

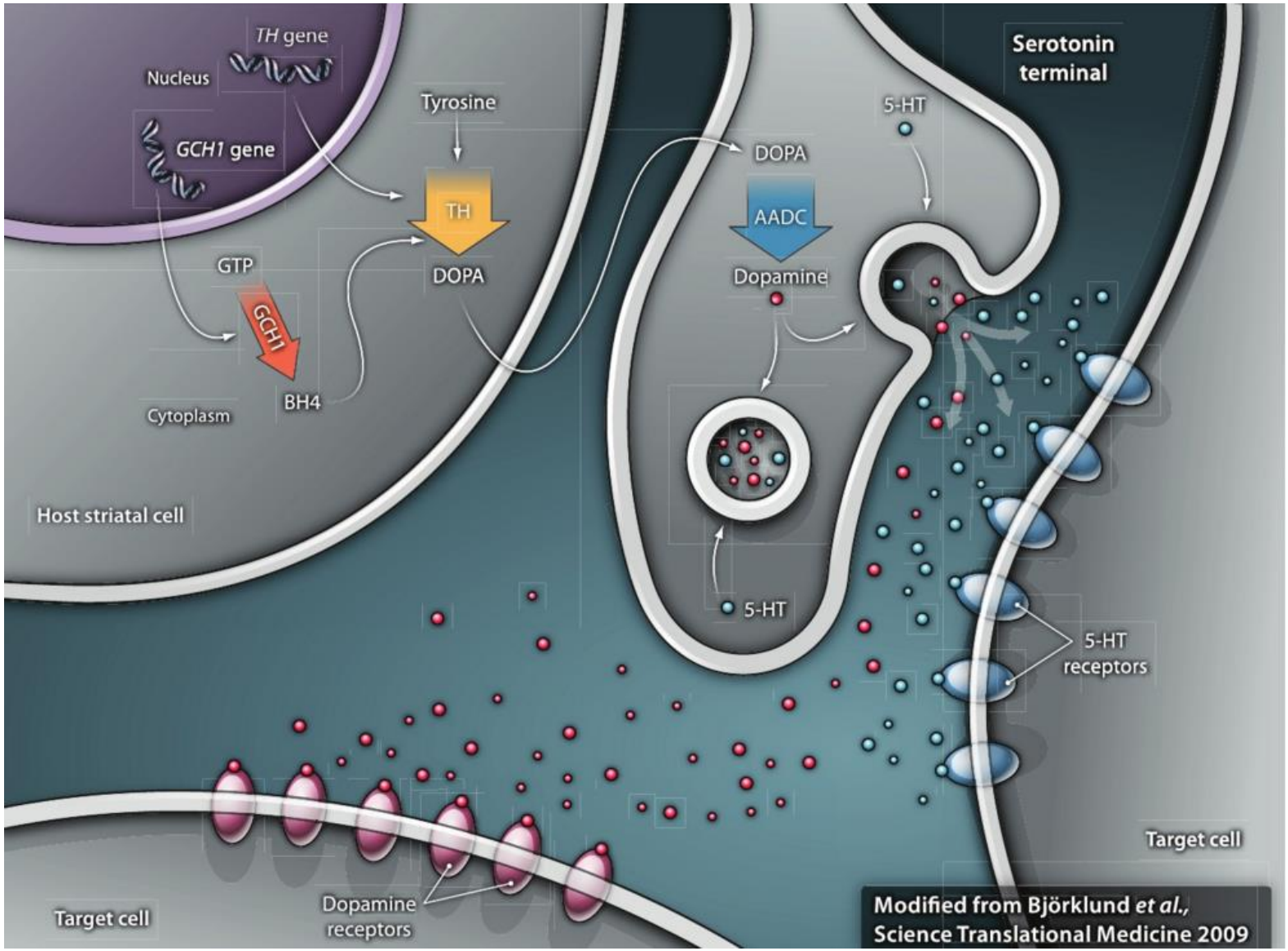
- 3-OMD might be associated with L-dopa-related motor dysfunction (AIMs)
  - The plasma 3-OMD from LID → significantly ↑ in LID v/s Non-LID (Tohgi et al, 1991).
  - ↑ Brain L-dopa → accumulation of 3-OMD in the brain (Marsden, 1994)
  - ↑ 3-OMD → wearing-off in PD (Tohgi et al, 1991a)

- ↑ 3-OMD → ↑ ROS → ↓ mitochondrial membrane potential → ↑ L-dopa-induced cytotoxic effects → ↑ rate of progression of the PD (Lee et al, 2008)

- In present study, IBHB showed normalization of 3-OMD in Striatum and Plasma → Beneficial effects in terms of:
  - LID symptoms (AIMs)
  - mitochondrial oxidative stress
  - progression of PD

Marsden (1994). Clin Neuropharmacol **17 Suppl 2: S32-44.**
Tohgi et al (1991). Neurosci Lett **127(2): 212-4.**
Tohgi (1991a) Neuroscience letters **132(1): 19-22.**
Lee et al (2008). Neurochem Res **33(3): 401-11.**

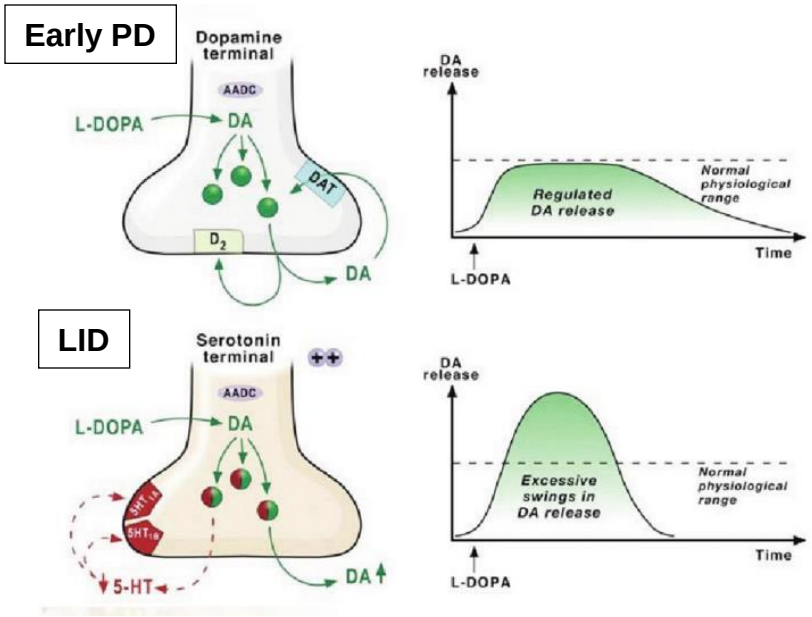
LID: DA acts as a ‘false transmitter’ in 5HT neurons



Björklund, A., Björklund, T. and Kirik, D. (2009). "Gene Therapy for Dopamine Replacement in Parkinson´ s Disease." Science translational medicine 1(2): 2ps2..

IBHB : restores serotonergic system balance

- In advance PD and LID
  - Hyper-innervation of 5HT
  - Increased 5HT and DA turnover
- Reduction of 5-HT turnover without DA decrease is important for normal 5-HT/DA balance (to prevent off condition of PD)



Carta et al (2007) Brain 130(Pt 7): 1819-33

- In present study, IBHB restored 5-HT to DA balance
  - Decreased 5-HT turnover in striatum without affecting DA turnover
  - Dopamine pathways related biochemical and gene expression markers are unaffected
  - Evident from 3-OMD levels restoration (indicator of normal motor functions)
  - 5HTc relation ?

IBHB on LID - Conclusions

- IBHB showed significant curative effects in animal model of LID
- IBHB restores the mitochondrial dysfunction
  - Increase in mitochondrial respiratory chain complexes activity in brain
  - Down-regulation of gene expression of CO-1 and Perkin
- IBHB reduces motor symptoms of dyskinesia
  - Reduction in abnormal involuntary movements (AIMs)
  - Reduces LID related behavioral symptoms (Catalepsy and grid test))
  - Reduces of 3-OMD levels in Striatum and Plasma
  - Indicative results towards slower progression of dyskinesia
    - Down-regulates gene expression of cFoS, Penk and Pdyn
    - Up-regulates gene expression of JunD
- IBHB restores serotonergic system balance
  - Decreases serotonin turn over in brain
  - Dopamine pathways related biochemical and gene expression markers are unaffected

Thank You