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The Role of Connexin36 in Dopamine D1/D2 Synergism and its Breakdown in Transgenic Mice

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THE ROLE OF CONNEXIN36 IN DOPAMINE D1/D2 SYNERGISM AND ITS BREAKDOWN IN TRANSGENIC MICE

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Abstract

Most behavioral, physiological and cellular effects of the neurotransmitter dopamine require concomitant activation of both D1 and D2 receptors, a phenomenon referred to as D1/D2 synergism. Since D1 and D2 receptors are located mostly on separate neurons, and since D1/D2 synergism occurs in the absence of action potentials, we have suggested that electrotonic coupling via gap junctions plays an important role in this phenomenon. A major constituent of gap junctions is connexin36 (Cx36), a protein that is abundant in neurons. The role Cx36 in D1/D2 synergism, as manifested behaviorally, was studied here in mice genetically engineered to express normal, reduced, or undetectable amounts of this protein. The results show that D1/D2 synergism and its breakdown were not affected by the presence or absence of Cx36. Unexpectedly, it was observed that the absence of Cx36 leads to resistance to the cataleptic effects of reserpine in a gene dosage-dependent manner.

Introduction

Gap junction channels are intercellular channels that allow the passage of ions and small molecules (~1kDa) between neighboring cells. These channels are dynamic, opening and closing in response to various intracellular and extracellular signals. Each hemichannel, called a connexon, is made up of six proteins called connexins. These connexins are synthesized in either the endoplasmic reticulum or the trans-Golgi network and from here they travel via microtubules to the plasma membrane. Once embedded in the plasma membrane connexins come together to form a connexon and two connexons that lie across from each other in adjacent cells form a gap junction (Ebihara, 2003). Initially gap junctions were thought to exist only in invertebrates, but since their discovery over 30 years ago, and with advances in technology, gap junctions are now known to be present in the mammalian central nervous system (Bennett, 1997).

To date there are over 20 different types of connexins found in mammals (Willecke et al., 2002). They are named for their predicted molecular weights; for example, connexin 36 (Cx36) has a mass of ~ 36 kDa. Most cells can express any number of connexons, and these connexons can be homomeric or heteromeric. However, Cx36, our connexin of interest, can only function as a homomeric channel (Al-Ubaidi, White, Ripps, Poras, Avner, Gomes, et al. 2000).

Location of Cx36 in the Brain

Using *in situ* hybridization Condorelli and colleagues (Condorelli, Belluardo, Salinaro-Trovato, & Mudo, 2000) analyzed the distribution of Cx36 mRNA in the rat central nervous system. They found that Cx36 is present in the lamina of the gray matter of the spinal cord and

in the dorsal root ganglia, the inferior olivary complex, cerebellum, hypothalamus, thalamus, hippocampus, cerebral cortex, olfactory bulb, retina and the basal ganglia.

We are interested in how the presence or absence of Cx36 affects the basal ganglia and in particular if the Cx36 gap junction has a role in the intriguing phenomenon whereby D1- and D2-type dopamine (DA) receptors interact synergistically.

Basal Ganglia

The basal ganglia are involved in many neuronal pathways that include movement, attention, affect, and learning and memory (Ring & Serra-Mestres, 2002). The basal ganglia include the striatum, which is composed of the caudate and putamen nuclei and the nucleus accumbens. (In rodents and other animals, the caudate and putamen are not distinct structures and are thus referred to as the caudate-putamen; CPu.) The striatum receives input from multiple cortical areas and projects through the globus pallidus and the substantia nigra pars reticulata (SNr) to the thalamus and, ultimately, back to the cortex. In addition, the striatum receives dense dopaminergic input from the substantia nigra pars compacta (SNc).

The striatum is the largest structure in the basal ganglia and can be functionally and anatomically divided into dorsal and ventral areas. The dorsal striatum (caudate and putamen) receives inputs from the association areas of the neocortex and the sensorimotor cortex. The ventral striatum (nucleus accumbens) receives input from the orbitofrontal cortex and other limbic cortical areas (Herrero, Barcia, & Navarro, 2002). Efferent striatal neurons consist mainly of GABAergic medium spiny neurons, which make up 90-95% of the striatal neuron population; the remaining 5-10% are local interneurons that are GABAergic or cholinergic (Gerfen, 1992). Efferent projections from the dorsal striatum are divided into two pathways, the

(a) direct, and (b) indirect pathways. Direct pathway neurons contain predominantly DA D1 receptors and synapse mainly in the SNr. Indirect pathway neurons leave the striatum and travel to the globus pallidus; from there the pathway continues to the subthalamic nucleus and then to the SNr. Striatopallidal neurons of this pathway contain predominantly DA D2 receptors. The movement disorder Parkinson's disease results from increased activity in the indirect pathway and decreased activity in direct pathway due to the loss of DA input (Herrero et al., 2002).

Dopamine Receptors

DA receptors belong to a group of signal transduction proteins called guanine nucleotide-binding proteins (G-proteins). DA receptors can be divided into two groups: D1 and D2 receptors. The D1 group consists of D1 and D5 receptors. D1 receptors are located in the SNr, caudate, putamen, nucleus accumbens, and olfactory tubercle. D5 receptors are located in the hypothalamus, thalamus, and hippocampus (Sibley and Monsma, 1992). D1 receptors greatly outnumber D5 receptors.

The D2 group consists of D2, D3, and D4 receptors. D2 receptors are located in the SNc, caudate, putamen, nucleus accumbens, and olfactory tubercle and bulb. D3 receptors are located in the nucleus accumbens and cerebellum and D4 receptors are located in the medulla, midbrain, hypothalamus, thalamus, amygdala, and olfactory bulbs (Sibley and Monsma, 1992). D2 receptors greatly outnumber D3 and D4 receptors except in nucleus accumbens where D3 receptors are plentiful.

The main difference between the D1- and D2-like receptors is in their signal transduction pathways. When DA binds with a D1-type receptor this leads to an increase in adenylyl cyclase

activity and an increase in cAMP production. When DA binds to a D2-type receptor this leads to a decrease in adenylyl cyclase activity (Kebabian and Calne, 1979).

Dopamine Pathways

DA constitutes about 80% of the total amount of catecholamines in the brain. Yet, the total number of DA cells present in the brain is very small when compared with the total number of cells present in the brain. DA nerve cells are found mainly in the midbrain, hypothalamus, and olfactory bulbs and from these areas there are both ascending and descending projection pathways (Roth, Wolf, and Deutch, 1987).

The mesostriatal system arises from neurons in the SNc (A9), ventral tegmental area (A10), and the retrorubral nucleus (A8). The dorsal projections from this system give rise to the nigrostriatal pathway. DA fibers that originate in the SNc project to the caudate, putamen, and to a much lesser degree to the globus pallidus. These neurons show numerous branching in the striatum and this pathway is very important in motor control. The loss of neurons in this pathway leads to Parkinson's disease (Fuxe, Aganti, Kalia, Goldstein, Andersson, & Harfstrand, 1985).

The ventral projections from the mesostriatal system give rise to the mesocorticolimbic pathway. This pathway arises from the A10 group of cells, and some of the A8 and A9 cells, and projects to the nucleus accumbens, septum, amygdala, hippocampus, nucleus of the diagonal band, anterior olfactory nucleus, and limbic cortical areas. This pathway is believed to be hyperactive in schizophrenia (Fuxe et al., 1985). Also, the DA projections to the nucleus accumbens have been linked to the addictive properties of drugs of abuse (Bozarth and Wise, 1983).

D1/D2 Synergism

D1- and D2-type receptors can be activated or inhibited by a variety of drug treatments and the effects of these drugs can be seen in motor behavior. In normal rats and mice, the administration of the mixed D1/D2 agonist apomorphine leads to locomotion at low to moderate doses and stereotypical motor behavior at higher doses. Typically, the rat or mouse will demonstrate continuous sniffing, licking, biting or gnawing and they will stand upright against a vertical grid (Braun and Chase, 1986).

Many of the behaviors that are controlled by DA are only observed by the activation of both D1 and D2 receptors. This is known as D1/D2 synergism. Both *in vivo* and *in vitro* research confirm this phenomenon. Lewis, Widerlov, Knight, Kilts, & Mailman (1983) showed that either a selective D1 or a selective D2 antagonist could block locomotion and motor stereotypy elicited by the nonselective DA agonist apomorphine. Research by Walters, Bergstrom, Carlson, Chase, & Braun (1987) showed that there has to be concomitant stimulation of both D1 and D2 receptors in order to see a disinhibition of globus pallidus neurons and an inhibition of caudate-putamen neurons. Importantly, Walters et al. (1987) also showed that endogenous DA acting at D1 receptors is sufficient to synergize with an exogenous D2 agonist. Thus, it is crucial in studies of D1/D2 synergism to control for endogenous DA. In doing this, LaHoste and Marshall (1992) showed that, in normal rodents, the behavioral effects of a selective DA agonist can be blocked by the heterotypic antagonist.

D1/D2 synergism can also be seen in the DA-stimulated expression of the immediate-early gene c-*fos* in striatal neurons. When D1 and D2 agonists are administered together this results in the expression of c-*fos* in the striatum (LaHoste, Yu, & Marshall, 1993). Furthermore,

either a selective D1 or a selective D2 antagonist can block the striatal c-*fos* response to amphetamine or cocaine (Graybiel, Mortalla, & Robertson, 1990).

Breakdown in D1/D2 Synergism

Under normal conditions co-stimulation of D1 and D2 receptors is necessary for motor behavior in rats and mice as described above. However, once DA has been depleted from the striatum this no longer holds true. Denervation of the nigrostriatal pathway by 6-hydroxydopamine (6-OHDA) or the administration of the monoamine depleting agent reserpine causes a breakdown in D1/D2 synergism (Rouillard and Bedard, 1988).

In 24-h reserpine (5mg/kg) treated mice, the sole stimulation of the D1 receptor by SKF 38393 restored the motor behavior, consisting of grooming, rearing and consistent locomotion, that was lost due to DA depletion. Similarly, D2 receptor agonists (RU 24213 and lisuride) could restore locomotor activity in reserpine-pretreated mice. Also, motor behavior stimulated by a D2 receptor agonist in reserpine-treated mice cannot be blocked by a heterotypic (D1) antagonist. This led to the conclusion that prior DA depletion elicits a change in the relationship between D1 and D2 receptor such that they each function independently of one another (Starr, Starr, & Kilpatrick, 1987).

In 6-OHDA treated rats, the death of the dopaminergic neurons along with the depletion of endogenous DA liberates D1 and D2 receptors from their independence with each other and induces profound receptor supersensitivity. After a unilateral 6-OHDA lesion, independent stimulation of D1 or D2 receptors will result in rotation away from the lesioned side (Arnt and Hyttel, 1984) indicative of an asymmetric breakdown in D1/D2 synergism. LaHoste and Marshall (1992) performed bilateral 6-OHDA lesions on rats and found that there was increase in locomotor activity and motor stereotypy in response to D1 or D2 agonist alone. Similar to what

occurs following destruction of DA neurons by 6-OHDA, depletion of synaptic DA by administration of reserpine, which disrupts vesicular storage of monoamines, also results in D1 and D2 independence and supersensitivity (LaHoste & Marshall, 1993).

D1/D2 Receptor Co-Localization

What is the mechanism of D1/D2 receptor synergism? One could hypothesize very different mechanisms depending on whether D1 and D2 receptors are located on the same neuron or on distinct neurons. This has been an issue of debate. *In situ* hybridization histochemistry studies show that the majority of D1 and D2 receptors are located on separate pathways. Most striatonigral pathway neurons (which project to the SNr and constitute the "direct pathway") express D1 receptor mRNA as well as dynorphin and substance P mRNA, but not D2 mRNA. Most striatopallidal pathway neurons (which project to the globus pallidus and constitute the "indirect pathway") neurons expressed both D2 receptor mRNA and enkephalin mRNA but not D1, dynorphin, or substance P mRNA (Gerfen, Engber, Mahan, Susel, Chase, Monsma et al. 1990). Thus, the vast majority of striatal neurons express only one DA receptor subtype.

Investigators using single-cell reverse-transcription polymerase chain reaction (RT-PCR) claim that D1 and D2 mRNA are co-expressed in half of the medium spiny striatal neurons (Surmeier, Song, & Yan, 1996). One drawback to this technique is that multiple rounds of PCR are performed, resulting in an amplification factor in the billions. However, when a single round of PCR was conducted versus multiple rounds, there was very little co-localization seen between D1 and D2 receptors. These results were similar to the results presented above by Gerfen using *in situ* hybridization.

Based on these results, Surmeier and colleagues raised the question that behavioral synergism may be due to interactions between D1 and D3, D1 and D4 receptors or D2 and D5 receptors located on the same neuron. LaHoste, Henry, & Marshall (2000) used newly developed antagonists that were selective for D2, D3, and D4 receptor subtypes to test this hypothesis. They found that specific D2 antagonists, but not D3 or D4 antagonists, were able to block DA agonist-induced expression of c-*fos*, a marker of D1/D2 synergism (see above).

Research by both proponents of D1/D2 co-localization and D1/D2 segregation, however, seems to show that the *majority* of D1 and D2 receptors are located on separate neurons and that these neurons belong to separate pathways that project to different areas of the basal ganglia. Thus, there must be some type of interneuronal communication between the two types of receptors that can explain D1/D2 synergism.

Interneuronal Communication

The most typical form of communication between neurons is through the synapse by action potential-induced release of neurotransmitter substance. If D1 and D2 receptors are on separate neurons and communicate using action potentials to effect D1/D2 synergism, then blockade of action potentials should block D1/D2 synergism. LaHoste et al. (2000) administered the fast sodium channel blocker tetrodotoxin (TTX) intrastriatally to rats. While this blocked action potentials, D1/D2 synergism remained intact (as seen indicated by c-*fos* expression).

Since D1/D2 synergism occurs under conditions in which separate D1- and D2-expressing neurons cannot communicate through action potentials, we offer an alternative hypothesis involving electrotonic communicating via gap junctions.

Electrotonic Coupling

Electrotonic coupling takes place among neurons via gap junctions. Gap junctions provide neurons with a means of intercellular communication in the retina and several brain nuclei in mammals including the striatum (Cepeda, Walsh, Hull, Buchwald, &Levine, 1989; Grace and Bunney 1983: Lasater and Dowling 1985). Furthermore, several studies have shown that DA acts to gate gap junction channels in the retina and striatum. For example, at the beginning of the light cycle, DA is released from the amacrine cells of the retina and acts on D1 receptors there to suppress electrotonic transmission between adjacent horizontal cells (Lasater and Dowling 1985; McMahon, Knapp, & Dowling, 1989).

Histochemically, coupling can also be illustrated using gap junction-permeable Lucifer Yellow dye, which can be injected into individual cells. If the injected cell is coupled to another, the dye will pass through the gap junction and fill the adjoining cell. This form of labeling is a reliable indicator of electrotonic coupling between neurons in the brain (Grace and Bunney, 1983). Research using the Lucifer Yellow dye has shown coupling between neurons in the ventral striatum (nucleus accumbens) that is modulated by DA. Stimulation of D1 receptors led to a decrease in coupling between neurons, whereas stimulation of D2 receptors did not change the coupling between cells from control levels (O'Donnell and Grace, 1993). Both electrolytic and neurochemical DA-depleting lesions in the dorsal striatum, which result in the breakdown of D1/D2 synergism and agonist supersensitivity, resulted in increased dye coupling between striatal neurons (Cepeda et al., 1989).

Dopaminergic neurons in the SNc have been shown to be electotonically coupled to one another (Vandecasteele, Glowinski, & Venance, 2005). Furthermore, the firing frequency of coupled DA neurons there is modulated by gap junctions. Also, when compared with chemical

synapses it appears that electrical synapses appear to have a greater impact in fast communication between DA neurons.

Previous research in our laboratory has investigated the role of Cx32 in D1/D2 synergism (Cline, 2000; McKenna, 2004). At the time that work was begun, Cx32 was the only known connexin present in neurons. Results from this work did not indicate that Cx32 was involved in D1/D2 synergism. Since then, Cx36 was cloned and shown to be present to a substantial degree in neurons. Cx36 is located throughout the brain including the dorsal and ventral striatum (Condorelli et al., 2000). Since, as stated earlier, the majority of D1 and D2 receptors are located on separate neurons, and since D1/D2 synergism occurs in the absence of action potentials, the present study aimed to elucidate the role of Cx36-constituted gap junctions in D1/D2 synergism.

Hypotheses and Rationale

Research suggests that connexins might play a role in D1/D2 synergism in the striatum. Since Cx36 is known to be present in the striatum, and since depletion of DA leads to a breakdown in D1/D2 synergism and an increase in gap junction dye coupling, we reasoned that mice that have been engineered to be incapable of expressing Cx36 protein should behave differently than their wild-type littermates on striatally-mediated behavioral tests after separate and concomitant administration of selective D1 and D2 agonists.

Hypothesis 1: Genetically engineered mice that differ in their ability to produce Cx36 protein will differ with respect to stereotyped motor behavior following separate or combined agonist stimulation of D1 and D2 receptors.

Hypothesis 2: Genetically engineered mice that differ in their ability to produce Cx36 protein will differ with respect to stereotyped motor behavior following separate or combined agonist stimulation of D1 and D2 receptors after reserpine pre-treatment.

Methods

Animals

Mice were bred in the University of New Orleans Department of Psychology rodent colony from male and female breeders kindly donated by Dr. David L. Paul (Harvard University). Original founder mice had been generated by inserting two reporter genes (β-galacotosidase and placental alkaline phosphatase) into the Cx36 coding region by means of homologous recombination (Deans, Gibson, Sellitto, Connors, & Paul, 2001). This resulted in mice whose Cx36 gene was "knocked-out." The breeding of heterozygous male and female Cx36 mice results in three genotypes, two homozygous and one heterozygous. The homozygous genotypes are: "wild type" (WT), in which both Cx36 alleles are normal, and "knockout" (KO) in which both Cx36 alleles have been altered to prevent transcription. Heterozygotes (Het) have one mutated and one normal allele.

From the resulting breeding colony, adult male mice, weighing 20-30g were chosen for used in the present study. Animals were kept in same-sex cages in groups of 6-10 with free access to food and water. Artificial lighting was provided from 0700 to 1900h. All mice were maintained and used in accordance with the guidelines for animal care and experimentation established by the National Institutes of Health and the University of New Orleans Institutional Animal Care and Use Committee (approved protocol #070).

Genotyping

On postnatal day (P) 30-35, mouse pups were genotyped using genomic DNA purified from tail biopsies obtained under general anesthesia (2% tribromoethanol, 0.10-0.15 ml/10 gram

body weight, i.p.). Biopsy tissue was digested overnight at 55^{o}C in proteinase K. After centrifugation, isopropanol was added to the supernatant to precipitate high molecular weight genomic DNA. Genomic DNA was amplified using PCR: 1) Cx36 genotyping solution was made of the following: 10X buffer, 25mM Mg^{++}, 2.5 mM dNTPs, a 3' primer that is common to both WT and KO alleles, a 5' WT-specific primer, and a 5' KO-specific primer; 2) 28μl of the Cx36 genotyping solution was distributed to individual tubes; 3) 1-3 μg of genomic DNA from each mouse was added to each tube, along with 0.5μl Taq polymerase; 4) the tubes were then placed in a thermocycler (for 30 cycles) to amplify the DNA. After amplification, the DNA was analyzed using electrophoresis in a 1% agarose gel.

Paradigm for Assessing D1/D2 Synergism and its Breakdown

Stereotyped motor behavior in rodents is elicited by high doses of DA agonists acting in the dorsal striatum. In normal animals, this behavior is only elicited following combined stimulation of D1- and D2-type receptors. By assessing the ability of separate or combined stimulation of D1 and D2 receptors to elicit stereotypy in mice genetically engineered to express different amounts of Cx36, we can test the hypothesis that this protein plays a role in the maintenance of D1/D2 synergism (*Hypothesis 1*). Following daily reserpine treatments, the same striatally-mediated stereotyped behaviors that normally require concomitant D1/D2 stimulation can now be elicited independently by either D1 or D2 agonist stimulation. By assessing the ability of independent stimulation of D1 or D2 receptors to elicit stereotypy in reserpine-pretreated Cx36 transgenic mice, we can test the hypothesis that this protein plays a role in the breakdown of D1/D2 synergism *(Hypothesis 2)*.

Drug Treatments

Experiment 1. To investigate D1/D2 synergism, we gave Cx36 KO (n = 8), WT (n = 7), and Het mice (n = 8) drug treatments that resulted in agonist stimulation of (a) D1 receptors, (b) D2 receptors, (c) D1 and D2 receptors, or (d) neither D1 nor D2 receptors. Each mouse received each of the drug treatments in counterbalanced order as determined by a Latin square (see Table 1). Drug treatments were separated by 72-96h intervals.

Table 1. Receptor(s) Stimulated

Order	**Test Day**			
	1	*2*	*3*	*4*
A	None	D1+D2	D2	D1
B	D1	D2	None	D1+D2
C	D2	D1	D1+D2	None
D	D1+D2	none	D1	D2

D1 and D2 receptors were stimulated by the mixed D1/D2 agonist apomorphine (3.0 mg/kg, i.p.). Selective agonist stimulation of individual receptor subtypes was achieved by prior (30 min.) selective blockade of the heterotypic receptor. Thus, D1 receptors were stimulated by apomorphine preceded by the selective D2 antagonist eticlopride (0.3 mg/kg, i.p.); D2 receptors were stimulated by apomorphine preceded by the selective D1 antagonist SCH 23390 (0.1 mg/kg, i.p.). Details of acute drug treatments are given in Table 2. Stereotyped motor behavior was recorded for one hour following each drug treatment (see below).

Table 2. Time Course of Acute Drug Treatments

Receptor(s) stimulated	**Time (min.)**	
	0	*30*
none	vehicle	vehicle
D1	eticlopride	apomorphine
D2	SCH 23390	apomorphine
D1+D2	vehicle	apomorphine

Experiment 2. To induce the breakdown in D1/D2 synergism, we gave mice reserpine (1 mg/kg, i.p.) once daily. DA depletion was assessed 24 hrs. after reserpine injection using catalepsy as the dependent variable. Forelimbs were placed on a horizontal bar and the latency to step down was recorded. If step-down latency was >180 sec., agonist testing followed; if latency was <180 sec., mice were returned their home cages and given an additional reserpine injection on the following day. Mice reaching a criterion of 180 seconds of catalepsy were tested for motor stereotypy in response to selective D1 or D2 activation. Each mouse was tested under each agonist condition (as described above) by testing on successive days, the order of which was counterbalanced within each genotype (WT: n = 5; Het: n = 8; KO: n = 9). Stereotyped motor behavior was recorded for one hour following stimulation of each receptor type (see below).

Behavioral Assay of D1/D2 Synergism

Mice were tested for motor behavior following each of the drug treatments. Each mouse was placed in a plastic cylinder (measuring 30 cm high, 16cm diameter) for one hour prior to drug treatment. The animals' behavior was recorded with a Sony digital video camera.

Stereotyped motor behavior was observed for 30 seconds every 5 minutes beginning 5 minutes prior to any drug injection and continuing until one hour after the second drug (agonist) had been administered. Behavior was rated using a scale of 0-5 that was modified for mice from LaHoste and Marshall (1993), and represents species-specific stereotypies elicited by increasing doses of DA agonists (Table 3). In addition, grooming behavior was quantified as the amount of time spent grooming during each 30-second observation period.

Table 3. Stereotyped Behavior Rating Scale

Score	Behavior
0	still
1	grooming
2	discontinuous stereotyped behavior (sniffing interrupted by grooming)
3	continuous unfocused stereotypy (sniffing/licking/chewing without spatial focusing)
4	continuous focused sniffing (sniffing that takes place at only one particular site)
5	continuous focused oral stereotypy (licking/chewing that takes place in one site)

Data Analysis

Behavior scores were initially subjected to a mixed-design three-way analysis of variance (ANOVA) with one between-subjects factor (Genotype) and two within-subjects factors (Drug Treatment and Time). Significant main effects were further analyzed by Newman-Keuls *post-hoc* tests. In some cases, data from each animal were averaged over Time, thus eliminating this variable and avoiding the often difficult interpretation of higher-order interactions.

Results

The Role of Connexin36 in D1/D2 Synergism

To investigate potential behavioral differences between genetically engineered mice that differ in their ability to produce Cx36 protein, we measured stereotyped motor behavior following different drug treatments that stimulated D1 and D2 receptors either separately or in combination. The results show that sustained motor stereotypy is observed only following combined stimulation of both D1 and D2 receptors, indicating D1/D2 synergism (Fig. 1). Stimulation of D2 receptors alone elicited an early burst of stereotypy that was quickly suppressed, an effect that is consistently observed in rats (e.g., LaHoste & Marshall, 1992).

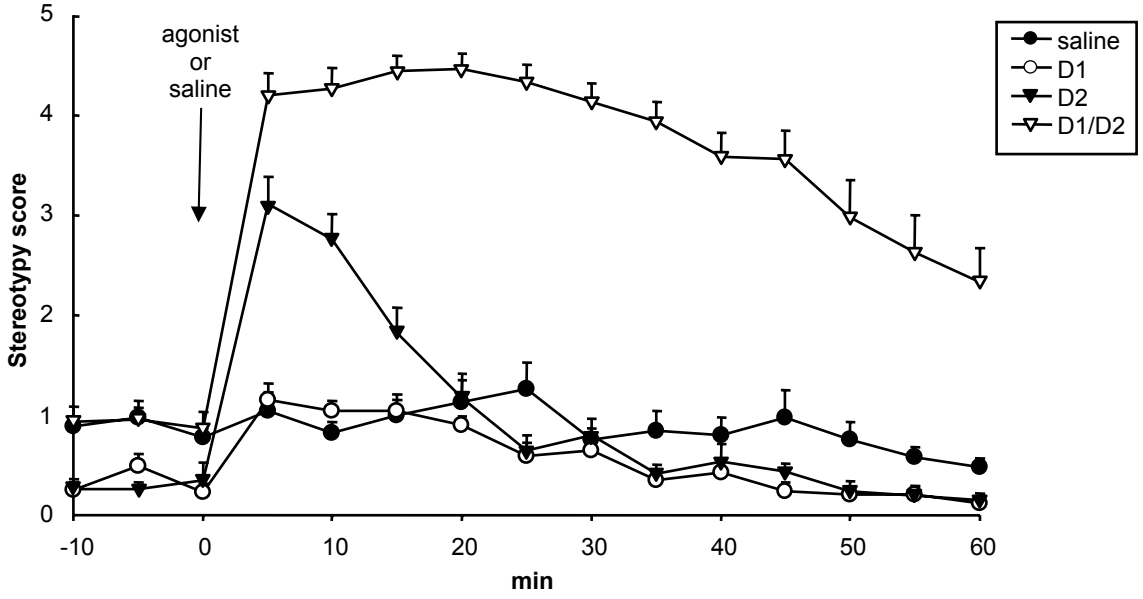

Figure 1. Mean stereotypy scores following separate or combined stimulation of D1 and D2 receptors illustrate D1/D2 synergism. *n* = 21-23. For description of stereotypy rating scale in this and subsequent figures, see Methods.

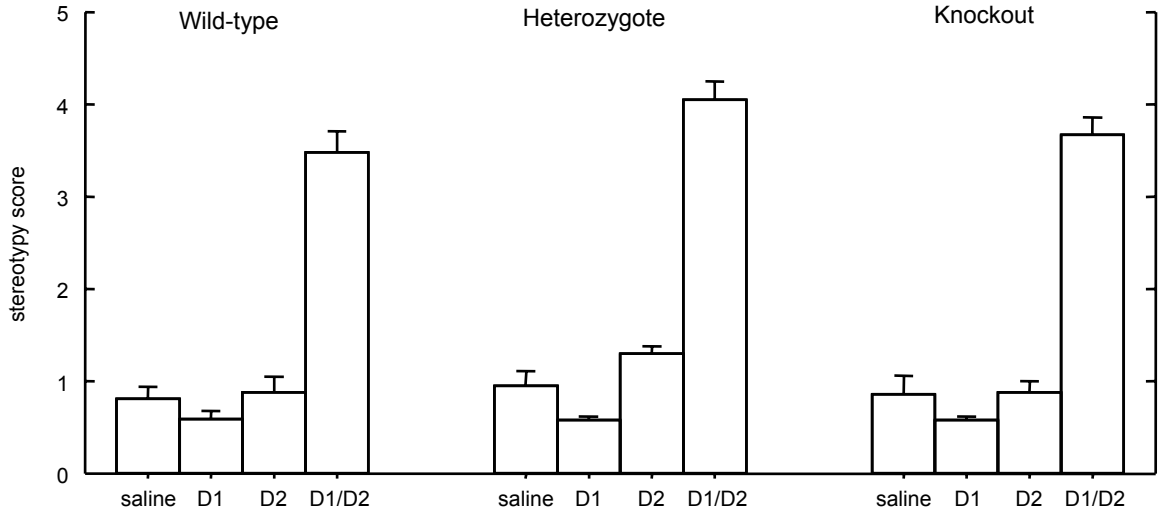

Figure 2. Mean stereotypy scores averaged over the 60-minute post-agonist time interval and grouped by genotype for all drug treatments. n = 7-8. Scores for the combined D1/D2 treatment are significantly higher ($p<.001$) than for any of the other treatments, which do not differ significantly from each other. The genotypes do not differ significantly.

The presence or absence of Cx36 did not affect the behavioral expression of D1/D2 synergism (Fig. 2). A 2-way ANOVA with one repeated measure (Drug Treatment) and one between-subjects measure (Genotype) revealed a significant main effect for Drug Treatment ($F_{3,60} = 245.1$, $p<.001$) but no significant main effect for Genotype ($F_{2,20} = 2.1$, ns) and no Drug Genotype interaction ($F_{6,60} = 0.8$, ns). *Post hoc* tests on the Drug Treatment effect showed that the combined agonist group differed significantly from all other agonist or vehicle treatments (Fig. 3). Therefore, D1 and D2 receptors synergize at the behavioral level in a normal manner in mice lacking Cx36.

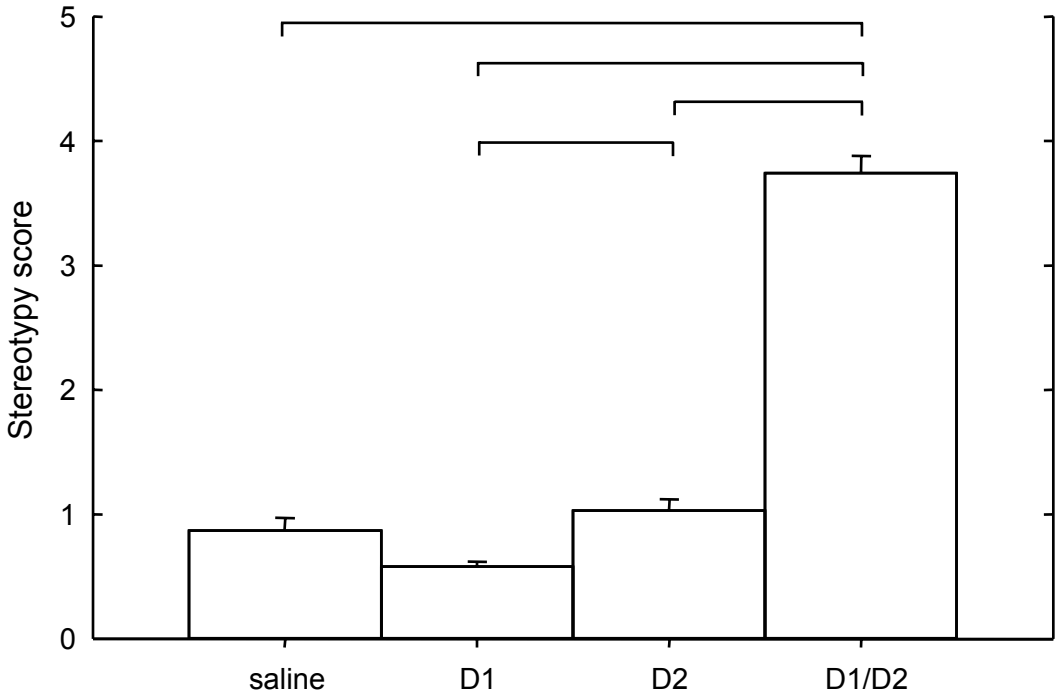

Figure 3. Mean stereotypy scores collapsed across genotypes and averaged over time. Combined D1/D2 treatment is significantly different from all other treatments. Brackets indicate significant differences as determined by Newman-Keuls *post hoc* tests, $p<.01$. $n = 21$-23.

The Role of Connexin36 in D1 Receptor–mediated Behavior

To further analyze the role of Cx36 in behaviors that are modulated by DA receptors, we examined differences between genotypes in D1 agonist-stimulated grooming, a behavior that does not require concomitant stimulation of D2 receptors (LaHoste & Marshall, 1992). Data were analyzed by a 2-way ANOVA with one repeated measure (Time: pre- vs. post-agonist) and one between-subjects factor (Genotype). As shown in Figure 4, D1 receptor stimulation elicited grooming behavior as indicated by a significant main effect for Time ($F_{1,20} = 58.9$, $p<.001$). There was no main effect for Genotype ($F_{2,20} =0.4$) nor significant Genotype X Time interaction. Therefore, D1 receptor-stimulated grooming is normal in mice lacking Cx36.

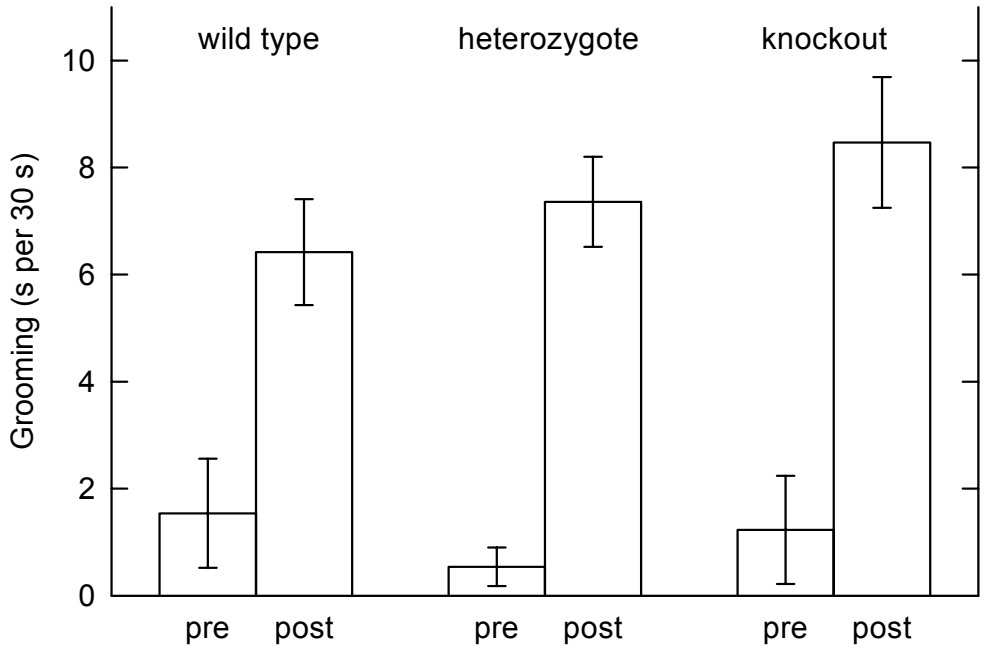

Figure 4. Mean grooming scores (number of seconds spent grooming per 30-sec. observation period) grouped by genotype and averaged across the 15-min. time period just prior to agonist injection and the 1-h time period following agonist injection. *n* = 7-8. D1 stimulation elicited significant grooming ($p<.001$). The genotypes do not differ significantly.

The Role of Connexin36 in D2 Receptor-mediated Behavior

As indicated above, stimulation of D2 receptors alone reliably elicits an early D1-independent burst of stereotypy that quickly subsides to baseline levels by 20 minutes post-agonist (see Fig. 1). To determine if there were differences between genotypes in D2 receptor stimulation alone we re-examined the stereotypy scores for the first 15 minutes after saline or selective stimulation of D2 receptors (D1 antagonist + apomorphine). A 2-way ANOVA revealed a significant Drug effect ($F_{1,20} = 38.0$, $p<.001$) but no Genotype effect ($F_{1,20} = 1.3$, ns) and no Drug X Genotype interaction ($F_{2,20} = 2.5$, ns) (Fig. 5). Therefore, the brief D2-

stimulated behavioral activation that is independent of D1 receptors is normal in mice lacking Cx36.

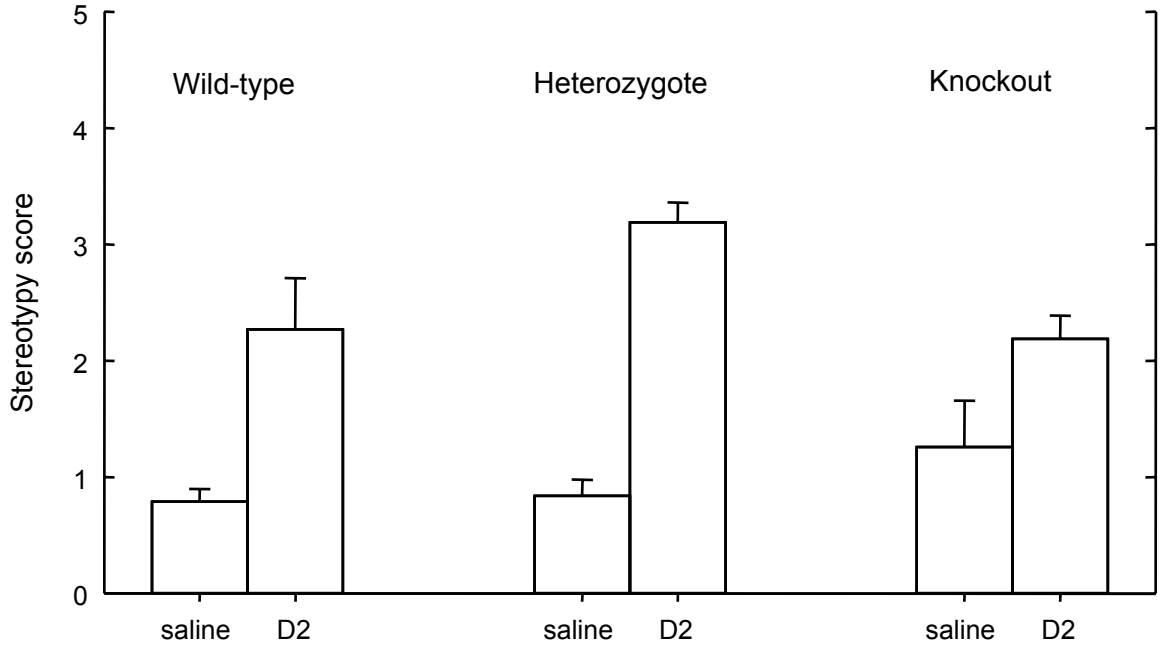

Figure 5. Mean stereotypy scores for the first 15 min. after apomorphine injection (following pre-treatment with a D1 antagonist) or following saline. n = 7-8. Selective D2 receptor activation produced a significant, short-lasting stereotypy ($p<.001$) that did not differ across genotypes.

The Role of Connexin36 in the Breakdown of D1/D2 Synergism

To study the role of Cx36 in the breakdown of D1/D2 synergism, we administered the DA-depleting drug reserpine, a treatment that is known to cause a breakdown in D1/D2 synergism in normal rodents. Behavioral results following separate D1 or D2 receptor stimulation are presented in Figure 6. The results show D1 and D2 receptor independence in reserpine-pretreated mice. That is, mice showed sustained and robust motor stereotypy following selective stimulation of *either* D1 *or* D2 receptors.

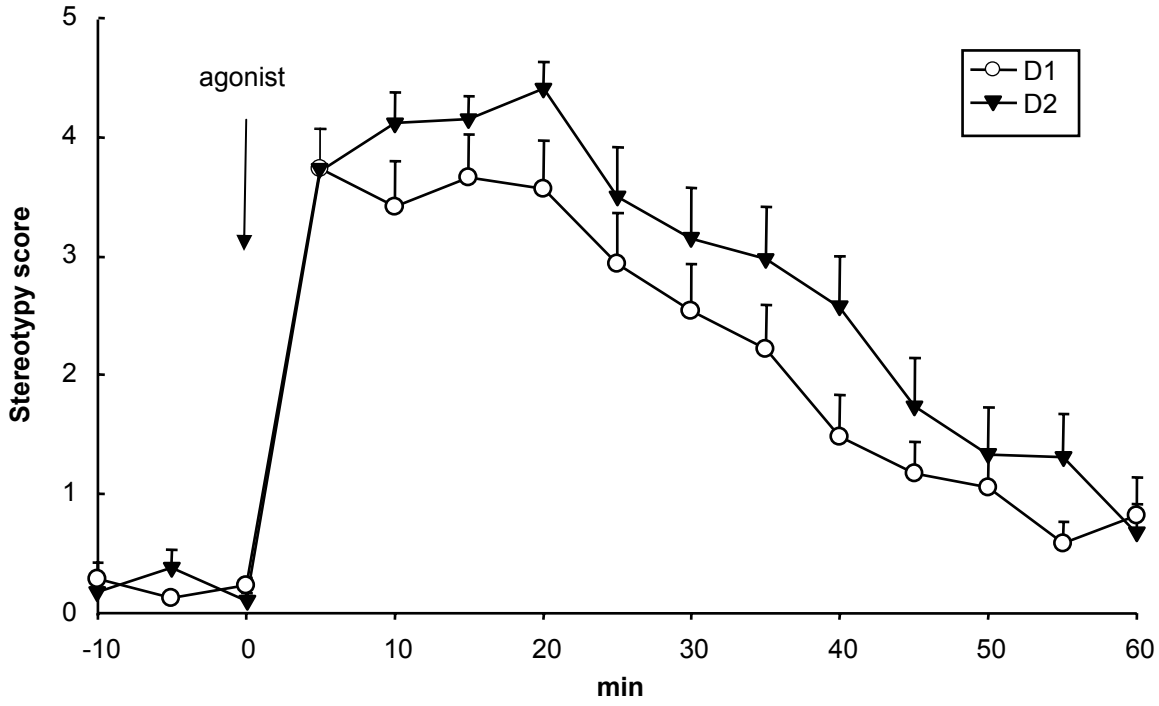

Figure 6. Mean stereotypy scores plotted across time in reserpine-treated mice reveal D1 and D2 independence. $n = 22$.

To assess this breakdown in D1/D2 synergism and its possible modulation by Cx36, we compared the behavioral responses of reserpine- and non-pretreated mice as a function of genotype (Fig. 7). Data were averaged over the one-hour post-agonist period and subjected to a 3-way ANOVA with one repeated measure (Drug Treatment: D1 vs. D2 stimulation) and two between-subjects factors (Pretreatment: reserpine or no reserpine; and Genotype). There were significant main effects for Drug Treatment ($F_{1,39} = 16.5$, $p<.001$) and Pretreatment ($F_{1,39} = 72.0$, $p<.001$), but not Genotype ($F_{2,39} = 0.04$, ns). However, there was a highly significant Drug X Genotype interaction ($F_{2,39} = 6.4$, $p=0.004$); no other interactions were significant.

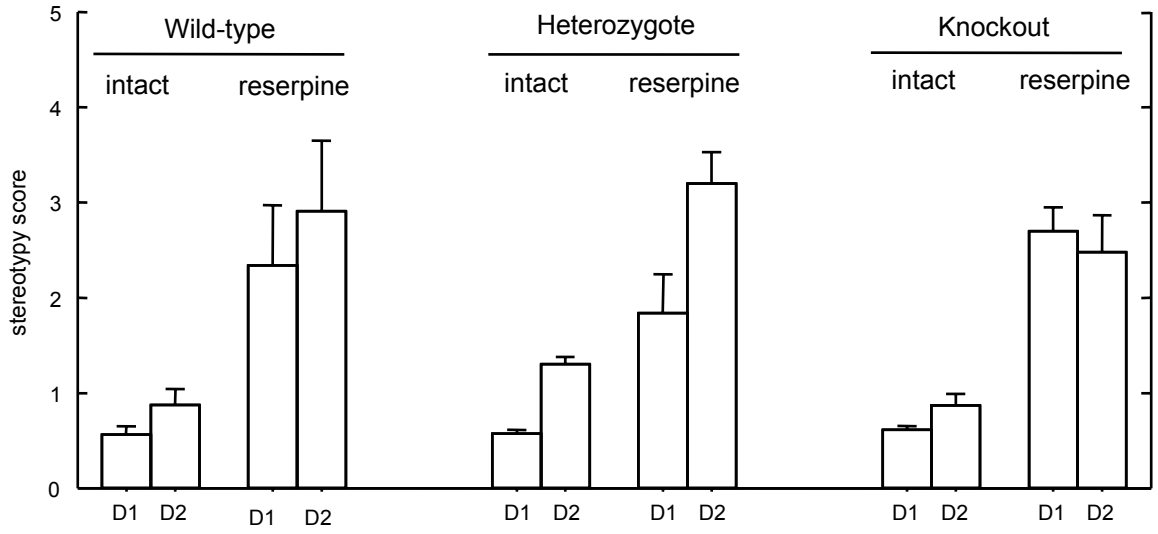

Figure 7. Mean stereotypy scores following selective D1 or D2 receptor stimulation in non-pretreated and reserpine-pretreated mice, averaged across the 1 h post-agonist time period. n = 5-9. Reserpine pretreatment caused a >3-fold increase in stereotypy scores. Genotypes did not differ.

To address the Drug X Genotype interaction we compared genotypes within drug treatments but found no significant differences across genotype (Fig. 8). The significant Drug X Genotype interaction likely reflects the fact that the pattern of mean differences across genotype differs for D1- and D2-stimulated behavior. In general, reserpine greatly potentiates the stereotypic response to selective D1 or D2 stimulation, but does so equally for D1 and D2 receptors and equally for all genotypes.

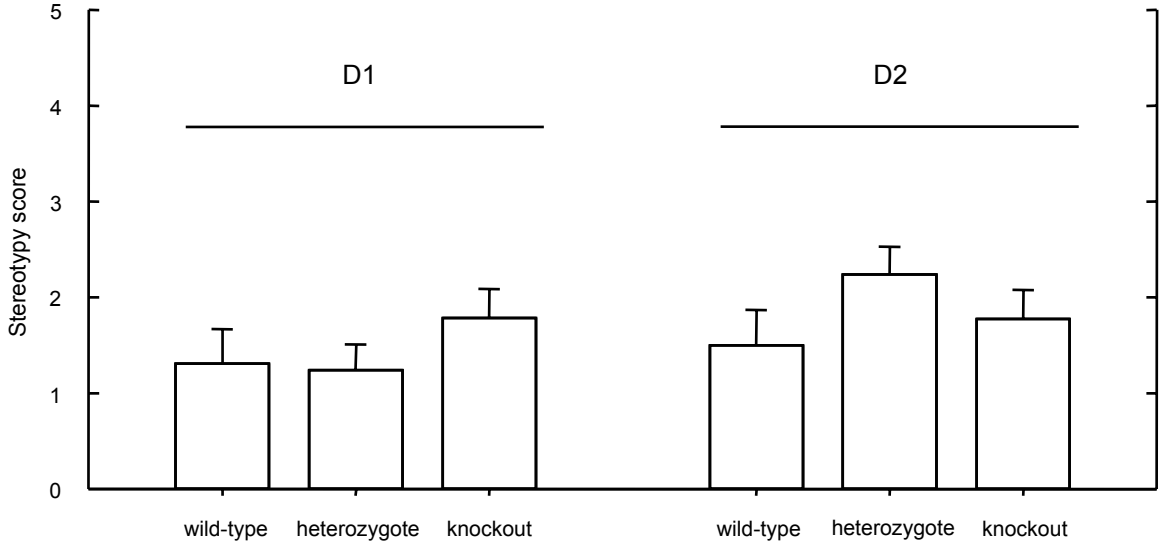

Figure 8. *Post hoc* analysis of data in Fig. 6. Mean stereotypy scores are collapsed across pretreatment for illustration of the significant Genotype × Drug interaction. There are no significant *post hoc* differences within either drug treatment. n = 13-16.

Catalepsy and Connexin36

While giving reserpine injections to the mice it was noticed that there were differences among the three genotypes in their response to the drug. Specifically, it was observed that Cx36 KO mice required much more reserpine to reach the criterion of 180 seconds of catalepsy compared to WT mice, while Hets required an intermediate amount. These differences were quantified, and are depicted in Figures 9 and 10. With respect to the number of daily injections of reserpine it took to induce 180 seconds of catalepsy, a 1-way ANOVA indicated a significant main effect for Genotype ($F_{2,18} = 39.2$, $p<.001$). *Post hoc* tests indicated that all groups were different from each other (Fig. 9) with the WT mice requiring the fewest number of injections and KO mice requiring the most. When cumulative dose-response data were analyzed using the Allfit computer program, the ED_{50}s for WT, Het, and KO mice were all significantly different

from each other (p<.001, Fig. 10). These data demonstrate an unexpected finding, namely that loss of Cx36 leads to resistance to the cataleptic effects of reserpine in a gene dosage-dependent manner.

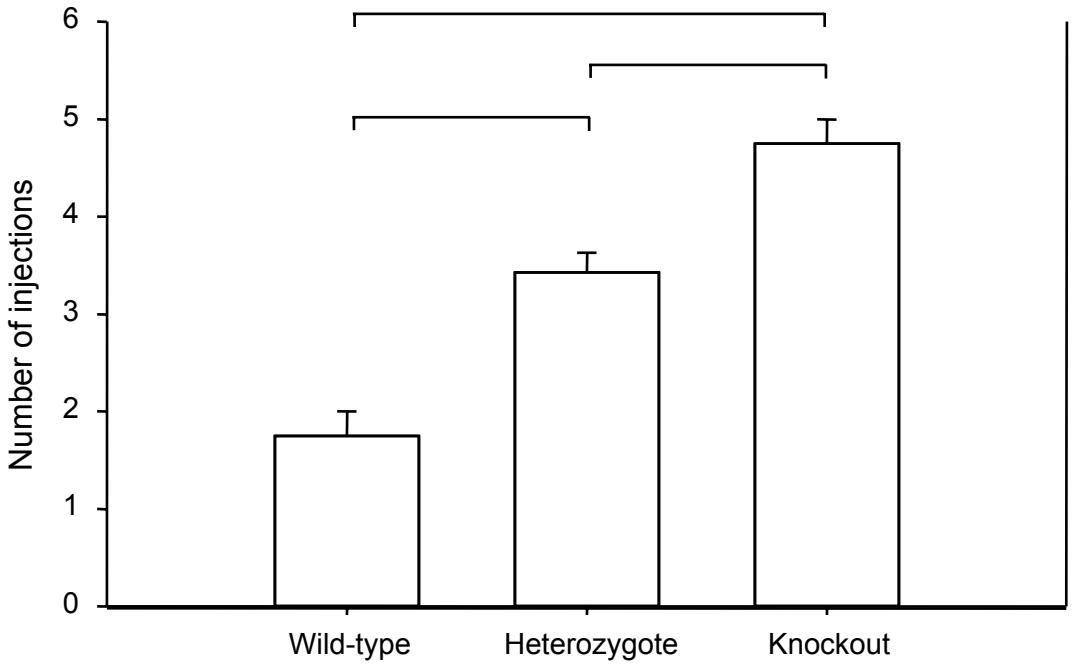

Figure 9. Mean number of 1 mg/kg reserpine injections required to reach 180 s of catalepsy. Brackets indicate significant differences as revealed by Newman-Keuls *post hoc* tests, p<.001. *n* = 6-9. All genotypes differ significantly, indicating a Cx36 gene dosage-dependent sensitivity to reserpine.

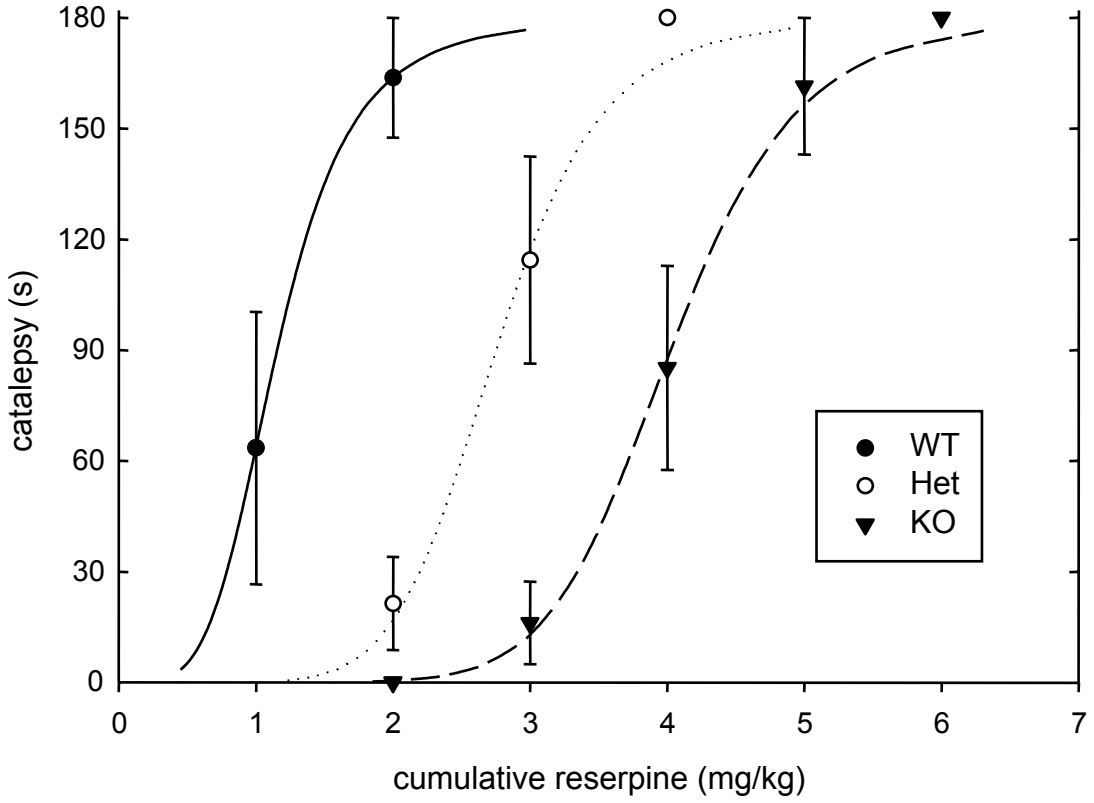

Figure 10. Computer-generated sigmoidal best-fit curves for mean catalepsy scores. Calculated ED_{50}s: wild-type, 1.2 mg/kg,; heterozygote, 2.8 mg/kg,; knockout, 4.0 mg/kg. *n*= 6-9. Increasing loss of Cx36 leads to lower sensitivity to reserpine.

Discussion

Much research over the past two decades has shown that many of the behavioral, physiological and cellular effects of DA require concomitant stimulation of D1 and D2 receptors to be manifested, a phenomenon known as D1/D2 synergism. The purpose of this study was to investigate a possible mechanism for D1/D2 synergism and its breakdown. Previous findings suggested that synergism could not be explained by either receptor co-localization within the same neuron or by classical interneuronal communication via action potentials. By using mice with targeted disruptions to the connexin gene Cx36, the present thesis investigated the possibility that electrotonic coupling between neurons via gap junctions plays a role in D1/D2 synergism.

The main findings of the present research suggest that Cx36 does not play a role in D1/D2 synergism or its breakdown. Synergism was assessed by testing for motor stereotypies following strong activation of D1 and D2 receptors alone or in combination. Similar to what has been found in rats, mice in the present study displayed intense, focused species-typical behaviors (focused sniffing, chewing or licking) when given the mixed D1/D2 agonist apomorphine. These agonist-elicited behaviors could be blocked by either a selective D1 or D2 antagonist, indicating that co-stimulation of the two receptor subtypes was required (Figs. 1 and 2). Of particular importance for the present thesis, this same pattern of response, demonstrating intact D1/D2 synergism, was observed in mice of all genotypes (Fig. 2). Thus, the degree to which Cx36 protein was present or absent had no effect on the outcome.

There are some exceptions to the rule that DA-stimulated behavior requires concomitant stimulation of D1 and D2 receptors. For example, D1 stimulation elicits grooming behavior that

is not blocked by a D2 antagonist. In addition, a very reliable finding in the primary behavioral paradigm used here is that there is an early component of D2-elicited stereotypy that is not blocked by a D1 antagonist (see, e.g., Fig. 1). Since the presence or absence of Cx36 had no effect on behaviors requiring co-stimulation, we asked whether independently mediated D1 or D2 behaviors differed across genotypes. As expected, independent activation of D1 receptors (apomorphine in the presence of D2 blockade) elicited grooming behavior; however, this effect was observed to the same degree in all genotypes (Fig. 4). When we re-examined the transient activation of motor stereotypy that is mediated independently by D2 receptors (apomorphine in the presence of D1 blockade), we found that this activation was present to similar degrees in all genotypes (Fig. 5).

Depletion of DA by the drug reserpine causes a breakdown in D1/D2 synergism. That is, the same behaviors that normally require co-activation of D1 and D2 receptors can now be elicited independently by agonist stimulation of either receptor subtype. When these transgenic mice in the present study were administered reserpine they showed D1 and D2 receptor independence (Fig. 6). In comparing the reserpine treated mice to the intact mice there was a three-fold increase in stereotypy scores in D1 and D2 receptor stimulation alone (Fig. 7). Nonetheless, there were no genotype differences in these results. Thus, Cx36 does not appear to be involved in the breakdown of D1/D2 synergism. There was one interesting observation that was made between the genotypes and that was their cataleptic response to the drug reserpine (Fig. 9 and 10), which was significant for each of the genotypes ($p < .001$). The WT mice required less cumulative reserpine to reach 180 seconds of catalepsy and the KO mice required more reserpine to reach the same criterion. Thus, the absence of Cx36 appears to have some protective effect against the cataleptic action of reserpine.

Why was there no effect of the loss of Cx36?

The standard method of generating knockout mice results in the loss of the "knocked-out" gene from the very beginning of development. Thus, interpretation of the results of studies using such mice must take into consideration the possibility of developmental compensation. There is evidence to suggest that this may incur in Cx36 knockout mice. In the inferior olive widespread electrical coupling gives rise to rhythmic subthreshold oscillations in membrane potential. Although electrotonic coupling in this nucleus is mediated almost exclusively by Cx36-constituted gap junctions, Long, Deans, Paul, & Connors (2002) found that these olivary oscillations were nonetheless preserved in Cx36 knockout mice. De Zeeuw, Chorev, Devor, Manor, Van Der Giessen, De Jeu et al. (2003) found that olivary neurons in the knockout mice had both structural and electrophysiological compensations that allowed normal oscillations that were no different from those of wild-type mice. Could there be some type of compensation that is taking place in our Cx36 mice that could explain our behavioral results? Perhaps D1/D2 synergism is normally mediated by Cx36, but the loss of this protein developmentally leads to compensatory structural or functional changes—perhaps the increased expression of other connexins—resulting in the appearance of normal behavior. We found that there *are* differences between WT and KO mice with respect to sensitivity to reserpine. Perhaps the compensatory changes that normalize DA-mediated behavior also lead to a resistance to reserpine.

There is also the possibility that, while electrotonic coupling via gap junctions may be involved in D1/D2 interactions, Cx36 may not be the critical connexin. Although few other known connexins are likely candidates, this is a large protein family with possibly as yet undiscovered members. Finally, one must consider the possibility that the small number of medium spiny neurons with co-localized D1 and D2 receptors mediate D1/D2 synergism. A

transgenic method that could test this hypothesis would be a Cre-*lox* system in which D1 receptors were conditionally knocked-out only in cells that also expressed D2 receptors (or vice versa).

In order to address the genotype differences in the response to the drug reserpine we would have to look at a number of issues. First, are there differences in how the drug reserpine is metabolized by the mice? This could be investigated by examining brain tissue reserpine levels using mass spectrometry. If there were differences found in the amount of striatal reserpine, then the Cx36 knockout mouse could serve as a model for *metabolic resistance* to reserpine. Second, are there differences in how dopamine is depleted due to reserpine in these mice? One could perform HPLC (High Performance Liquid Chromatograph) to assess the amount of dopamine that is present in brain tissue of the mice after reserpine treatment. If there were differences found in the amount of striatal dopamine then the Cx36 knockout mouse could serve as a model for *neurochemical resistance* to dopamine depleting agents. If there were no genotype differences in the amount of striatal reserpine or dopamine, then the Cx36 knockout mouse could serve as a model for *behavioral resistance* to dopamine depletion. These could prove to be useful models in research on Parkinson's disease, the symptoms of which are due to depletion of striatal dopamine.

In summary, the present thesis found DA D1/D2 synergism to be intact in mice with targeted deletions of Cx36 genes. Nor was the induced breakdown in D1/D2 synergism affected by the absence of Cx36 gene. However, Cx36 does play a role in the action of the drug reserpine. This difference could be due to any number of causes including structural or electrophysiological compensations, gene regulation or reserpine metabolism.

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Vita

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